In the Light of Evolution

Volume I: Adaptation and Complex Design

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JOHN C. AVISE and FRANCISCO J. AYALA, *Editors*

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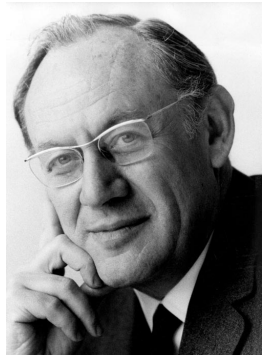
Arthur M. Sackler, M.D.
1913-1987



Born in Brooklyn, New York, Arthur M. Sackler was educated in the arts, sciences, and humanities at New York University. These interests remained the focus of his life, as he became widely known as a scientist, art collector, and philanthropist, endowing institutions of learning and culture throughout the world.

He felt that his fundamental role was as a doctor, a vocation he decided upon at the age of four. After completing his internship and service as house physician at Lincoln Hospital in New York City, he became a resident in psychiatry at Creedmoor State Hospital. There, in the 1940s, he started research that resulted in more than 150 papers in neuroendocrinology, psychiatry, and experimental medicine. He considered his scientific research in the metabolic basis of schizophrenia his most significant contribution to science and served as editor of the *Journal of Clinical and Experimental Psychobiology* from 1950 to 1962. In 1960 he started publication of *Medical Tribune*, a weekly medical newspaper that reached over one million readers in 20 countries. He established the Laboratories for Therapeutic Research in 1938, a facility in New York for basic research that he directed until 1983.

As a generous benefactor to the causes of medicine and basic science, Arthur Sackler built and contributed to a wide range of scientific institutions: the Sackler School of Medicine established in 1972 at Tel Aviv University, Tel Aviv, Israel; the Sackler Institute of Graduate Biomedical Science at New York University, founded in 1980; the Arthur M. Sackler Science Center dedicated in 1985 at Clark University, Worcester, Massachusetts; and the Sackler School of Graduate Biomedical Sciences, established in 1980, and the Arthur M. Sackler Center for Health Communications, established in 1986, both at Tufts University, Boston, Massachusetts.

His pre-eminence in the art world is already legendary. According to his wife Jillian, one of his favorite relaxations was to visit museums and art galleries and pick out great pieces others had overlooked. His interest in art is reflected in his philanthropy; he endowed galleries at the Metropolitan Museum of Art and Princeton University, a museum at Harvard

University, and the Arthur M. Sackler Gallery of Asian Art in Washington, D.C. True to his oft-stated determination to create bridges between peoples, he offered to build a teaching museum in China, which Jillian made possible after his death, and in 1993 opened the Arthur M. Sackler Museum of Art and Archaeology at Peking University in Beijing.

In a world that often sees science and art as two separate cultures, Arthur Sackler saw them as inextricably related. In a speech given at the State University of New York at Stony Brook, *Some reflections on the arts, sciences and humanities,* a year before his death, he observed: "Communication is, for me, the *primum movens* of all culture. In the arts. . . I find the emotional component most moving. In science, it is the intellectual content. Both are deeply interlinked in the humanities." The Arthur M. Sackler Colloquia at the National Academy of Sciences pay tribute to this faith in communication as the prime mover of knowledge and culture.

Contents

Preface to the *In the Light of Evolution* Series

Biodiversity—the genetic variety of life—is an exuberant product of the evolutionary past, a vast human-supportive resource (aesthetic, intellectual, and material) of the present, and a rich legacy to cherish and preserve for the future. Two urgent challenges, and opportunities, for 21st-century science are to gain deeper insights into the evolutionary processes that foster biotic diversity, and to translate that understanding into workable solutions for the regional and global crises that biodiversity currently faces. A grasp of evolutionary principles and processes is important in other societal arenas as well, such as education, medicine, sociology, and other applied fields including agriculture, pharmacology, and biotechnology. The ramifications of evolutionary thought also extend into learned realms traditionally reserved for philosophy and religion.

In 1973, Theodosius Dobzhansky penned a short commentary entitled "Nothing in biology makes sense except in the light of evolution." Most scientists agree that evolution provides the unifying framework for interpreting biological phenomena that otherwise can often seem unrelated and perhaps unintelligible. Given the central position of evolutionary thought in biology, it is sadly ironic that evolutionary perspectives outside the sciences have often been neglected, misunderstood, or purposefully misrepresented.

The central goal of the *ILE* series will be to promote the evolutionary sciences through state-of-the-art colloquia—in the series of Arthur M. Sackler colloquia sponsored by the National Academy of Sciences—and their published proceedings. Each installment will explore evolutionary

perspectives on a particular biological topic that is scientifically intriguing but also has special relevance to contemporary societal issues or challenges. Individually and collectively, the *ILE* series will aim to interpret phenomena in various areas of biology through the lens of evolution, address some of the most intellectually engaging as well as pragmatically important societal issues of our times, and foster a greater appreciation of evolutionary biology as a consolidating foundation for the life sciences.

The organizers and founding editors of this effort (Avise and Ayala) are the academic grandson and son, respectively, of Theodosius Dobzhansky, to whose fond memory this *ILE* series is dedicated. May Doby's words and insights continue to inspire rational scientific inquiry into nature's marvelous operations.

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Preface to *In the Light of Evolution, Volume I: Adaptation and Complex Design*

Darwin's elucidation of natural selection as a creative evolutionary force was one of the monumental intellectual achievements in the history of science, revolutionizing thought not only across the biological sciences but also fundamentally impacting much discourse in the social sciences, philosophy, and religion. No longer were explanations for the origin and marvelous adaptations of organisms necessarily to be sought solely in the context of supernatural causation. Instead, biological outcomes could now be interpreted within the critical scientific framework of natural processes governed by natural laws.

As a young man, Charles Darwin (like most biologists of his era, and before) was a natural theologian steeped in the notion that an attentive study of organisms in nature would ineluctably serve to document and further glorify the infinite creative powers of the Almighty. Darwin read and greatly admired William Paley's 1802 *Natural Theology*, which eloquently developed the "argument from design" that biological complexity was *prima facie* evidence for an intelligent engineer. This age-old idea had an illustrious intellectual pedigree. For example, it had been one of the "Five Ways" that St. Thomas Aquinas (an influential Dominican scholar of the 13th century) purported to prove God's existence. In 1779, the Scottish philosopher David Hume again encapsulated conventional wisdom when he wrote:

> the curious adapting of means to ends, throughout all of nature, resembles exactly, though it much exceeds, the productions of human contriv-

> ance, of human design, thought, wisdom, and intelligence. . . . By this argument *a posteriori*, and by this argument alone, do we perceive at once the existence of a Deity, and his similarity to human mind and intelligence (*Dialogues Concerning Natural Religion*).

The link between adaptation, biological complexity, and omnipotent design was apparent not only to philosophers and theologians. As phrased in the 1600s by the Christian scholar and scientist John Ray:

> You may hear illiterate persons of the lowest Rank of the Commonality affirming, that they need no Proof of the being of God, for that every Pile of Grass, or Ear of Corn, sufficiently proves that. . . . To tell them that it made it self, or sprung up by chance, would be as ridiculous as to tell the greatest Philosopher so (*The Wisdom of God Manifested in the Works of Creation*).

When Darwin boarded the *HMS Beagle* in 1831, he had no inkling that his voyage of discovery would eventually lead him to a revolutionary concept: that a purely natural process—natural selection—can yield biological outcomes that otherwise seem to have the earmarks of intelligent craftsmanship. Natural selection is an inevitable process of nature whenever organisms show heritable variation in their capacity to survive and reproduce in particular environments, but the operation has no more consciousness or intelligence than do natural physical forces such as gravity or weather. Thus, Darwin's key legacy is not the mere demonstration that evolution occurs (several of Darwin's predecessors were aware that species evolve), but rather the stunning revelation that a natural rather than a supernatural directive agent can orchestrate the evolutionary emergence of biological adaptations.

Nevertheless, 150 years after Darwin the challenge of understanding nature's complexity remains in many regards in its infancy. Only recently has science developed the necessary laboratory tools for delving deep within the molecular structure and function of genes that underlie particular complex adaptations (such as the eye, or the body plans of vertebrate animals). Only recently has it become possible to conduct genomic analyses in ways that permit the discovery of heretofore unspecified structural and regulatory genes that contribute to the molecular assembly of complex organismal phenotypes. Only recently have phylogenetic methods progressed to the point where the histories of complex phenotypes can be reliably elucidated. Scientific progress is occurring on many related fronts as well. For example, recent developments in evolutionary genetic theory (such as formal network analysis) have opened exciting new avenues for exploring the geneses and maintenance of biological complexity at the levels of genetic and metabolic pathways.

This book is the outgrowth of the Arthur M. Sackler colloquium on "Adaptation and Complex Design," which was sponsored by the National Academy of Sciences on December 1–2, 2006, at the Academy's Arnold and Mabel Beckman Center in Irvine, California. It is the first in a proposed series of Sackler colloquia under the umbrella title "In the Light of Evolution." The chapters that follow illustrate a wide variety of current scientific perspectives and methodological approaches directed toward understanding the origin and maintenance of complex biological adaptations.

In the opening chapter of this volume, Francisco Ayala develops the thesis that the Darwinian Revolution in effect completed the Copernican Revolution by extending from physics to biology a notion that the universe operates by natural laws that fall within the purview of rational scientific inquiry. In 1543, Nicolaus Copernicus published *De revolutionibus orbium celestium* (On the Revolutions of the Celestial Spheres), which introduced the idea that the earth is not at the center of creation and that natural laws govern the motion of structures in the physical universe. This thesis was bolstered and elaborated by the scientific discoveries of Galileo, Kepler, Newton, and others during the 16th and 17th centuries, but it was left to Darwin in the 19th century to discover that natural laws and processes also govern the emergence of apparent design in biological systems.

Subsequent chapters in this volume then illustrate the wide variety of current scientific avenues for exploring the nature of complex adaptations. Chapters are arranged into three parts, each immediately preceded by a brief editorial introduction. Authors in Part II set a conceptual stage by addressing epistemological issues related to biotic complexity from several disparate scientific perspectives including population genetics, information theory, and systems biology. In Part III, authors address the evolution of biotic complexity in a hierarchy of contexts, from the ontogenetic programs underlying particular phenotypes to the cooperation and conflicts often associated with multicellularity, social behaviors, and symbiotic associations. Chapters in Part IV provide additional case studies of how genetic, developmental, ecological, and other biological phenomena are now being dissected for complex phenotypes ranging from beetle horns to human adaptations for high-altitude hypoxia. Overall, the collection of ideas and data in this volume is highly eclectic but nonetheless broadly illustrative of modern scientific attempts to understand the evolution of complex adaptations.

These scientific endeavors are coming at a time of resurgent societal interest in supernatural explanations for biological complexity. Especially in the United States, proponents of intelligent design (ID)—the latest reincarnation of religious creationism—argue that biotic complexity can only be the product of a supreme intelligence (i.e., God). In the closing chapter of this volume, Eugenie Scott and Nicholas Matzke examine the

history of the ID movement and they conclude that although its scientific merit is nil, the crusade itself is of consequence to broader society because it represents a serious assault on the integrity of science education.

Perhaps there is a middle ground for scientific and theological interpretations of complex biological design. In his 1973 commentary entitled "Nothing in Biology Makes Sense Except in the Light of Evolution," Theodosius Dobzhansky famously proclaimed "I am a creationist *and* an evolutionist. Evolution is God's, or Nature's method of creation." Regardless of what our personal philosophical persuasion may be, let us rejoice in biotic complexity and in the scientific efforts to understand its geneses.

Part I

INTRODUCTORY ESSAY

1

Darwin's Greatest Discovery: Design Without Designer

FRANCISCO J. AYALA

Darwin's greatest contribution to science is that he completed the Copernican Revolution by drawing out for biology the notion of nature as a system of matter in motion governed by natural laws. With Darwin's discovery of natural selection, the origin and adaptations of organisms were brought into the realm of science. The adaptive features of organisms could now be explained, like the phenomena of the inanimate world, as the result of natural processes, without recourse to an Intelligent Designer. The Copernican and the Darwinian Revolutions may be seen as the two stages of the one Scientific Revolution. They jointly ushered in the beginning of science in the modern sense of the word: explanation through natural laws. Darwin's theory of natural selection accounts for the "design" of organisms, and for their wondrous diversity, as the result of natural processes, the gradual accumulation of spontaneously arisen variations (mutations) sorted out by natural selection. Which characteristics will be selected depends on which variations happen to be present at a given time in a given place. This in turn depends on the random process of mutation as well as on the previous history of the organisms. Mutation and selection have jointly driven the marvelous process that, starting from microscopic organisms, has yielded orchids, birds, and humans. The theory of evolution conveys chance and

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necessity, randomness and determinism, jointly enmeshed in the stuff of life. This was Darwin's fundamental discovery, that there is a process that is creative, although not conscious.

There is a version of the history of the ideas that sees a parallel between the Copernican and the Darwinian revolutions. In this view, the Copernican Revolution consisted in displacing the Earth from its previously accepted locus as the center of the universe and moving it to a subordinate place as just one more planet revolving around the sun. In congruous manner, the Darwinian Revolution is viewed as consisting of the displacement of humans from their exalted position as the center of life on earth, with all other species created for the service of humankind. According to this version of intellectual history, Copernicus had accomplished his revolution with the heliocentric theory of the solar system. Darwin's achievement emerged from his theory of organic evolution.

What this version of the two revolutions says is correct but inadequate, because it misses what is most important about these two intellectual revolutions, namely that they ushered in the beginning of science in the modern sense of the word. These two revolutions may jointly be seen as the one Scientific Revolution, with two stages, the Copernican and the Darwinian.

The Copernican Revolution was launched with the publication in 1543, the year of Nicolaus Copernicus' death, of his *De revolutionibus orbium celestium* (*On the Revolutions of the Celestial Spheres*), and bloomed with the publication in 1687 of Isaac Newton's *Philosophiae naturalis principia mathematica* (*The Mathematical Principles of Natural Philosophy*). The discoveries by Copernicus, Kepler, Galileo, Newton, and others, in the 16th and 17th centuries, had gradually ushered in a conception of the universe as matter in motion governed by natural laws. It was shown that Earth is not the center of the universe but a small planet rotating around an average star; that the universe is immense in space and in time; and that the motions of the planets around the sun can be explained by the same simple laws that account for the motion of physical objects on our planet, laws such as $f = m \times a$ (force = mass × acceleration) or the inverse-square law of attraction, $f = g(m_1 m_2)/r^2$ (the force of attraction between two bodies is directly proportional to their masses, but inversely related to the square of the distance between them).

These and other discoveries greatly expanded human knowledge. The conceptual revolution they brought about was more fundamental yet: a commitment to the postulate that the universe obeys immanent laws that account for natural phenomena. The workings of the universe were brought into the realm of science: explanation through natural laws. All

physical phenomena could be accounted for as long as the causes were adequately known.

The advances of physical science brought about by the Copernican Revolution had driven mankind's conception of the universe to a split-personality state of affairs, which persisted well into the mid-19th century. Scientific explanations, derived from natural laws, dominated the world of nonliving matter, on the Earth as well as in the heavens. However, supernatural explanations, which depended on the unfathomable deeds of the Creator, were accepted as explanations of the origin and configuration of living creatures. Authors, such as William Paley, argued that the complex design of organisms could not have come about by chance or by the mechanical laws of physics, chemistry, and astronomy but was rather accomplished by an Intelligent Designer, just as the complexity of a watch, designed to tell time, was accomplished by an intelligent watchmaker.

It was Darwin's genius to resolve this conceptual schizophrenia. Darwin completed the Copernican Revolution by drawing out for biology the notion of nature as a lawful system of matter in motion that human reason can explain without recourse to supernatural agencies. The conundrum faced by Darwin can hardly be overestimated. The strength of the argument from design to demonstrate the role of the Creator had been forcefully set forth by philosophers and theologians. Wherever there is function or design, we look for its author. It was Darwin's greatest accomplishment to show that the complex organization and functionality of living beings can be explained as the result of a natural process—natural selection—without any need to resort to a Creator or other external agent. The origin and adaptations of organisms in their profusion and wondrous variations were thus brought into the realm of science.

Darwin accepted that organisms are "designed" for certain purposes, that is, they are functionally organized. Organisms are adapted to certain ways of life and their parts are adapted to perform certain functions. Fish are adapted to live in water, kidneys are designed to regulate the composition of blood, and the human hand is made for grasping. But Darwin went on to provide a natural explanation of the design. The seemingly purposeful aspects of living beings could now be explained, like the phenomena of the inanimate world, by the methods of science, as the result of natural laws manifested in natural processes.

Darwin occupies an exalted place in the history of Western thought, deservedly receiving credit for the theory of evolution. In *The Origin of Species*, published in 1859, he laid out the evidence demonstrating the evolution of organisms. Darwin did not use the term "evolution," which did not have its current meaning, but referred to the evolution of organisms by the phrase "common descent with modification" and similar expres-

sions. However, Darwin accomplished something much more important for intellectual history than demonstrating evolution. Indeed, accumulating evidence for common descent with diversification may very well have been a subsidiary objective of Darwin's masterpiece. Darwin's *Origin of Species* is, first and foremost, a sustained effort to solve the problem of how to account scientifically for the design of organisms. Darwin seeks to explain the design of organisms, their complexity, diversity, and marvelous contrivances, as the result of natural processes. Darwin brings about the evidence for evolution because evolution is a necessary consequence of his theory of design.

INTELLIGENT DESIGN: THE ORIGINAL VERSION

William Paley (1743–1805), one of the most influential English authors of his time, argued forcefully in his *Natural Theology* (1802a) that the complex and precise design of organisms and their parts could be accounted for only as the deed of an Intelligent and Omnipotent "Designer." The design of organisms, he argued, was incontrovertible evidence of the existence of the Creator.

Paley was an English clergyman intensely committed to the abolition of the slave trade. By the 1780s, Paley had become a much sought-after public speaker against slavery. Paley was also an influential writer of works on Christian philosophy, ethics, and theology. *The Principles of Moral and Political Philosophy* (1785) and *A View of the Evidences of Christianity* (1794) earned him prestige and well-endowed ecclesiastical benefices, which allowed him a comfortable life. In 1800, Paley gave up his public speaking career for reasons of health, providing him ample time to study science, particularly biology, and to write *Natural Theology; or, Evidences of the Existence and Attributes of the Deity* (1802a), the book by which he has become best known to posterity and which would greatly influence Darwin. With *Natural Theology*, Paley sought to update the work of another English clergyman, John Ray's *Wisdom of God Manifested in the Works of the Creation* (1691). But Paley could now go much beyond Ray by taking advantage of one century of additional biological knowledge. Paley's keystone claim is that "There cannot be design without a designer; contrivance, without a contriver; order, without choice; . . . means suitable to an end, and executing their office in accomplishing that end, without the end ever having been contemplated" (1802a, pp. 15–16).

Natural Theology is a sustained argument for the existence of God based on the obvious design of humans and their organs, as well as the design of all sorts of organisms, considered by themselves, as well as in their relations to one another and to their environment. The argument has two parts: first, that organisms give evidence of being designed; second,

that only an omnipotent God could account for the perfection, multitude, and diversity of the designs.

There are chapters dedicated to the complex design of the human eye; to the human frame, which displays a precise mechanical arrangement of bones, cartilage, and joints; to the circulation of the blood and the disposition of blood vessels; to the comparative anatomy of humans and animals; to the digestive tract, kidneys, urethras, and bladder; to the wings of birds and the fins of fish; and much more. For 352 pages, *Natural Theology* conveys Paley's expertise: extensive and accurate biological knowledge, as detailed and precise as was available in the year 1802. After detailing the precise organization and exquisite functionality of each biological entity, relationship, or process, Paley draws again and again the same conclusion, that only an omniscient and omnipotent Deity could account for these marvels of mechanical perfection, purpose, and functionality and for the enormous diversity of inventions that they entail.

Paley's first model example in *Natural Theology* is the human eye. Early in chapter 3, Paley points out that the eye and the telescope "are made upon the same principles; both being adjusted to the laws by which the transmission and refraction of rays of light are regulated" (1802a, p. 20). Specifically, there is a precise resemblance between the lenses of a telescope and "the humors of the eye" in their figure, their position, and the ability of converging the rays of light at a precise distance from the lens—on the retina in the case of the eye.

Paley makes two remarkable observations, which enhance the complex and precise design of the eye. The first observation is that rays of light should be refracted by a more convex surface when transmitted through water than when passing out of air into the eye. Accordingly, "the eye of a fish, in that part of it called the crystalline lens, is much rounder than the eye of terrestrial animals. What plainer manifestation of design can there be than this difference? What could a mathematical instrument maker have done more to show his knowledge of this principle . . . ?" (Paley, 1802a, p. 20).

The second remarkable observation made by Paley that supports his argument is dioptric distortion: "Pencils of light, in passing through glass lenses, are separated into different colors, thereby tinging the object, especially the edges of it, as if it were viewed through a prism. To correct this inconvenience has been long a desideratum in the art. At last it came into the mind of a sagacious optician, to inquire how this matter was managed in the eye, in which there was exactly the same difficulty to contend with as in the telescope. His observation taught him that in the eye the evil was cured by combining lenses composed of different substances, that is, of substances which possessed different refracting powers" (Paley, 1802a, pp. 22–23). The telescope maker accordingly corrected the dioptic distor-

tion "by imitating, in glasses made from different materials, the effects of the different humors through which the rays of light pass before they reach the bottom of the eye. Could this be in the eye without purpose, which suggested to the optician the only effectual means of attaining that purpose?" (Paley, 1802a, p. 23).

ARGUMENT AGAINST CHANCE

Paley summarizes his argument by stating the complex functional anatomy of the eye. The eye consists "first, of a series of transparent lenses—very different, by the by, even in their substance, from the opaque materials of which the rest of the body is, in general at least, composed" (Paley, 1802a, p. 48). Second, the eye has the retina, which as Paley points out is the only membrane in the body that is black, spread out behind the lenses, so as to receive the image formed by pencils of light transmitted through them, and "placed at the precise geometrical distance at which, and at which alone, a distinct image could be formed, namely, at the concourse of the refracted rays" (1802a, p. 48). Third, he writes, the eye possesses "a large nerve communicating between this membrane [the retina] and the brain; without which, the action of light upon the membrane, however modified by the organ, would be lost to the purposes of sensation" (1802a, p. 48).

Could the eye have come about without design or preconceived purpose, as a result of chance? Paley had set the argument against chance in the very first paragraph of *Natural Theology* (1802a, p. 1), reasoning rhetorically by analogy: "In crossing a heath, suppose I pitched my foot against a *stone*, and were asked how the stone came to be there, I might possibly answer, that for any thing I knew to the contrary it had lain there for ever; nor would it, perhaps, be very easy to show the absurdity of this answer. But suppose I had found a *watch* upon the ground, and it should be inquired how the watch happened to be in that place, I should hardly think of the answer which I had before given, that for any thing I knew the watch might have always been there. Yet why should not this answer serve for the watch as well as for the stone; why is it not as admissible in the second case as in the first? For this reason, and for no other, namely, that when we come to inspect the watch, we perceive—what we could not discover in the stone—that its several parts are framed and put together for a purpose, e.g., that they are so formed and adjusted as to produce motion, and that motion so regulated as to point out the hour of the day; that if the different parts had been differently shaped from what they are, or placed after any other manner or in any other order than that in which they are placed, either no motion at all would have been carried on in the machine, or none which would have answered the use that is now served

by it.'' In other words, the watch's mechanism is so complicated it could not have arisen by chance.

PALEY'S IRREDUCIBLE COMPLEXITY

The strength of the argument against chance derives, Paley tells us, from what he names ''relation,'' a notion akin to what some contemporary authors have named ''irreducible complexity'' (Behe, 1996). This is how Paley formulates the argument for irreducible complexity: ''When several different parts contribute to one effect, or, which is the same thing, when an effect is produced by the joint action of different instruments, the fitness of such parts or instruments to one another for the purpose of producing, by their united action, the effect, is what I call *relation*; and wherever this is observed in the works of nature or of man, it appears to me to carry along with it decisive evidence of understanding, intention, art'' (Paley, 1802a, pp. 175–176). The outcomes of chance do not exhibit relation among the parts or, as we might say, they do not display organized complexity. He writes that ''a wen, a wart, a mole, a pimple'' could come about by chance, but never an eye; ''a clod, a pebble, a liquid drop might be,'' but never a watch or a telescope.

Paley notices the ''relation'' not only among the component parts of an organ, such as the eye, the kidney, or the bladder, but also among the different parts, limbs, and organs that collectively make up an animal and adapt it to its distinctive way of life: ''In the *swan*, the web-foot, the spoon bill, the long neck, the thick down, the graminivorous stomach, bear all a relation to one another. . . . The feet of the mole are made for digging; the neck, nose, eyes, ears, and skin, are peculiarly adapted to an under-ground life. [In a word,] this is what I call relation'' (Paley, 1802a, pp. 180, 183).

Throughout *Natural Theology*, Paley displays extensive and profound biological knowledge. He discusses the fish's air bladder, the viper's fang, the heron's claw, the camel's stomach, the woodpecker's tongue, the elephant's proboscis, the bat's wing hook, the spider's web, insects' compound eyes and metamorphosis, the glowworm, univalve and bivalve mollusks, seed dispersal, and on and on, with accuracy and as much detail as known to the best biologists of his time. The organized complexity and purposeful function reveal, in each case, an intelligent designer, and the diversity, richness, and pervasiveness of the designs show that only the omnipotent Creator could be this Intelligent Designer.

Paley was not the only proponent of the argument from design in the first half of the 19th century. In Britain, a few years after the publication of *Natural Theology*, the eighth Earl of Bridgewater endowed the publication of treatises that would set forth ''the Power, Wisdom and Goodness of God as manifested in the Creation.'' Eight treatises were published dur-

ing 1833–1840, several of which artfully incorporate the best science of the time and had considerable influence on the public and among scientists. One of the treatises, *The Hand, Its Mechanisms and Vital Endowments as Evincing Design* (1833), was written by Sir Charles Bell, a distinguished anatomist and surgeon, famous for his neurological discoveries, who became professor of surgery in 1836 at the University of Edinburgh. Bell follows Paley's manner of argument, examining in considerable detail the wondrously useful design of the human hand but also the perfection of design of the forelimb used for different purposes in different animals, serving in each case the particular needs and habits of its owner: the human's arm for handling objects, the dog's leg for running, and the bird's wing for flying. "Nothing less than the Power, which originally created, is equal to the effecting of those changes on animals, which are to adapt them to their conditions."

Paley and Bell are typical representatives of the intellectual milieu prevailing in the first half of the 19th century in Britain as well as on the Continent. Darwin, while he was an undergraduate student at the University of Cambridge between 1827 and 1831, read Paley's *Natural Theology*, which was part of the university's canon for nearly half a century after Paley's death. Darwin writes in his *Autobiography* of the "much delight" and profit that he derived from reading Paley: "To pass the B.A. examination, it was also necessary to get up Paley's *Evidences of Christianity*, and his *Moral Philosophy*. . . . The logic of . . . his *Natural Theology* gave me as much delight as did Euclid. . . . I did not at that time trouble myself about Paley's premises; and taking these on trust, I was charmed and convinced by the long line of argumentation" (Darwin, 1887a).

Later, however, after he returned from his 5-year voyage around the world in the *HMS Beagle*, Darwin would discover a scientific explanation for the design of organisms. Science, thereby, made a quantum leap.

DARWIN'S "MY THEORY"

Darwin considered natural selection, rather than his demonstration of evolution, his most important discovery and designated it as "my theory," a designation he never used when referring to the evolution of organisms. The discovery of natural selection, Darwin's awareness that it was a greatly significant discovery because it was science's answer to Paley's argument from design, and Darwin's designation of natural selection as "my theory" can be traced in Darwin's "Red Notebook" and "Transmutation Notebooks B to E," which he started in March 1837, not long after returning (on October 2, 1836) from his 5-year voyage on the *Beagle*, and completed in late 1839 (see Eldredge, 2005).

The evolution of organisms was commonly accepted by naturalists in the middle decades of the 19th century. The distribution of exotic species in South America, in the Galápagos Islands, and elsewhere and the discovery of fossil remains of long-extinguished animals confirmed the reality of evolution in Darwin's mind. The intellectual challenge was to explain the origin of distinct species of organisms, how new ones adapted to their environments, that "mystery of mysteries," as it had been labeled by Darwin's older contemporary, the prominent scientist and philosopher Sir John Herschel (1792–1871).

Early in the Notebooks of 1837 to 1839, Darwin registers his discovery of natural selection and repeatedly refers to it as "my theory." From then until his death in 1882, Darwin's life would be dedicated to substantiating natural selection and its companion postulates, mainly the pervasiveness of hereditary variation and the enormous fertility of organisms, which much surpassed the capacity of available resources. Natural selection became for Darwin "a theory by which to work." He relentlessly pursued observations and performed experiments to test the theory and resolve presumptive objections.

WALLACE: A DISTINCTION WITH A DIFFERENCE

Alfred Russel Wallace (1823–1913) is famously given credit for discovering, independently of Darwin, natural selection as the process accounting for the evolution of species. On June 18, 1858, Darwin wrote to Charles Lyell that he had received by mail a short essay from Wallace such that "if Wallace had my [manuscript] sketch written in [1844] he could not have made a better abstract." Darwin was thunderstruck.

Darwin and Wallace had started occasional correspondence in late 1855. At the time Wallace was in the Malay Archipelago collecting biological specimens. In his letters, Darwin would offer sympathy and encouragement to the occasionally dispirited Wallace for his "laborious undertaking." In 1858, Wallace came upon the idea of natural selection as the explanation for evolutionary change and he wanted to know Darwin's opinion about this hypothesis, because Wallace, as well as many others, knew that Darwin had been working on the subject for years, had shared his ideas with other scientists, and was considered by them as the eminent expert on issues concerning biological evolution.

Darwin was uncertain how to proceed about Wallace's letter. He wanted to credit Wallace's discovery of natural selection, but he did not want altogether to give up his own earlier independent discovery. Eventually, Sir Charles Lyell and Joseph Hooker proposed, with Darwin's consent, that Wallace's letter and two of Darwin's earlier writings would be presented at a meeting of the Linnean Society of London. On July 1, 1858,

three papers were read by the society's undersecretary, George Busk, in the order of their date of composition: Darwin's abbreviated abstract of his 230-page essay from 1844; an "abstract of abstract" that Darwin had written to the American botanist Asa Gray on September 5, 1857; and Wallace's essay, "On the Tendency of Varieties to Depart Indefinitely from Original Type; Instability of Varieties Supposed to Prove the Permanent Distinctness of Species" (Wallace, 1858).

The meeting was attended by some 30 people, who did not include Darwin or Wallace. The papers generated little response and virtually no discussion, their significance apparently lost to those in attendance. Nor was it noticed by the president of the Linnean Society, Thomas Bell, who, in his annual address the following May, blandly stated that the past year had not been enlivened by "any of those striking discoveries which at once revolutionize" a branch of science.

Wallace's independent discovery of natural selection is remarkable. But the lesser credit given to Wallace than to Darwin for this discovery may not be misplaced. Wallace was not interested in explaining design but rather in accounting for the evolution of species, as indicated in his paper's title: "On the Tendency of Varieties to Depart Indefinitely from the Original Type." Wallace thought that evolution proceeds indefinitely and is progressive. Wallace (1858) writes: "We believe that there is a tendency in nature to the continued progression of certain classes of varieties further and further from the original type—a progression to which there appears no reason to assign any definite limits. This progression, by minute steps, in various directions . . . ."

Darwin, on the contrary, did not accept that evolution would necessarily represent progress or advancement, nor did he believe that evolution would always result in morphological change over time; rather, he knew of the existence of "living fossils," organisms that had remained unchanged for millions of years. For example, "some of the most ancient Silurian animals, as the Nautilus, Lingula, etc., do not differ much from living species" (Darwin, 1859b, p. 306). In 1858, Darwin was at work on a multivolume treatise, intended to be titled "On Natural Selection." Wallace's paper stimulated Darwin to write *The Origin*, which would be published the following year. Darwin saw *The Origin* as an abbreviated version of the much longer book he had planned to write.

DARWIN'S EXPLANATION OF DESIGN

Darwin's focus in *The Origin* was the explanation of design, with evolution playing the subsidiary role of supporting evidence. The Introduction and chapters I–VIII of *The Origin* explain how natural selection accounts for the adaptations and behaviors of organisms, their "design."

The extended argument starts in chapter I, where Darwin describes the successful selection of domestic plants and animals and, with considerable detail, the success of pigeon fanciers seeking exotic "sports." The success of plant and animal breeders manifests how much selection can accomplish by taking advantage of spontaneous variations that occur in organisms but happen to fit the breeders' objectives. A sport (mutation) that first appears in an individual can be multiplied by selective breeding so that after a few generations, that sport becomes fixed in a breed, or "race." The familiar breeds of dogs, cattle, chickens, and food plants have been obtained by this process of selection practiced by people with particular objectives.

The ensuing chapters (II–VIII) of *The Origin* extend the argument to variations propagated by natural selection for the benefit of the organisms themselves rather than by artificial selection of traits desired by humans. As a consequence of natural selection, organisms exhibit design, that is, exhibit adaptive organs and functions. The design of organisms as they exist in nature, however, is not "intelligent design," imposed by God as a Supreme Engineer or by humans; rather, it is the result of a natural process of selection, promoting the adaptation of organisms to their environments. This is how natural selection works: Individuals that have beneficial variations, that is, variations that improve their probability of survival and reproduction, leave more descendants than individuals of the same species that have less beneficial variations. The beneficial variations will consequently increase in frequency over the generations; less beneficial or harmful variations will be eliminated from the species. Eventually, all individuals of the species will have the beneficial features; new features will arise over eons of time.

Organisms exhibit complex design, but it is not, in current language, "irreducible complexity," emerging all of a sudden in full bloom. Rather, according to Darwin's theory of natural selection, the design has arisen gradually and cumulatively, step by step, promoted by the reproductive success of individuals with incrementally more adaptive elaborations.

It follows from Darwin's explanation of adaptation that evolution must necessarily occur as a consequence of organisms becoming adapted to different environments in different localities and to the ever-changing conditions of the environment over time, and as hereditary variations become available at a particular time that improve, in that place and at that time, the organisms' chances of survival and reproduction. *The Origin*'s evidence for biological evolution is central to Darwin's explanation of design, because this explanation implies that biological evolution occurs, which Darwin therefore seeks to demonstrate in most of the remainder of the book (Darwin, 1859b, chapters IX–XIII).

In the concluding chapter XIV of *The Origin*, Darwin returns to the dominant theme of adaptation and design. In an eloquent final paragraph, Darwin asserts the "grandeur" of his vision:

> It is interesting to contemplate an entangled bank, clothed with many plants of many kinds, with birds singing on the bushes, with various insects flitting about, and with worms crawling through the damp earth, and to reflect that these *elaborately constructed* forms, *so different* from each other, and dependent on each other *in so complex a manner*, have all been produced by laws acting around us. . . . Thus, from the war of nature, from famine and death, the most exalted object which we are capable of conceiving, namely, the production of the higher animals, directly follows. There is grandeur in this view of life, with its several powers, having been originally breathed into a few forms or into one; and that, whilst this planet has gone cycling on according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved (Darwin, 1859b, pp. 489–490; emphasis added).

Darwin's *Origin* addresses the same issue as Paley: how to account for the adaptive configuration of organisms and their parts, which are so obviously designed to fulfill certain functions. Darwin argues that hereditary adaptive variations ("variations useful in some way to each being") occasionally appear, and that these are likely to increase the reproductive chances of their carriers. The success of pigeon fanciers and animal breeders clearly shows the occasional occurrence of useful hereditary variations. In nature, over the generations, Darwin's argument continues, favorable variations will be preserved, multiplied, and conjoined; injurious ones will be eliminated. In one place, Darwin avers: "I can see no limit to this power [natural selection] in slowly and beautifully *adapting* each form to the most complex relations of life" (Darwin, 1859b, p. 469).

In his *Autobiography*, Darwin wrote, "The old argument of design in nature, as given by Paley, which formerly seemed to me so conclusive, falls, now that the law of natural selection has been discovered. We can no longer argue that, for instance, the beautiful hinge of a bivalve shell must have been made by an intelligent being, like the hinge of a door by a man" (Barlow, 1958).

Natural selection was proposed by Darwin primarily to account for the adaptive organization, or design, of living beings; it is a process that preserves and promotes adaptation. Evolutionary change through time and evolutionary diversification (multiplication of species) often ensue as by-products of natural selection fostering the adaptation of organisms to their milieu. Evolutionary change is not directly promoted by natural selection, however, and therefore it is not its necessary consequence.

Indeed, some species may remain unchanged for long periods of time, as Darwin noted. Nautilus, Lingula, and other so-called "living fossils" are Darwin's examples of organisms that have remained unchanged in their appearance for millions of years.

MUTATION AND NATURAL SELECTION

Evolution affects all aspects of an organism's life: morphology (form and structure), physiology (function), behavior, and ecology (interaction with the environment). Underlying these changes are changes in the hereditary materials. Hence, in genetic terms, evolution consists of changes in the organisms' hereditary makeup.

Evolution can be seen as a two-step process. First, hereditary variation arises by mutation; second, selection occurs by which useful variations increase in frequency and those that are less useful or injurious are eliminated over the generations. "Useful" and "injurious" are terms used by Darwin in his definition of natural selection. The significant point is that individuals having useful variations "would have the best chance of surviving and procreating their kind" (Darwin, 1859b, p. 81). As a consequence, useful variations increase in frequency over the generations, at the expense of those that are less useful or injurious.

The process of mutation provides each generation with many new genetic variations, in addition to those carried over from previous generations. Thus, it is not surprising to see that, when new environmental challenges arise, species are able to adapt to them. More than 200 insect and rodent species, for example, developed resistance to DDT, Warfarin, and other pesticides in places where spraying was intense. Although these animals had never before encountered these synthetic compounds, mutations allowed some individuals to survive in their presence. These individuals reproduced and, thus, the mutations providing resistance increased in frequency over the generations, so that eventually the population was no longer susceptible to the pesticide. The adaptation had come about by the combined processes of mutation and natural selection.

The resistance of disease-causing bacteria and parasites to antibiotics and other drugs is a consequence of the same process. When an individual receives an antibiotic that specifically kills the bacteria causing a disease—say, tuberculosis—the immense majority of the bacteria die, but one in several million may have a mutation that provides resistance to the antibiotic. These resistant bacteria survive, multiply, and spread from individual to individual. Eventually, the antibiotic no longer cures the disease in most or all people because the bacteria are resistant. This is why modern medicine treats bacterial diseases with cocktails of antibiotics. If the incidence of a mutation conferring resistance to a given antibiotic is

one in a million, the probability of one bacterium carrying three mutations, each conferring resistance to one of three antibiotics, is one in a quintillion (one in a million million million). Even at the peak of infection, when billions or trillions of bacteria exist in a sick person, it is not likely, if not altogether impossible, that any bacteria resistant to all three antibiotics will occur in any infected individual.

Natural selection is much more than a "purifying" process, for it is able to generate novelty by increasing the probability of otherwise extremely improbable genetic combinations. Natural selection in combination with mutation becomes, in this respect, a creative process. Moreover, it is a process that has been occurring for many millions of years in many different evolutionary lineages and a multitude of species, each consisting of a large number of individuals. Evolution by mutation and natural selection has produced the enormous diversity of the living world with its wondrous adaptations.

Several hundred million generations separate modern animals from the early animals of the Cambrian geological period (542 million years ago). The number of mutations that can be tested, and those eventually selected, in millions of individual animals over millions of generations is difficult for a human mind to fathom, but we can readily understand that the accumulation of millions of small, functionally advantageous changes could yield remarkably complex and adaptive organs, such as the eye.

Natural selection is an incremental process, operating over time and yielding organisms better able to survive and reproduce than others. Individuals of a given species differ from one another at any one time only in small ways; for example, the difference between bacteria that have or lack an enzyme able to synthesize the sugar lactose or between moths that have light or dark wings. These differences typically involve one or only a few genes, but they can make the difference between survival or death, as in the resistance to DDT or to antibiotics. Consider a different sort of example. Some pocket mice (*Chaetodipus intermedius*) live in rocky outcrops in Arizona. Light, sandy-colored mice are found in light-colored habitats, whereas dark (melanic) mice prevail in dark rocks formed from ancient flows of basaltic lava. The match between background and fur color protects the mice from avian and mammal predators that hunt guided largely by vision. Mutations in one single gene (coding for the melanocortin-1-receptor, represented as *MC1R*) account for the difference between light and dark pelage (Nachman *et al.,* 2003).

Adaptations that involve complex structures, functions, or behaviors involve numerous genes. Many familiar mammals, but not marsupials, have a placenta. Marsupials include the familiar kangaroo and other mammals native primarily to Australia and South America. Dogs, cats, mice, donkeys, and primates are placental. The placenta makes it pos-

sible to extend the time the developing embryo is kept inside the mother and thus make the newborn better prepared for independent survival. However, the placenta requires complex adaptations, such as the suppression of harmful immune interactions between mother and embryo, delivery of suitable nutrients and oxygen to the embryo, and the disposal of embryonic wastes. The mammalian placenta evolved more than 100 million years ago and proved a successful adaptation, contributing to the explosive diversification of placental mammals in the Old World and North America.

The placenta also has evolved in some fish groups, such as *Poeciliopsis*. Some *Poeciliopsis* species hatch eggs. The females supply the yolk in the egg, which furnishes nutrients to the developing embryo (as in chicken). Other *Poeciliopsis* species, however, have evolved a placenta through which the mother provides nutrients to the developing embryo. Molecular biology has made possible the reconstruction of the evolutionary history of *Poeciliopsis* species. A surprising result is that the placenta evolved independently three times in this fish group. The required complex adaptations accumulated in each case in <750,000 years (Reznick *et al.*, 2002; Avise, 2006).

Natural selection produces combinations of genes that would seem highly improbable because natural selection proceeds stepwise over long periods of time. Consider the evolution of the eye in humans and other vertebrates. Perception of light, and later vision, were important for the survival and reproductive success of their ancestors, because sunlight is a predominant feature of the environment. Accordingly, natural selection favored genes and gene combinations that increased the functional efficiency of the eye. Such mutations gradually accumulated, eventually leading to the highly complex and efficient vertebrate eye.

How complex organs, such as the human eye, may arise stepwise from a very simple structure can be observed in living mollusks (Fig. 1.1). The mollusks (squids, clams, and snails) are a very ancient group of organisms, older than the vertebrates. Marine organisms have variable visual needs, depending on their lifestyle. Limpets have the simplest imaginable eye; just an eye spot consisting of a few pigmented cells with nerve fibers attached to them. Slit-shell mollusks have a slightly more advanced organ, consisting of some pigmented cells shaped as a cup, which allow these mollusks some perception of the direction of light. *Nautilus*, a group of open ocean mollusks that have remained virtually unchanged for millions of years, have an extended and nearly closed cup, with a pinhole opening but without a lens. *Murex*, a group of marine snails, have eyes with a primitive refractive lens protected by a layer of skin cells serving as cornea. Octopuses and squids have eyes just as complex as the human eye, with cornea, iris, refractive lens, retina, vitreous internal substance, optic nerve, and muscle.

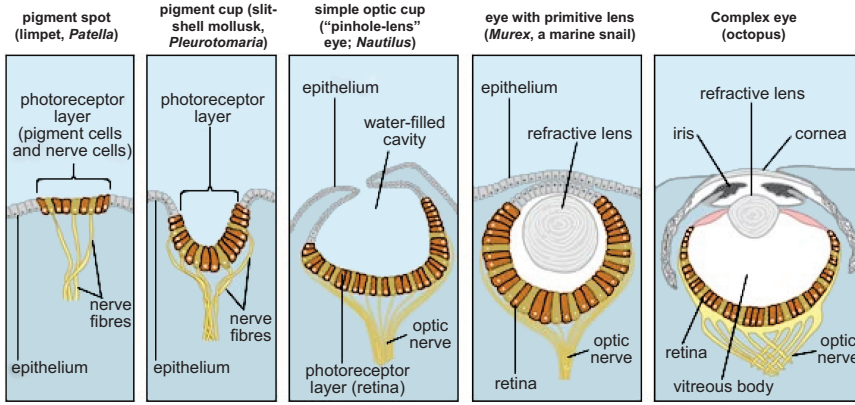

FIGURE 1.1 Steps in the evolution of eye complexity in living mollusks. The simplest eye is found in limpets (far left), consisting of only a few pigmented cells, slightly modified from typical epithelial (skin) cells. Slit-shell mollusks (second from the left) have a slightly more advanced organ, consisting of some pigmented cells shaped as a cup. The octopus eye (far right) is quite complex, with components similar to those of the human eye such as cornea, iris, refractive lens, and retina. (Adapted from "Evolution, The Theory of." By courtesy of Encyclopaedia Britannica, Inc.)

DESIGN WITHOUT DESIGNER

Natural selection sorting out spontaneously arising mutations is a creative process because it causes favorable mutations to combine and accumulate, yielding a great diversity of organisms over eons of time. But there are important features that distinguish the kind of "design" achieved by natural selection, namely the adaptations of organisms, from the kind of design produced by an intelligent designer, an engineer.

An engineer has a preconception of what the design is supposed to achieve and will select suitable materials and arrange them in a preconceived manner so that it fulfills the intended function. On the contrary, natural selection does not operate according to some preordained plan. It is a purely natural process resulting from the interacting properties of physicochemical and biological entities. Natural selection is simply a consequence of the differential survival and reproduction of living beings. It has some appearance of purposefulness because it is conditioned by the environment: which organisms survive and reproduce more effectively depends on which variations they happen to possess that are useful or beneficial to them in the place and at the time where they live.

Natural selection does not have foresight; it does not anticipate the environments of the future. Drastic environmental changes may introduce obstacles that are insuperable to organisms that were previously thriving. In fact, species extinction is a common outcome of the evolutionary process. The species existing today represent the balance between the origin of new species and their eventual extinction. The available inventory of living species describes nearly 2 million species, although at least 10 million are estimated to exist. But we know that perhaps more than 99% of all species that have ever lived on Earth have become extinct.

Increased complexity is not a necessary consequence of natural selection, but it does emerge occasionally, when mutations that increase complexity are favored over mutations that do not. That complexity-increasing mutations do not necessarily accumulate over time is apparent in many evolutionary lineages. For example, the longest living organisms on Earth are the microscopic bacteria, which have existed continuously on our planet for ≈3.5 billion years. Yet, modern bacterial species appear to exhibit no greater complexity than their ancient ancestors. More complex organisms came about much later, without the elimination of their simpler relatives. Nevertheless, over the eons, multitudes of complex organisms have arisen on Earth. Some groups of complex organisms came into existence only recently (on the evolutionary scale). The primates appeared on Earth only 50 million years ago; our species, *Homo sapiens*, less than 200,000 years ago.

In evolution, there is no entity or person who is selecting adaptive combinations. These combinations select themselves because the organisms possessing them reproduce more effectively than those with less adaptive variations. Therefore, natural selection does not strive to produce predetermined kinds of organisms but only organisms that are adapted to their present environments. As pointed out, which characteristics will be selected depends on which variations happen to be present at a given time in a given place. This, in turn, depends on the random process of mutation as well as on the previous history of the organisms (that is, on the genetic makeup they have as a consequence of their previous evolution). Natural selection is an opportunistic process. The variables determining the direction in which natural selection will proceed are the environment, the preexisting constitution of the organisms, and the randomly arising mutations.

Thus, adaptation to a given habitat may occur in a variety of different ways. For example, many plants have adapted to a desert climate. Their fundamental adaptation is to the condition of dryness, which holds the danger of desiccation. During most of the year, and sometimes for several years in succession, there is no rain. Plants have adapted to the scarcity of water in different ways. Cacti have transformed their leaves into spines

and thus avoid the evaporation that occurs in the leaves; photosynthesis is performed on the surface of the stem instead. In addition, their stems have evolved into barrel-like structures that store a reserve of water. A second mode of adaptation occurs in desert plants that have no leaves during the dry season, but after it rains, they burst into leaves and flowers and quickly produce seeds. A third mode of adaptation is that of desert ephemeral plants, which germinate from seeds, grow, flower, and produce seeds, all within the few weeks of the year when rainwater is available; at other times, the seeds lie quiescent in the soil.

CHANCE AND NECESSITY: NATURAL SELECTION AS A CREATIVE PROCESS

The fossil record shows that life has evolved in a haphazard fashion. The radiations of some groups of organisms, the numerical and territorial expansions of other groups, the replacement of some kinds of organisms by other kinds, the occasional but irregular occurrence of trends toward increased size or other sorts of change, and the ever-present extinctions are best explained by natural selection of organisms subject to the vagaries of genetic mutation, environmental challenge, and past history. The scientific account of these events does not necessitate recourse to a preordained plan, whether imprinted from the beginning or through successive interventions by an omniscient and almighty Designer. Biological evolution differs from a painting or an artifact in that it is not the outcome of preconceived design. The design of organisms is not intelligent but imperfect and, at times, outright dysfunctional.

Natural selection accounts for the "design" of organisms because adaptive variations tend to increase the probability of survival and reproduction of their carriers at the expense of maladaptive, or less adaptive, variations. The arguments of intelligent design proponents that state the incredible improbability of chance events, such as mutation, to account for the adaptations of organisms are irrelevant because evolution is not governed by random mutations. Rather, there is a natural process (namely, natural selection) that is not random but oriented and able to generate order or "create." The traits that organisms acquire in their evolutionary histories are not fortuitous but rather determined by their functional utility to the organisms, designed, as it were, to serve their life needs.

Chance is, nevertheless, an integral part of the evolutionary process. The mutations that yield the hereditary variations available to natural selection arise at random. Mutations are random or chance events because (*i*) they are rare exceptions to the fidelity of the process of DNA replication and because (*ii*) there is no way of knowing which gene will mutate in a particular cell or in a particular individual. However, the meaning

of "random" that is most significant for understanding the evolutionary process is (*iii*) that mutations are unoriented with respect to adaptation; they occur independently of whether or not they are beneficial or harmful to the organisms. Some are beneficial, most are not, and only the beneficial ones become incorporated in the organisms through natural selection.

The adaptive randomness of the mutation process (as well as the vagaries of other processes that come to play in the great theater of life) is counteracted by natural selection, which preserves what is useful and eliminates what is harmful. Without hereditary mutations, evolution could not happen because there would be no variations that could be differentially conveyed from one to another generation. But without natural selection, the mutation process would yield disorganization and extinction because most mutations are disadvantageous. Mutation and selection have jointly driven the marvelous process that, starting from microscopic organisms, has yielded orchids, birds, and humans.

The theory of evolution conveys chance and necessity jointly enmeshed in the stuff of life; randomness and determinism interlocked in a natural process that has spurted the most complex, diverse, and beautiful entities that we know of in the universe: the organisms that populate the Earth, including humans who think and love, endowed with free will and creative powers, and able to analyze the process of evolution itself that brought them into existence. This is Darwin's fundamental discovery, that there is a process that is creative although not conscious. And this is the conceptual revolution that Darwin completed: the idea that the design of living organisms can be accounted for as the result of natural processes governed by natural laws. This is nothing if not a fundamental vision that has forever changed how mankind perceives itself and its place in the universe.

Part II

EPISTEMOLOGICAL APPROACHES TO BIOCOMPLEXITY ASSESSMENT

The sphere of biological phenomena interpretable in the light of evolution is vast, so perhaps it is not surprising that researchers from many different scientific backgrounds and orientations have weighed in on how best to approach the study of complex adaptations. The chapters in Part II will illustrate some of this diversity.

In Chapter 2, Robert Hazen, Patrick Griffin, James Carothers, and Jack Szostak raise two important related questions: What actually is meant by biological "complexity" and how might complexity be quantified? The authors suggest that a hallmark of any complex system (physical or biological) is its potential to perform a quantifiable operation. Starting with that premise, they formally define a metric—functional information—that basically describes the fraction of all possible configurations of the system that possess a specified degree of function. Although this metric may be difficult to apply in the real world (because it requires knowledge of all possible configurations and the degree of function of each), it nonetheless may have heuristic merit for studying the properties of complex systems. The authors illustrate this approach using their virtual world of computer programs that self-replicate, mutate, and adapt by natural selection.

In 1975, Mary-Claire King and Allan Wilson popularized an earlier idea by Roy Britten and Eric Davidson (1969) that evolutionary changes in gene regulation—rather than DNA sequence mutations in protein-coding exons *per se*—were largely responsible for phenotypic evolution and the emergence of complex adaptations. This sentiment has since become mainstream, as reflected in several papers in the current volume.

In Chapter 3, John Gerhart and Marc Kirschner accept the notion that regulatory changes are of central importance, and indeed they argue that most key phenotypic evolution over the past 600 million years has resulted from altered usage patterns in a large set of otherwise conserved core genetic components that direct organismal development and physiology. In the "theory of facilitated variation" by Gerhart and Kirschner, several regulatory features of the genome collude to foster more phenotypic evolution with less genetic change than would otherwise have been possible.

In Chapter 4, Adam Wilkins examines the converse of evolutionary plasticity: phenotypic constraint. It has long been evident that phylogenetic legacies and developmental contingencies restrict (albeit to a debatable degree) the suite of evolutionary pathways potentially available to any species. Wilkins proposes that in addition to these conventionally recognized inhibitors of phenotypic evolution, inherent constraints also operate at the levels of interacting genes and complex genetic networks. If molecular biologists can illuminate the genetic biases that constrain as well as promote the evolution of particular phenotypes, it might become possible, Wilkins argues, to specify the relative probabilities of alternative evolutionary trajectories (at least over the short term) for particular lineages. Traditionally, this kind of predictability about evolutionary futures had been regarded as essentially impossible.

In the final chapter of Part II, Michael Lynch reminds us that mechanistic explanations of phenotypic evolution that emerge from the fields of developmental biology and molecular genetics cannot violate the fundamental dynamics of the evolutionary process as elucidated by a century of work in theoretical population genetics. Regardless of which genes underlie complex or other phenotypes, their microevolutionary dynamics remain governed by the forces of mutation, gene flow, natural selection, recombination, and random genetic drift. The point, however, is not to claim priority for one discipline over another, but rather to emphasize that any evolutionary model that disregards population genetic reality does so at its peril. To illustrate his argument, Lynch examines the ineluctable consequences of genetic drift, especially in small populations, and he highlights a wide assortment of genic and genomic phenomena that make sense only after accounting for variation among taxa in the relative power of nonadaptive evolutionary forces.

2

Functional Information and the Emergence of Biocomplexity

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Complex emergent systems of many interacting components, including complex biological systems, have the potential to perform quantifiable functions. Accordingly, we define "functional information," $I(E_x)$, as a measure of system complexity. For a given system and function, x (e.g., a folded RNA sequence that binds to GTP), and degree of function, E_x (e.g., the RNA–GTP binding energy), $I(E_x) = -\log_2[F(E_x)]$, where $F(E_x)$ is the fraction of all possible configurations of the system that possess a degree of function $> E_x$. Functional information, which we illustrate with letter sequences, artificial life, and biopolymers, thus represents the probability that an arbitrary configuration of a system will achieve a specific function to a specified degree. In each case we observe evidence for several distinct solutions with different maximum degrees of function, features that lead to steps in plots of information versus degree of function.

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Complex emergent systems, in which interactions among numerous components or "agents" produce patterns or behaviors not obtainable by individual components, are ubiquitous at every scale of the physical universe, for example in neural networks (Deamer and Evans, 2006), turbulent fluids (Frisch, 1995), insect colonies (Camazine *et al.*, 2001), and spiral galaxies (Carlberg, 1992). Complex systems also appear in a range of artificial symbolic contexts, including genetic algorithms (Mitchell, 1996), cellular automata (Wolfram, 2002), artificial life (Adami, 1995), and models of market economies (Holland, 1995).

Life, with its novel collective behaviors at the scale of molecules, genes, cells, and organisms, is the quintessential emergent complex system. Furthermore, the ancient transition from a geochemical world to a living planet may be modeled as a sequence of emergent events, each of which increased the chemical complexity of the prebiotic world (De Duve, 1995; Morowitz, 2002; Hazen, 2005).

Given this ubiquity and diversity, it is desirable to understand the characteristics of emergent complex systems, as well as the factors that might promote complexity in evolving systems. However, complexity has proven difficult to define or measure with precision (Gell-Mann, 1995; Adami, 2003; Shalizi, 2006). A central objective of this study, therefore, is to examine "functional information" (Szostak, 2003) as a quantitative measure of complexity that may be applicable to the analysis and prediction of attributes of a wide range of phenomena in physical and symbolic systems, including evolving biological systems.

An extensive literature explores historical developments and recent advances in the study of complexity and information (Kåhre, 2002; Gell-Mann and Lloyd, 2003; Von Baeyer, 2003; Shalizi, 2006) as well as their application to understanding biological systems (Morowitz, 1978; Bell, 1997; Allen *et al.*, 1998; Solé and Goodwin, 2000; Camazine *et al.*, 2001; Adami, 2003; Avery, 2003; Ricard, 2003). Despite this rich literature, previous discussions of complexity have not generally focused on the relationship between information content and function (Lehman *et al.*, 2000). We propose to measure the complexity of a system in terms of functional information, the information required to encode a specific function.

SYSTEMS AND THEIR FUNCTIONS

In this chapter we consider the functional information of both symbolic systems (letter sequences and Avida artificial life genomes) and biopolymers (RNA aptamers). These systems share several characteristics: first, they consist of numerous individual components or "agents"; second, the agents can combine in a combinatorially large number of different configurations; and third, some configurations display functions that

are not characteristic of the individual agents. Analyses of these systems address fundamental questions about the relationship between information content and function. For example, How much information does it take to encode a function? Are there multiple distinct solutions? How are solutions distributed in configuration space? How much more information does it take to encode a given improvement in function? What environmental factors might influence these relationships?

The function of some emergent systems is obvious: a sequence of letters communicates a specific idea, a computer algorithm performs a specific computation, and an enzyme catalyzes at least one specific reaction. Less obvious are the functions of systems of many interacting inanimate particles, such as molecules, sand grains, or stars, but these systems may also be described quantitatively in terms of function, for example, in terms of their ability to dissipate energy or to maximize entropy production through patterning (e.g., Bertalanffy, 1968; Nicolis and Prigogine, 1977; Swensen and Turvey, 1991; Emanuel, 2006). Living systems, by contrast, typically display multiple essential functions (Allen *et al.,* 1998; Ayala, 1999; McShea, 2000). This consideration of complexity in terms of the function of a system, as opposed to some intrinsic measure of its patterning or structural intricacy, distinguishes our treatment from many previous efforts.

QUANTIFYING COMPLEXITY

Development of a quantitative measure of complexity has proven difficult for at least three reasons, each of which relates to the diversity of systems that may be labeled ''complex.''

1. Systems may be complex in terms of information content, physical structure, and/or behavior. Consider three stages in the life cycle of a multicellular organism: a fertilized egg, a live adult, and a postmortem adult. All three states are complex, but they are complex in different respects. All three states possess the sequence information (a genome) necessary to grow a living organism. Living and dead adult organisms also display complex anatomical structures, but only living organisms possess behavioral complexity. Any universal definition of complexity must thus have the potential to quantify complexity independently in terms of information, structure, or behavior.

2. It has been difficult to define complexity in terms of a metric that applies to all complex systems. No obvious common thread exists in comparing the complexity of symbolic systems, such as language, with those of physical agents, such as cells. Parameters useful in characterizing symbolic systems (e.g., algorithm- or information-based complexity metrics)

generally differ from those used to analyze systems of interacting particles (e.g., Newtonian dynamics or maximum entropy models). Gell-Mann (1995) concludes, "A variety of different measures would be required to capture all our intuitive ideas about what is meant by complexity."

3. Complex emergent systems are diverse in terms of their dimensionality. Sequences of letters, computer code, or bipolymers can be treated as one-dimensional strings of symbolic information (or as points in a high-dimensional sequence space). On the other hand, many physical emergent systems, including those composed of many interacting sand grains, cells, organisms, or stars, exhibit time-dependent behaviors in two or three spatial dimensions. It is desirable for a complexity formalism to apply to this range of dimensionalities.

Despite this diversity, a common thread is present: All complex systems alter their environments in one or more ways, which we refer to as functions (Bigelow and Pargetter, 1998). In the words of von Baeyer (2003), "Information gathering by itself, without observable effects on the gatherer's behavior, is a pointless pursuit." Function is thus the essence of complex systems. Accordingly, we focus on function in our operational definition of complexity. Therefore, although many previous investigators have explored aspects of biological systems in terms of information (e.g., Schneider *et al.*, 1986), we adopt a different approach and explore information in terms of the function of a system (including biological systems).

Szostak and coworkers (Szostak, 2003; Carothers *et al.*, 2004) introduced "functional information" as a measure of complexity. They proposed that the complexity of an information-rich system, such as RNA aptamers (RNA structures that bind a target molecule), can be quantified in the context of specific functions of the system, in contrast to prior formalisms based on genomic, sequence, or algorithmic information (e.g., Lenski *et al.*, 1999; Adami, 2003). Here we examine applications of this formalism to letter sequences, the artificial life platform Avida (Adami, 1998), and RNA aptamers.

FUNCTIONAL INFORMATION AS A MEASURE OF SYSTEM COMPLEXITY

Many emergent systems of interacting agents can be described in terms of their potential to accomplish one or more quantifiable tasks. Consider a system that can exist in a combinatorially large number of different configurations (i.e., a 100-nt RNA strand comprised of four different nucleotides, A, U, G, and C, with 4^{100} different possible sequences). Assume that a small fraction of these configurations accomplishes a specified function x to a high degree (corresponding to a high information

content). Typically, a significantly greater number of configurations will prove somewhat less efficient in accomplishing function x (corresponding to lower information content), whereas the majority of configurations will display little or no function (Lenski *et al.*, 2003; Carothers *et al.*, 2004).

Accordingly, "degree of function x" (E_x) is a measure of a configuration's ability to perform the function x. For example, in an enzymatic system E_x might be defined as the increase in a specific reaction rate that is achieved by the enzyme. In the case of a sequence of letters, E_x might represent the probability that the sequence conveys a desired message to a particular recipient. And in a system with water flowing over sand ripples, E_x might be defined as the rate of energy dissipation by turbulence, compared with flow over a smooth, unpatterned surface. The units or scale of E_x may be somewhat arbitrary and will depend on the nature of function x. Thus, for example, catalytic efficiency might be recorded in terms of rate enhancement or in terms of decreased activation energy (proportional to the log of the rate enhancement).

In the formalism of Szostak (2003; see also Morowitz, 1978, p. 252), functional information [$I(E_x)$] is calculated with reference to a specific degree of function x, designated E_x. Typically, a small fraction, $F(E_x)$, of all possible configurations of a system achieves at least the specified degree of function, $\geq E_x$. Accordingly, we define functional information in terms of $F(E_x)$:

$$I(E_x) = -\log_2[F(E_x)].$$

Thus, in a system with N possible configurations (e.g., a sequence of n RNA nucleotides, which has $N = 4^n$ discrete possible sequences):

$$I(E_x) = -\log_2[M(E_x)/N],$$

where $M(E_x)$ is the number of different configurations that achieves or exceeds the specified degree of function x, $\geq E_x$

In every system, the fraction of configurations, $F(E_x)$, capable of achieving a specified degree of function will generally decrease with increasing E_x (Szostak, 2003). The largest possible functional information of a system is exhibited in the case of a single configuration that displays the highest possible degree of function, E_{max}:

$$I(E_{max}) = -\log_2[1/N] = \log_2 N,$$

where I is measured in bits. This maximum functional information is thus equivalent to the maximum number of bits necessary and sufficient to specify any particular configuration of the system.

Alternatively, the minimum functional information of a system is zero for configurations with the lowest degree of function, E_{min}, because all possible states have $E_x \geq E_{min}$:

$$I(E_{min}) = -\log_2(N/N) = -\log_2(1) = 0 \text{ bits.}$$

In this formulation, functional information increases with degree of function, from zero for no function (or minimum function) to a maximum value corresponding to the number of bits necessary and sufficient to specify completely any configuration of that system.

Functional information is defined only in the context of a specific function x. For example, the functional information of a ribozyme may be greater than zero with respect to its ability to catalyze one specific reaction but will be zero with respect to many other reactions. Functional information therefore depends on both the system and on the specific function under consideration. Furthermore, if no configuration of a system is able to accomplish a specific function x [i.e., $M(E_x) = 0$], then the functional information corresponding to that function is undefined, no matter how structurally intricate or information-rich the arrangement of its agents.

It is important to emphasize that functional information, unlike previous complexity measures, is based on a statistical property of an entire system of numerous agent configurations (e.g., sequences of letters, RNA oligonucleotides, or a collection of sand grains) with respect to a specific function. To quantify the functional information of any given configuration, we need to know both the degree of function of that specific configuration and the distribution of function for all possible configurations in the system. This distribution must be derived from the statistical properties of the system as a whole [as opposed, for example, to the statistical properties of populations evolving in a fitness landscape (Wright, 1942)]. Any analysis of the functional information of a specific functional sequence or object, therefore, requires a deep understanding of the system's agents and their various interactions.

Three examples (letter sequences, the artificial life platform Avida, and RNA aptamers) serve to illustrate the concept of functional information.

THE FUNCTIONAL INFORMATION OF LETTER SEQUENCES

Systems of many interacting components can occur in a combinatorially large number of different configurations. Functional information depends on the fraction of all possible configurations that achieve at least a specified degree of function. Sequences of letters provide a conceptually familiar example. Consider various sequences of n letters that convey the message: "A fire has just started in a house at the corner of Main Street

and Maple Street.'' Many different sequences of letters are capable of conveying that information. To determine the functional information of any particular sequence we must specify three parameters:

1. n, the number of letters in the sequence.
2. E_x, the degree of function x of that sequence. In the case of the fire example cited above, E_x might represent the probability that a local fire department will understand and respond to the message (a value that might, in principle, be measured through statistical studies of the responses of many fire departments). Therefore, E_x is a measure (in this case from 0 to 1) of the effectiveness of the message in invoking a response.
3. $M(E_x)$, the total number of different letter sequences that will achieve the desired function, in this case, the threshold degree of response, $\geq E_x$.

The functional information, $I(E_x)$, for a system that achieves a degree of function, $\geq E_x$, for sequences of exactly n letters is therefore

$$I(E_x) = -\log_2[M(E_x)/26^n].$$

Note that 26^n is the total number of possible arrangements of a 26-letter alphabet in a sequence of n letters, and in this treatment we assign equal probability to all possible sequences. The important more general case of configurations of unequal probabilities is a straightforward extension of the treatment of Shannon (Shannon, 1948; Klir, 2006), as discussed by Carothers *et al.* (2004). Greater clarity of expression can be added through additional characters such as ''space,'' ''capital,'' and ''period''; however, in this example we use only 26 letters. As in all combinatorially large emergent systems, most sequences convey no information (i.e., have no discernable function). Functional information is determined by identifying the fraction of all sequences that achieve a specified outcome.

Consider, for example, sequences of 10 letters that have a high probability ($E_x \simeq 1$) of evoking a positive response from the fire department. Such sequences might include ''FIREONMAIN,'' ''MAINSTFIRE,'' or ''MAPLENMAIN.'' Additionally, some messages containing phonetic misspellings (FYRE or MANE), mistakes in grammar or usage (FIREOFMAIN), or typing errors (MAZLE or NAPLE) may also yield a significant but lower probability of response ($0 << E_x < 1$). Given these variants, on the order of 1,000 combinations of 10 letters might initiate a rapid response to the approximate location of the fire. Thus,

$$I(1) \approx -\log_2[1000/26^{10}] \approx 36 \text{ bits}.$$

Numerous additional 10-letter sequences convey some relevant information but would result in a lower probability of response ($0 < E_x < 1$): "FIREHELPME," "DANGERFIRE," or "BURNINGNOW." A lower degree of function, E_x, will generally correspond to a larger number of effective letter sequences, $M(E_x)$.

The formulation of functional information also applies to systems in which sequences of varied lengths are combined. For letter sequences of any length from 1 to n letters,

$$I(E_x) = -\log_2\{M(E_x)/[\Sigma_{1 \text{ to } n}\,(26^n)]\}.$$

Varying the maximum length, n, of the letter sequence has a significant effect on the maximum possible degree of function, E_x, as well as the number of states, $M(E_x)$, that achieve that degree of function. Sequences of 1, 2, or 3 letters are unlikely to convey sufficient information to achieve any response. With 4 letters, however, a few suggestive configurations exist (FIRE, MAIN, or MAPL), although all such sequences possess a high degree of ambiguity (i.e., $E_x << 1$).

On the other hand, with longer letter sequences ($n >> 10$), the number of messages of a given degree of function increases dramatically, with new opportunities for explicit instructions (and hence maximum degree of function, $E_x = 1$). With a sufficient number of letters, any arbitrary degree of accuracy and precision in a message can be communicated. Note, however, that arbitrarily long sequences are not necessarily more effective at conveying information and thus may not increase the functional information of a system. For example, consider sequences of letters that begin with the following 22 letters:

FIREATMAINSTANDMAPLEST . . .

Such a sequence should invariably summon the fire department, no matter what or how many additional letters are placed at the end of the sequence. Thus, for this admittedly contrived fire department scenario, the fraction of sequences that achieve the desired outcome attains a maximum value at ≈20 letters. In competitive systems, notably genetic information constrained by length-selective pressure in living systems (e.g., Mills *et al.,* 1967; Andersson and Andersson, 1999; Shigenobu *et al.,* 2000; Nakabuchi *et al.,* 2006), longer sequences may prove inefficient and do not necessarily confer an advantage. (Indeed, in the case of reporting a fire, an overly long and detailed message might delay response time.)

Note that in this formulation of functional information the maximum possible value, $I(E_{max})$, arises when a message is so specific that only a single letter sequence out of all possible letter sequences achieves a desired

outcome. In the case of a sequence of n letters, that maximum functional information occurs when $M(E_x) = 1$:

$$I(E_{\max}) = -\log_2[1/26^n] = \log_2 26^n \approx 4.7n \text{ bits.}$$

Although this conceptual example is qualitative, it introduces key concepts that are required to quantify functional information in any emergent system with numerous configurations. Of special interest is the relationship between information and degree of function. Letter sequences point to the existence of discrete ''classes'' of functional configurations, based in this case on the appearance of familiar words (''FIRE'' and ''MAIN'') as well as their mutations (''FYRE'' and ''MANE''). We explore the role of such multiple classes of solutions in the subsequent sections on Avida and RNA aptamers.

We conclude that rigorous analysis of the functional information of a finite system with respect to a specified function x requires knowledge of two attributes: (*i*) all possible configurations of the system (e.g., all possible sequences of a given length in the case of letters or RNA nucleotides) and (*ii*) the degree of function x for every configuration.

These two requirements are difficult to meet in many systems. In the case of letter sequences, for example, the sequence is obvious, but it is difficult to determine quantitatively the degree of function of many sequences. By contrast, it is relatively straightforward to determine the degree of function (for example, the ligand affinity) of any given RNA sequence, but impossible with present technology to measure all sequences in a large population, e.g., $\approx 10^{14}$ randomly generated 100-mers as used in some aptamer evolution studies (although single-molecule methods may ultimately provide a technical solution to this challenge). However, these concepts may be placed on a firmer footing in the case of computational systems, such as the artificial life platform Avida.

THE FUNCTIONAL INFORMATION OF AVIDA POPULATIONS

We have adapted the artificial life platform Avida (Adami, 1998; Lenski *et al.*, 2003) to explore the distribution of function in an emergent system. The digital organisms that populate the virtual world of Avida are ''computer programs that self-replicate, mutate, and adapt by natural selection'' (Lenski *et al.*, 1999) and as such share many (although not all) of the attributes ascribed to biological life. Accordingly, artificial life models have been used as a means of exploring ideas about organic biology that are not readily amenable to experimentation. Here we explore the functional information of randomly generated populations of Avida organisms. Understanding the origin and evolution of complex biologi-

cal systems motivates this work; however, the first task is to demonstrate an approach for quantifying the relationship between information and functional behavior in a well characterized emergent system, whether or not unambiguous biological insight is immediately revealed.

Avida organisms consist of multiple lines of machine instructions, termed its ''genome.'' Each organism operates as a formal computer similar to that outlined by Turing (1936), and the computational properties of each organism are determined by the sequence of machine instructions stored in its memory. A population of Avida organisms can be thought of as a multitude of identical computers running many different simple programs, where differences between any two members of the population arise solely from the differences in the programs being run.

This research focuses on the ability of a small fraction of all randomly generated Avida organisms to perform computational tasks that arise through the coordinated execution of multiple machine instructions (Lenski *et al.,* 2003). None of these computational tasks can be performed by the execution of a single instruction; indeed, the shortest functional program requires five instructions. The computational ability (function) of Avida organisms thus emerges from the interaction of instructions (the agents), making Avida an ideal model for characterizing complex emergent systems.

In a typical Avida experiment, we generate 10^7 random instruction sequences (i.e., 10^7 different individual genomes), each sequence 100–500 instructions in length, from the default set of 26 different machine instructions. Although most sequences display no function, a small subset of sequences code for the ability to compute logic operations (such as ''not'' or ''and'') or arithmetic functions (addition and subtraction).

The set of computational tasks Avida organisms can perform allows for varied solutions, analogous to variations seen in nature. This characteristic is underscored by the fact that in its evolution apparatus Avida does not consider how a task is accomplished but only the resulting function, i.e., whether or not it is executed. The Avida platform does not specify preferred approaches to problem solving, which allows novel solutions to appear through evolution. There may be great variety among these solutions, and they may be very different from those that might have been arrived at by design (Lenski *et al.,* 1999).

MEASURES OF AVIDA FUNCTION

Just as there is no unique measure of function in natural systems, there is no unique measure of the degree of function in an Avida sequence population. We chose to consider three distinct measures of function: (*i*) the number of times a sequence is able to compute a specific task, for example,

addition or not/and; (*ii*) the total number of all tasks the sequence is able to compute, because many sequences can perform multiple distinct operations; and (*iii*) the total number of different tasks the sequence is capable of computing.

Each of these measures of function correlates to strategies that biological organisms employ to increase their fitness. Some organisms rely on the ability to perform one action very well, others rely on the ability to perform multiple actions moderately well, and still others take advantage of flexibility, the ability to do many different tasks (Wilson, 1992). However, unlike with living organisms, quantifying the extent of these traits in Avida is straightforward and unambiguous. Most of the discussion that follows, however, focuses on execution of a single task.

Functional sequences constitute a tiny minority of the Avida genome space. Therefore, to explore fully the distribution of function within a sequence space, a large number of randomly generated sequences (i.e., equal probability) must be surveyed (see *Methods*). Such random explorations of genome space are similar to the strategies used in the directed evolution of RNA structures (e.g., Ellington and Szostak, 1990; Wilson and Szostak, 1999). Note, however, that this type of random sampling is not possible with living organisms because the portion of genome space explored in an evolution experiment will be constrained by the topology of the underlying fitness landscape and the particular configuration of the environment maxima (van Nimwegen *et al.,* 1999; Lehman *et al.,* 2000; Taverna and Goldstein, 2000; Sasaki and Nowak, 2003).

AVIDA RESULTS

Random sampling of genome space has yielded several interesting results related to the frequency and distribution of functional configurations. By using Avida's default set of 26 machine instructions, a randomly generated sequence with length of a magnitude of $\approx 10^2$ lines was found to be functional (i.e., was able to perform at least one logic or arithmetic operation at least once) with probability $P \approx 10^{-3}$. The functional fraction of a population decreases with decreasing sequence length until it reaches zero for populations with sequences of a length of four machine instructions or less.

We observe regular, reproducible structure in the distribution of task execution frequency, for example, in the number of not/and or addition operations executed (E_x) versus functional information (Fig. 2.1). This plot, which illustrates the distribution of function for 10^7 randomly generated 300-instruction genomes, is continuous over most values of E_x, for example, between 2 and 48. However, at several values of E_x, discontinuities appear. At $E_x > 73$ these discontinuities point to isolated individual

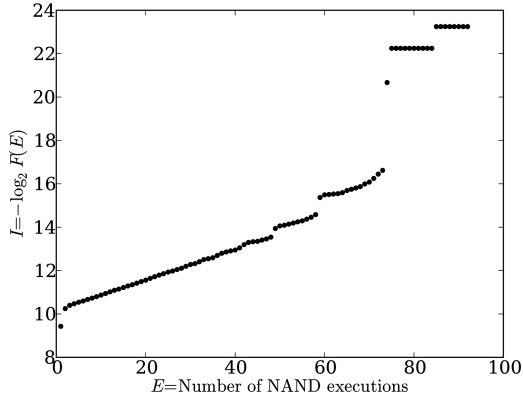

FIGURE 2.1 Distribution of the not/and (NAND) function in 300-line Avida genomes in a randomly generated sample of 10^7 genomes. The degree of function, E, is the number of times NAND is executed by the genome, whereas functional information, I (in bits), is $-\log_2$ of the fraction of all sequences that achieves at least that degree of function, $F(E)$. Note the discontinuities, which are a recurrent feature in these experiments.

genomes of high functionality; such outliers always appear, but they may occur at different values of E_x for repetitions of this experiment. However, other discontinuities (notably those between 48/49 and 58/59) are robust, always appearing in experiments on 300-instruction genomes. Thus these gap-like features reflect an intrinsic behavior of Avida genomes.

We also find that the number and specific location of these gaps, as well as the maximum values of $I(E_x)$ and E_x, depend on the length of the sequences being studied (Fig. 2.2). For example, we examined the number of executions of the addition function for 10^6 randomly generated genomes of 100, 200, 300, and 500 instructions. We find that the maximum number of addition executions, E_x, increases with genome length. We often observe discrete highly functional genomes, representing outlier solutions, as well as reproducible gaps. For randomly generated genomes of 100, 200, 300, and 500 instructions, the first significant gap in addition execution frequency occurs at 19, 39, 59, and 69 executions, respectively.

ISLANDS OF FUNCTION

What is the source of the reproducible discontinuities in Figs. 2.1 and 2.2? We suggest that the population of random Avida sequences contains

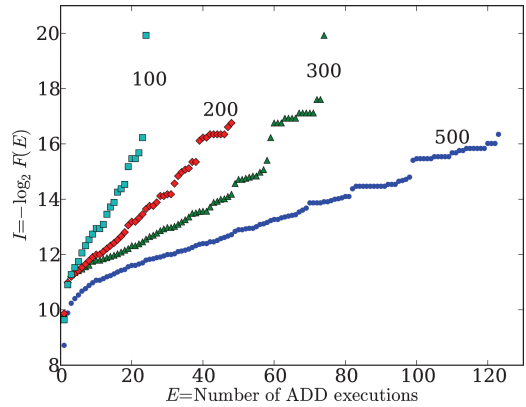

FIGURE 2.2 The frequency of the ADD function in 100-, 200-, 300-, and 500-line linear Avida genomes in randomly generated samples of 10^6 genomes. Degree of function, E, is the number of times the ADD function is executed by the genome, whereas functional information, I (in bits), is $-\log_2$ of the fraction of all sequences that achieves at least that degree of function, $F(E)$. Note that maximum E increases with genome length.

multiple distinct classes of solutions, perhaps with conserved sequences of machine instructions similar to those of words in letter sequences or active RNA motifs (Knight and Yarus, 2003). Each class has a maximum possible degree of function; therefore, the discontinuities occur at degrees of function below which a major class of sequences is represented and above which it is not represented.

Fig. 2.3 demonstrates one possible model for this stepped behavior, based on discrete ''islands'' of solutions. In Fig. 2.3, the islands, each of which represents a specific distinct set of solutions to the function [i.e., fitness (z axis)], are conceptually represented as being close to each other in sequence space (projected on the x–y plane). Note, however, that these islands are a visual simplification. For example, in the case of RNA sequences, any given ''island'' of closely related functional solutions may be more realistically represented by a densely interconnected network that spans all of sequence space (Huynen *et al.,* 1996; Lehman *et al.,* 2000; Reidys *et al.,* 2001). Similar consideration of function topologies has been applied to neural network connections (Ebner *et al.,* 2002) and to viroid solutions infecting the same plant host (Codoñer *et al.,* 2006). Avida may be similar, because the commands relevant to a given solution do not necessarily need to appear sequentially at a specific location

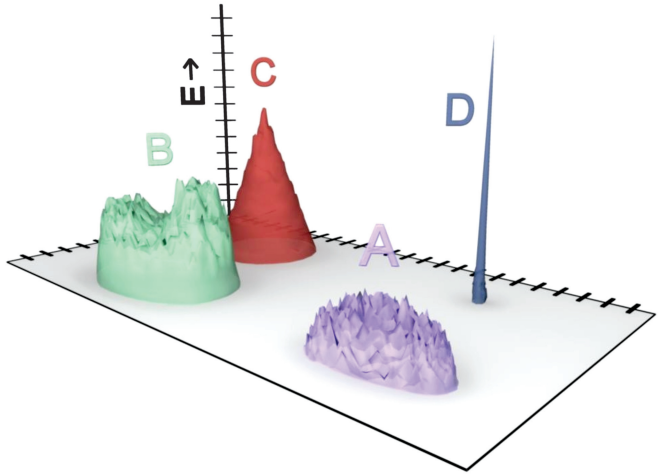

FIGURE 2.3 Schematic representation of four discrete functional classes, or "islands," of solutions that display function. The vertical axis is degree of function, *E*, whereas the horizontal plane represents a two-dimensional projection in sequence space. The number of sequences with degree of function ≥ *E* corresponds to the area intersected by the horizontal plane at that height along the *E* axis. Increasing *E* above the heights of the flat-topped islands A and B will result in discontinuities in the function *E* versus *I*, as illustrated in Figs. 2.1 and 2.2. Island C is a cone-shaped distribution, and island D represents a discrete solution of the type that might not be discovered in random sampling experiments.

in the string but can occur in different registers and can be spread apart by neutral commands.

Consider a case where four classes of solutions for the same function, labeled A–D, occur in the population (Fig. 2.3). Each class may contain a normal distribution of degrees of function, but each has a different topology in sequence space and a different maximum degree of function, E_x. For relatively low values of E_x, all four islands contribute functional sequences. As the value of E_x increases to just above the heights of flat-topped islands A and then B, discontinuities in the plot of E_x versus $I(E_x)$ will occur (i.e., in Fig. 2.1 the height corresponding to island A would be $E_x = 48$ and the height of island B would be $E_x = 58$). This model also matches the observation that the continuous stretches of E_x versus $I(E_x)$ are longest for populations of long sequences: Longer sequences allow for a greater number of distinct solutions whose superposition would serve to drown out individual discontinuities.

This model for generating discontinuities is plausible because multiple distinct solutions may exist in sequence space for a given task. For example, the shortest possible sequence (“gene”) for accomplishing subtraction is five lines long (Lenski *et al.*, 2003). However, an alternative unrelated subtraction gene 10 lines long can be constructed within the Avida language using two’s-complement arithmetic (Zarowski, 2004). This second class of solutions reinforces the concept of “islands” of function in sequence space, where two or more types of solutions exist that achieve the same task but do so in an unrelated fashion.

We note, by contrast, that purely random statistical functions do not display steps. For example, if the degree of function is defined as the frequency of the appearance of the number “1” in randomly generated sequences of 100 digits, then functional information follows a well behaved smooth curve (Fig. 2.4). Maximum functional information arises for the solitary state with 100 consecutive 1s, whereas an obvious uniform distribution follows for lesser degrees of function. This statistically random case is not stepped. By comparison, the structures depicted in Figs. 2.1 and 2.2 suggest that the tasks being considered as functions are neither trivial, nor are they achieved by essentially arbitrary or random, albeit rare, configurations of the system. The interactions in the Avida system, and perhaps many other complex systems, lead to distributions of function that prove far richer than in systems possessing statistically

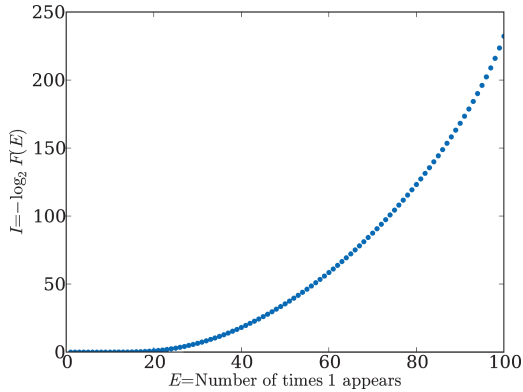

FIGURE 2.4 $I(E)$ versus E for the statistically random system, where E is the number of times the digit 1 appears at least that many times in a sequence of 100 digits. This statistically random case is not stepped, in contrast to the topology of Avida genomes.

trivial function. It remains to be seen, however, whether the observed stepped relationship between $I(E_x)$ and E_x is a general feature of functional information or an idiosyncratic characteristic of Avida genomes.

FUNCTIONAL INFORMATION AND RNA POLYMERS

The previous two examples, sequences of letters and Avida machine commands, illustrate the utility of the functional information formalism in characterizing the properties of symbolic systems that can occur in combinatorially large numbers of configurations. Functional information also has applicability to complex biological and biochemical systems; indeed, it was originally developed (Szostak, 2003; Carothers *et al.*, 2004) to analyze aptamers (RNA structures that bind target ligands) and ribozymes (RNA structures that catalyze specific reactions). Thus, the degree of function, E_x, of these linear sequences of RNA letters (A, C, G, and U) can be defined quantitatively as the binding energy to a particular molecule or the catalytic increase in a specific reaction rate. We can easily specify every possible RNA sequence of length n, and we can (at least in principle) synthesize RNA strands and measure the degree of function of any given sequence. The behavior of aptamers and ribozymes thus lends itself to the type of quantitative analysis that we applied previously to letter sequences and Avida populations (Carothers *et al.*, 2004).

In general, a single RNA nucleotide will display minimal catalytic or binding function, $x_{\min}$. It follows that a minimum sequence length ($n_{\min}$ nucleotides) will be required to achieve any significant degree of ribozyme or aptamer function, $E_x > E_{\min}$. Increasing the number of nucleotides ($n > n_{\min}$) will generally lead to many more functional sequences, some of which will have a greater degree of function. Furthermore, for any given catalytic or binding function there exists an optimal RNA sequence of length n_{opt} that attains the maximum possible degree of function, $E_{\max}$. That sequence thus possesses the maximum possible functional information:

$$I_{\max}(E_{\max}) = -\log_2\{1/[\textstyle\sum_{1-n_{\text{opt}}}(4^n)]\}.$$

For degrees of function less than the maximum ($E_x < E_{\max}$), an intermediate functional information obtains $[I(E_x) < I_{\max}(E_{\max})]$.

The *in vitro* evolution of RNA aptamers (e.g., Ellington and Szostak, 1990; Wilson and Szostak, 1999) provides a dramatic illustration of the evolution and selection of systems with high functional complexity. Aptamer evolution experiments begin with large populations (up to 10^{16} randomly generated RNA sequences), which are subjected to a selective environment, a test tube coated with a target molecule, for example. A small fraction of

the random RNA population will selectively bind to the target molecules. Those RNA strands are recovered, amplified with mutations (through reverse transcription, PCR, and transcription), and the process is repeated several times. Each cycle yields a more restricted RNA population with improved binding specificity (i.e., a higher degree of function, E_x).

Carothers *et al.* (2004), who analyzed the distribution of functional RNA aptamers in a random population, provide data on a specific example. They identify 11 distinct classes of GTP-binding RNAs, which are distinguished from each other both by nucleotide sequences (RNA motifs) (Knight and Yarus, 2003) and secondary stem–loop structures. The degree of function of these aptamers can be defined by a solution dissociation constant, a measure of the binding strength between GTP and the folded aptamer. Carothers and coworkers find that a 10-fold increase in GTP binding strength requires ≈10 additional bits of information (i.e., a 1,000-fold decrease in abundance in a population of random sequences). Such a finding is in accord with studies of biopolymers (Aronson *et al.*, 1994; Wang and Unrau, 2005) that show functionally similar peptides with dissimilar primary structures, as well as reports of many distinct classes of protease enzymes (Rawlings and Barrett, 1993; Rawlings *et al.*, 2006).

Furthermore, although the data of Carothers *et al.* (2004) are too few to draw definitive conclusions, there is a suggestion of a stepped relationship between binding strength (E_x) and functional information (I), a relationship analogous to that displayed by populations of Avida organisms (e.g., Fig. 2.1). These steps, if real, are likely caused by the existence of separate classes of GTP-binding solutions. Functional classes with greater numbers of stems represent a significantly smaller fraction of all RNA sequences, but they have the potential to display greater GTP-binding affinities.

FUNCTIONAL INFORMATION IN HIGHER-DIMENSIONAL SYSTEMS

Functional information provides a measure of complexity by quantifying the probability that an arbitrary configuration of a system of numerous interacting agents (and hence a combinatorially large number of different configurations) will achieve a specified degree of function. This concept was originally discussed in the context of biopolymer sequences that perform specific binding or catalytic functions (Szostak, 2003; Carothers *et al.*, 2004). In the preceding sections we demonstrated that the extension of functional information analysis to one-dimensional systems of letters or Avida computer code is conceptually straightforward, requiring only specification of the degree of function of each possible sequence.

We suggest that the functional information formalism may also be applicable to complex physical structures in higher-dimensional systems.

Of special interest in this regard are biological systems that display complex emergent behavior, for example, through long-range chemical signaling among a collection of cells in social amoebas (Goldbeter, 1996; Brännström and Dieckmann, 2005; Schaap *et al.,* 2006), cooperation among consortia of host organisms and symbionts (Moran, 2007, Chapter 9, this volume), or colonies of social insects (Solé and Goodwin, 2000; Camazine *et al.,* 2001; Strassmann and Queller, 2007, Chapter 8, this volume). We propose that functional information can be applied, at least in principle, to any such emergent system that has the ability to perform a function.

Many emergent systems can be analyzed in terms of their ability to dissipate energy or maximize entropy production (Nicolis and Progogine, 1977; Lorenz, 2003; Whitfield, 2005; Emanuel, 2006). For example, consider the functional information of an assemblage of sand grains subjected to a steady flow of wind or water (e.g., Bagnold, 1988; Hansen *et al.*, 2001). The formation of periodic sand dunes or ripples serves to initiate turbulent flow and thus increase energy dissipation. Functional information of the system can thus be measured as the fraction of all possible sand configurations, $F(E_x)$, that achieve at least the corresponding energy dissipation, E_x. Such a problem might be analyzed with Monte Carlo simulations of numerous gravitationally stable sand configurations. The analytical challenge remains to determine the degree of function of a statistically significant random fraction of all possible configurations of the system so that the relationship between $I(E_x)$ and E_x can be deduced.

CONCLUSIONS

A complexity metric is of little utility unless its conceptual framework and predictive power result in a deeper understanding of the behavior of complex systems. Analysis of complex systems in terms of functional information reveals several characteristics that are important in understanding the behavior of systems composed of many interacting agents. Letter sequences, Avida genomes and biopolymers all display degrees of functions that are not attainable with individual agents (a single letter, machine instruction, or RNA nucleotide, respectively). In all three cases, highly functional configurations comprise only a small fraction of all possible sequences. Furthermore, these three examples reveal that several discrete classes of functional configurations exist, a situation that can lead to distinctive step features in plots of information versus function.

The functional information formalism may also point to key factors in the origin and emergence of biocomplexity. In particular, functional information quantifies the probability that, for a particular system, a configuration with a specified degree of function will emerge. Furthermore, analysis of the relationship between information and function may reveal

how much more information is required to encode a given improvement in function. The formalism also points to strategies, such as increasing the concentration and/or diversity of molecular agents, that might maximize the effectiveness of chemical experiments that attempt to replicate steps in the origin of life.

METHODS

Determination of the computational properties of a randomly generated instruction sequence is accomplished within Avida's analyze mode. The trace feature in analyze mode generates detailed information on the state of the virtual computer at each step in the processing of a genome, including a notation of when a recognized function has been executed. An automated script parsed these logs to collect all of the data necessary to determine the functional properties of each sequence and cataloged the genomes found to be functional to permit later study. Detailed documentation of the Avida software, including descriptions of the trace function and analyze mode, can be found online at the Digital Evolution Laboratory at Michigan State University web site (http://devolab.cse.msu.edu/software/avida/doc).

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3

The Theory of Facilitated Variation

JOHN GERHART* and MARC KIRSCHNER†

This theory concerns the means by which animals generate phenotypic variation from genetic change. Most anatomical and physiological traits that have evolved since the Cambrian are, we propose, the result of regulatory changes in the usage of various members of a large set of conserved core components that function in development and physiology. Genetic change of the DNA sequences for regulatory elements of DNA, RNAs, and proteins leads to heritable regulatory change, which specifies new combinations of core components, operating in new amounts and states at new times and places in the animal. These new configurations of components comprise new traits. The number and kinds of regulatory changes needed for viable phenotypic variation are determined by the properties of the developmental and physiological processes in which core components serve, in particular by the processes' modularity, robustness, adaptability, capacity to engage in weak regulatory linkage, and exploratory behavior. These properties reduce the number of regulatory changes needed to generate viable selectable phenotypic variation, increase the variety of regulatory targets, reduce the lethality of genetic change, and increase the amount of genetic variation retained by a population. By such reductions and increases, the conserved core processes facilitate the generation of phenotypic

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variation, which selection thereafter converts to evolutionary and genetic change in the population. Thus, we call it a theory of facilitated phenotypic variation.

We will discuss the means by which animals have generated developmental and physiological variation since Cambrian times. In the course of their descent from a common ancestor, animals have diverged in their anatomy and physiology by the gradual accumulation of selected heritable modifications, their phenotypic variations. Although such variation is indispensable to evolution, Darwin conceded that "our ignorance of the laws of variation is profound" (Darwin, 1859b, p. 167), and 150 years later the mode of its generation remains largely unknown. Phenotypic variation is thought to affect all aspects of an animal's phenotype and to be "copious in amount, small in extent, and undirected" with regard to selective conditions (Gould, 2002). Most of these characterizations go back to Darwin himself. As Gould has noted (2002), they accord well with selection's primacy as the creative force in evolution, refining chaotic, profligate variation into exquisite adaptations. However, they afford little insight into the generation of phenotypic variation, and they raise questions about how copious, small, and undirected variation really is. Although small in extent, heritable phenotypic variations need be significant enough to be selected, and, if complex change entails numerous sequential phenotypic variations, evolution may be impeded. An example we will pursue later is that of the species of Darwin's finches that diverged in the Galapagos from a common ancestor. The beaks of some species are large and nutcracker-like, and those of others are small and forceps-like. As Darwin did, we too might imagine that many small heritable beak variations accrued slowly in the different species to create large observable differences. Small variations are arguably the only viable and selectable ones, because they would allow the upper and lower beaks, the adjacent skull bones, and head muscles to coevolve with each other in small selected steps, thereby maintaining viable intermediate beaks along the paths to the nutcracker and forceps forms. Repeated selections would be needed to coordinate the numerous, small, independent beak and head changes, all requiring genetic change. Is this an accurate appraisal of the paths of change? Or might the finch's own means of beak development coordinate many changes, allowing larger viable variations and a simpler, more rapid beak evolution? Insight into the mode of generation of variation could answer such questions about the size, abundance, and directedness of phenotypic variations.

Research of the modern era has revealed that heritable phenotypic variation requires genetic change, that is, DNA sequence change. Changes

occur throughout the genome, although perhaps not at uniform frequency, and include changes of single bases or short sequences or even long segments of DNA (Feuk *et al.*, 2006). Some genetic changes are lethal, some are neutral, and fewer are viable and selectable. Furthermore, the understanding of variation has advanced with the knowledge that DNA sequences encode RNA and protein, because the latter two would bear the marks of DNA sequence change and, in principle, alter the phenotype. Also, discoveries of gene regulation have opened the possibility of important evolutionary changes in nontranscribed DNA sequences, as well. Still, there are no "laws of variation" regarding its generation, only a black box of chaotic accidents entered by genetic variation and occasionally exited by selectable phenotypic variation.

In the past 20 years, enormous insights have been gained about the development and physiology of animals, namely, about the generation of their phenotype from their genotype, the kind of information eventually needed to explain and predict phenotypic change from genetic change. From these advances, can something now be said about the nature of phenotypic variation and its dependence on genetic change? What is really modified in descent with modification? Have all components of a new trait been modified a little, or a few elements a lot while others not at all? Are many genetic changes needed for a modification of phenotype or only a few? Are there preferred targets for change? Are there cryptic sources of variation? These questions require concrete answers that can come only from in-depth studies of the phenotype, that is, the animal's development and physiology.

We propose that the phenotype of the organism plays a large role in (*i*) providing functional components for phenotypic variation and (*ii*) facilitating the generation of phenotypic variation from genetic change. We outline a set of concepts from others and ourselves, organized in a theory of facilitated variation, to connect genetic and phenotypic variation (see Kirschner and Gerhart, 2005, for a longer presentation). Like other theories (King and Wilson, 1975; Carroll *et al.*, 1995; Davidson, 2006), it identifies regulatory changes as ones particularly important for animal evolution, but unlike others it also emphasizes the targets of regulation.

We include four steps from genetic variation to viable phenotypic variation of anatomy and physiology, and we wish to show at which steps the facilitation of variation occurs, and how it occurs. First, as widely accepted, genetic variation arises from recent mutations and rearrangements of the genome and from standing genetic differences arranged in new combinations by sexual reproduction. Second, particular genetic variations then lead to regulatory changes, namely (*i*) changes of DNA sequences at cis-regulatory sites; (*ii*) changes of DNA at sites transcribed into RNA regulatory regions, such as those for RNA stability, trans-

latability, and splicing (including microRNA processing); or (*iii*) DNA sequences transcribed and translated into protein regulatory regions, such as those for posttranslational modification, protein activation or inactivation, stability and degradation, or for binding regulatory agents and transducing their effects. Third, these regulatory changes impact "what is regulated," namely, the large set of conserved core components functioning in the animal's development and physiology. New regulation specifies new combinations, amounts, and functional states of those components to act at particular times and places in the animal. And fourth, the altered combinations, amounts, and states of the conserved components function to develop and operate a new trait on which selection acts. Of course the entire process is repeated in successive rounds of phenotypic variation and selection in an evolving trait.

The theory implies that new traits contain very little that is new in the way of functional components, whereas regulatory change is crucial. However, is a prohibitive number of regulatory changes needed to express thousands of genes at the new place and time of the new trait, and to operate thousands of encoded gene products (proteins and RNAs) at specific rates and in specific states? What quantity and quality of regulatory changes are needed? In answer, the theory of facilitated variation posits that core functional components, and the processes in which they serve, have special properties that greatly reduce the need for regulatory change, in ways that (*i*) reduce the number of necessary genetic changes, (*ii*) increase the variety of regulatory targets for change, (*iii*) reduce the amount of lethality due to genetic change, and (*iv*) increase the amount of genetic variation carried in the population. All of these effects facilitate the generation of viable phenotypic variation by regulatory change, and therefore we call it a theory of facilitated variation.

We will address three points of the proposals. What are the conserved core components and processes, what are their special properties that facilitate the generation of phenotypic variation by regulatory change, and what, in turn, are the regulatory innovations that have facilitated the use of core processes?

CONSERVED CORE COMPONENTS: RAW MATERIAL OF PHENOTYPIC VARIATION

These components generate and operate the animal's phenotype. Most are conserved across diverse phyla of the animal kingdom. Most operate in multicomponent processes that we call "conserved core processes." They comprise an enormous toolkit, and the genes encoding them comprise the majority of the genetic repertoire of the animal. They have changed very little in the course of animal evolution since the Cambrian, even

though animal anatomy and physiology have changed. These conserved functional components comprise that which is regulated in the animal; regulation of them has changed in animal evolution.

To indicate their diverse indispensable contributions to the phenotype, we enumerate core processes in Table 3.1, associating each with one of four major episodes of pre-Cambrian functional innovation (mostly protein evolution). These biochemical, molecular genetic, cell biological, physiological, and developmental components (which fill the textbooks of these fields) were carried forward, unchanged, in all bilateral animals. This, we argue, was such a powerful and versatile toolkit that post-Cambrian animals could largely omit further functional innovation at the gene product level (protein and functional RNA evolution) and instead exploit regulatory innovation to diversify anatomy, physiology, and development. What is remarkable about the processes, as a large set, is that they can be

TABLE 3.1 The Metazoan Toolkit of Conserved Functional Components and Processes: When Did They First Arise in Evolution?

First Arose in Evolution	Conserved Functional Components and Processes
Three billion years ago, in early prokaryotic organisms	Components of energy metabolism, biosynthesis of the 60 building blocks, DNA replication, DNA transcription to RNA, translation of RNA to protein, lipid membrane synthesis, transmembrane transport
Two billion years ago, in early eukaryotic cells	Components of the formation of microfilament and microtubule cytoskeletons, motor proteins moving materials along the cytoskeletons, contractility processes, movement of the cell by cilia and ruffling membrane action, shuttling of materials between intracellular organelles, phagocytosis, secretion, chromosome dynamics, a complex cell cycle driven by protein kinases and protein degradation, sexual reproduction with meiosis and cell fusion
One billion years ago, in early multicellular animal life forms	Components of 15–20 cell–cell signaling pathways, cell adhesion processes, apical basal polarization of cells, junction formation, epithelium formation, specialization of cells toward physiological ends, some developmental processes of the single-celled egg to the multicellular adult
Near pre-Cambrian, in animals with early body axes	Components of complex developmental patterning, such as anteroposterior axis formation (Wnt/Wnt antagonist gradients) and dorsoventral axis formation (Bmp/antagonist gradients), inductions, complex cell competence, additional specialized cell types, formation of the body plan's map of selector gene compartments (both transcription factors and signaling proteins), various regulatory processes

used in so many contexts toward so many ends. They define the envelope of possibilities of what regulatory change can achieve.

Parenthetically, though, some core components and processes have admittedly evolved since the Cambrian, and these, too, have become conserved. Appendage and limb formation (arthropods and tetrapods, respectively) would be developmental examples. These complex processes are, we argue, combinations of different conserved core processes linked in new regulatory configurations, conserved in their entirety. Others appear to entail protein evolution and new functions combined with old conserved processes, such as the SCPP proteins of bone formation, or keratins of hair and skin cells, or various myelin proteins of glial cells, or neural crest cells, or the adaptive immune system, all evolving in early vertebrates. These entail significant additions to the toolkit. And of course, protein evolution was very important in the four episodes of pre-Cambrian innovation described previously. For the most part, though, animals since the Cambrian have repeatedly reused the processes and components that had been evolved long beforehand to generate novel traits of anatomy and physiology.

Recent genome analysis has brought quantification to the impressions about conservation. More than 80 metazoan genomes have now been sequenced, and a typical case is the mouse (Mouse Genome Sequencing Consortium *et al.,* 2002). Of its total set of gene sequences, 23% are shared with prokaryotes, a further 29% are shared with non-animal eukaryotes (protists, fungi, and plants), and a further 27% are shared with nonchordate animals. Thus, 79% of mouse genes retain pre-Cambrian sequences. Reciprocally stated, only 21% of its functional components are unique to chordates, much less vertebrates, mammals, or mice. Such DNA sequence conservation among life forms conveniently allows the rapid identification of genes in new genomes by equating them with proteins or RNAs of other animals or yeast or bacteria where their function has been elucidated. As examples, the actins and β-tubulins of yeast and humans are 91% and 86% identical in amino acid sequence, respectively, and the otoferlins (a sensory cilium protein) of human hearing and *Drosophila* sensilla are 80% identical.

A complementary finding of genomics is the less-than-expected number of genes in animal genomes compared with bacteria and single-celled eukaryotes. The gene range from sea anemone (*Nematostella*) to human is 20–25,000 (Putnam et al., 2007), with some exceptions reflecting gene loss (honey bee, 10,000; *Drosophila*, 13,600). These numbers are but two to five times the inventory of *Escherichia coli* (4,600) or yeast (6,400), even though animals seem much more complex in their anatomy and physiology. One way out of the seeming paradox both of an embarrassingly small gene number in animals and of the widespread sharing of gene sequences with

other organisms is combinatorics (John and Miklos, 1988; Gerhart and Kirschner, 1997), the use of subsets of the same components in different combinations to get different outcomes, an interpretation we favor.

Why are such sequences conserved? All functioning proteins have specialized surface sites for precise interactions. At these sites, nonsynonymous amino acid substitutions are almost always detrimental to function and are eliminated by purifying selection, whereas synonymous substitutions are not (neutral or nearly neutral DNA changes), indicating that the conserved genes did undergo sequence change, like other DNA regions. For evolution, this deep conservation overwhelmingly documents the descent of animals from ancestors and has helped clarify phylogenetic relationships.

Functional conservation might seem to constrain phenotypic change because most sequence changes of those DNA regions encoding functional proteins and RNAs are lethal. (Note that the regulatory parts of proteins and RNAs are, we think, more changeable.) These DNA regions are effectively excluded from the list of targets at which genetic change could generate viable selectable phenotypic variation. They just cannot be tinkered with. Was evolution impeded by this vast functional conservation? We suggest that so much gene sequence is precluded from viable change that we should even revise our question about phenotypic variation to ask: what are the special properties of animals' phenotypes that allow phenotypic variation to be generated in seemingly copious amounts and great anatomical and physiological variety? These conserved processes have, we think, facilitated or deconstrained evolution because of their special properties of robustness and adaptability, their modularity and compartmentalization, their capacity for weak regulatory linkage, and their exploratory behavior. These properties make regulatory change efficacious and phenotypic variation copious and varied. We subsequently consider these properties and their consequences for regulation.

WEAK REGULATORY LINKAGE

Linkage, which denotes the connecting of processes to each other or to particular conditions, is central to our theory because different core processes must become linked, by regulatory means, in different combinations, and operated in different amounts, states, times, and places for the generation of new anatomical and physiological traits. Regulatory linkage pervades development and physiology. In general, a regulatory signal or input from one process or condition impinges on another process, which gives a response or output. The two are linked. Can regulatory linkages be made and changed easily, or do they require multiple complex instructions and precise stereochemical complementarity of the input and output? We

argue that conserved core processes have a special capacity for weak regulatory linkage (Gerhart and Kirschner, 1997; Kirschner and Gerhart, 2005), which reduces such demands and therefore facilitates the generation of phenotypic variation. In defining weak regulatory linkage, we stress two points: (*i*) the signal input and response output interact indirectly through an intermediate agency and hence do not require stereochemical complementarity to each other, and (*ii*) the output can be much more complex than the regulatory input because it has been previously built into the core process, independent of the nature of the signal. Although the signal seems superficially to control the response, it invariably turns out that the responding core process can produce the output by itself but inhibits itself from doing so. This self-regulation is built into the process. The signal, then, merely interferes with the self-inhibition (the intermediate agency), thus releasing the output, which may be much more complex than the signal and needs little instruction from it. In evolution, the signal is selectable just for its regulatory value, without regard to its chemical relationship to the response or to its instructive capacity. The regulatory input and functional output need not coevolve. Conceptually, the alternative is ''strong linkage'' (e.g., cofactors and substrates), which, we argue, requires more complex, precise, informative, and direct interactions from the input to make a process give a particular output. Constraint to change would be greater; more genetic change seems required.

Allosteric proteins, also known as switch proteins, are the simplest examples. These pervade metabolism, signal transduction pathways, neuronal excitation, transcriptional regulation, and physiology (e.g., hemoglobin). The protein's intrinsic activity is self-inhibited by a change of conformation of the protein and/or repacking of its subunits. The protein spontaneously switches between on and off states of activity but, on its own, strongly favors the off state. Regulatory agents select one or the other state by binding more strongly to it. This binding stabilizes the state, increasing its frequency in the protein population. Any regulator binding better to the on-state is an activator; any binding better to the off-state is an inhibitor. It is important to note that activity and inactivity are built into the protein, without instruction from the regulator, which only performs a state selection. Control of the protein is minimal. The regulator does not bind near the functional sites of the protein and need not be structurally compatible with them. They do not coevolve. Regulatory linkages can evolve with little constraint.

Neuronal transmission is a more complex two-state example, a physiological process comprising several core processes. The neuron connects inputs (received neurotransmitters) to distant outputs (the secretion of other neurotransmitters). To do this, the neuron generates two states, resting and active, which differ in their membrane potential. The resting state with

a more negative potential blocks the secretion of neurotransmitters. The active state with a less negative potential permits secretion. The received neurotransmitter initiates a local opening of allosteric ion channels, and local depolarization, at one end of the resting neuron. Weak linkage is provided by the propagated change of membrane potential, activating the entire neuron. When the other end becomes activated, it initiates secretion. The input (receptors and ion channels) is largely independent of the output (the secretory mechanism), connected only by the propagated depolarization. Receptors and ion channels can be installed or removed without reconfiguring secretion, membrane polarization, or impulse propagation, which are all conserved. They do not have to coevolve. In this case weak linkage has probably facilitated the evolution of the large variety of receptors, ion channels, and nerve cell types.

A still more complex example of weak linkage is embryonic induction, a developmental process first described in 1924 by Spemann and Mangold. Here a small group of cells, the ''organizer,'' induces the development of the central nervous system in nearby cells of the rest of the vertebrate embryo. At the time, it was thought this induction must entail detailed instructions to the responding cells. A surprising discovery of the past decade is that the organizer acts by secreting a few inhibitors (antagonists) that do not even bind to the responding cells (De Robertis, 2006). Instead, they antagonize an inhibitory signal secreted and received by the nearby cells in a self-inhibitory circuit to block their development of the nervous system. The organizer, via its antagonist, disrupts the self-inhibition, and neurogenesis commences. Thus, a simple signal, which can easily be moved, replaced, or modulated, regulates the time, place, and amount of the very complex developmental response. The ease with which simple signals can entrain complex processes reflects the capacity of core processes to engage in weak regulatory linkage.

Finally, the action of enhancer binding proteins in eliciting or repressing transcription (a complex specific output) is an excellent example of weak linkage. Transcription factors bind to the genome and mobilize enzymes that modify chromatin; the factors do not directly contact the core transcriptional machinery and play no role in transcript elongation, only in the initiation decision. Because of weak linkage, cis-regulatory DNA sites at which transcription factors bind can be far from the transcription start site, in either orientation, and composed of numerous independently acting regions (Levine and Tjian, 2003).

Weak regulatory linkage is important in developmental plasticity, which West-Eberhard has persuasively argued is a frequent substrate for heritable regulatory cooption (West-Eberhard, 2003). This plasticity entails the choosing of alternative developmental pathways according to environmental inputs. Examples include male–female differences, learning,

and alternate jaw structures. In her view, if the capacity to develop large phenotypic differences already exists in the organism as self-inhibited alternate states, and these can be elicited by simple signals (weak linkage), then large evolutionary steps can be made with a modicum of genetic change. In such cases, the distinction blurs between evolutionary gradualism and saltation (the generation of significant traits by single mutations). As an example, sex in some vertebrates (fish and reptiles) is determined environmentally (temperature, crowding, or social interactions) but in others, heritably (sex chromosomes). The underlying mechanisms for sex determination are similar in all vertebrates. It is just that an environmental stimulus (acting via weak linkage) has been replaced by a genetic one in the sex chromosome case. Neither provides much information about the outcome but just acts on the conserved switch.

To summarize, the relevant point of these examples is that regulatory change is easily effected when conserved core processes have an inherent capacity for weak regulatory linkage, that is, when switch-like behavior and alternative states of function are already built into them. The regulator need not inform the response or be stereochemically compatible with it. Regulation does not need to coevolve with the functional response. The requirements for regulation and regulatory change are reduced.

EXPLORATORY PROCESSES

As the name implies, some conserved core processes appear to search and find targets in large spaces or molecular populations. Specific connections are eventually made between the source and target. These processes display great robustness and adaptability and, we think, have been very important in the evolution of complex animal anatomy and physiology. Examples include the formation of microtubule structures, the connecting of axons and target organs in development, synapse elimination, muscle patterning, vasculogenesis, vertebrate adaptive immunity, and even behavioral strategies like ant foraging. All are based on physiological variation and selection. In the variation step, the core process generates not just two output states, but an enormous number, often at random and at great energetic expense. In the selective step, separate agents stabilize one or a few outputs, and the rest disappear. Although that agent seems to signal the distant process to direct outputs to it, it actually only selects locally via weak linkage among the many outputs independently generated by the process. Components of the variation and selection steps of the process are highly conserved.

Microtubules, for example, adopt vastly different spatial arrays in different cells. First, the tips of numerous microtubule polymers grow outward from a nucleation center, in random directions (the variation event).

Each polymer is unstable and, after a short time, by chance, shrinks back from the tip (Kirschner and Mitchison, 1986). They probe all regions of the cell in a futile cycle of outgrowth and shrinkage. If one by chance encounters a stabilizing agent at the cell periphery, its end is trapped, preventing shrinkage (the selection event). The entire length of microtubule leading to the agent is preserved. As more microtubules are selectively stabilized in one location, the cell's anatomy becomes polarized. This process is very adaptable and robust, providing microtubules no matter where stabilizers are located. It can therefore accommodate to placement errors or changing needs of the cell and can serve diverse roles, as in cilia, axons, and the mitotic spindle. Although the process of outgrowth and shrinkage is strongly conserved, and hence internally constrained in its own change, it generates diverse arrays each time it is used. In any particular cell, most outcomes are wasted, but they can be put to new uses in evolution simply by other cells' placing selective agents in new locations.

Wiring of the nervous system also draws heavily on exploratory processes. Excess axons extend from the central nervous system and randomly explore the body's periphery. Some accidentally hit target organs, such as muscles, and receive a dose of stabilizing protein (nerve growth factor); they persist, while others, failing contact, shrink back to the central nervous system.

ROBUSTNESS AND ADAPTABILITY

Weak regulatory linkage, state selection, and exploratory behavior underlie the robustness and adaptability of conserved core processes, that is, their capacity to produce functional (viable) outcomes despite physiological, developmental, environmental, or even evolutionary change. Robustness implies that a process remains the same because of tolerance or resistance to changing conditions, and adaptability implies that a process changes with the conditions in ways still to achieve the objective. Related to such properties, several authors have discussed the positive role of phenotypic plasticity in evolution (Schlichting and Pigliucchi, 1998; West-Eberhard, 2003); we feel that plasticity reflects the robustness and adaptability of core processes linked in complex assemblies. Robustness and adaptability are essential to the kind of evolution we have described, wherein core processes are used in different combinations, amounts, and states to produce new traits. They strongly reduce the requirements for regulatory change, and hence genetic change, and increase the frequency of viable phenotypic variations.

Adaptable robust processes can support nonlethal phenotypic variation in other processes, a situation called "accommodation" by West-Eberhard (2003). A specific example is the evolution of the tetrapod forelimb to a

bird or bat wing. Not only did the length and thickness of bones change, but also the associated musculature, nerve connections, and vasculature. Did many regulatory changes occur in parallel, coordinated by selection, to achieve the coevolution of all these tissues in the limb evolving to a wing? The answer comes from studies of limb development showing that muscle, nerve, and vascular founder cells originate in the embryonic trunk and migrate into the developing limb bud, which initially contains only bone and dermis precursors. Muscle precursors are adaptable; they receive signals from developing dermis and bone (Kardon *et al.,* 2003) and take positions relative to them, wherever they are. Then, as noted previously, axons in large numbers extend into the bud from the nerve cord; some fortuitously contact muscle targets and are stabilized, and the rest shrink back. Finally, vascular progenitors enter. Wherever limb cells are hypoxic, they secrete signals that trigger nearby blood vessels to grow into their vicinity (Ferrara *et al.,* 2003). This self-regulating vasculogenesis operates not just in the limb but throughout the body, accommodating to growing tissues, to exceptional demands such as pregnancy, and alas to growing tumors. The adaptability and robustness of normal muscle, nerve, and vascular development have significant implications for evolution, for these processes accommodate to evolutionary change as well. In the case of the evolving wing, if bones undergo regulatory change (driven by genetic change) in length and thickness, the muscles, nerves and vasculature will accommodate to those changes without requiring independent regulatory change. Coevolution is avoided. Selection does not have to coordinate multiple independently varying parts. Hence, less genetic change is needed, lethality is reduced, larger phenotypic changes are viable, and phenotypic variation is facilitated.

Finally, as Schmalhausen, Waddington, and others (Waddington, 1953; Schmalhausen, 1986; Gibson and Dworkin, 2004) have argued, physiological and developmental robustness reduces lethality because of undirected genetic variation. Less genetic variation is eliminated from the population, leaving it available for new trials of regulatory combinations and effects.

FAVORABLE SOURCES AND PATHS OF PHENOTYPIC CHANGE

Several authors tried in the past to connect long-term evolutionary change to short-term physiological change. As well known, Lamarck postulated that animals undergo anatomical and physiological changes in response to the environment, and then their offspring inherit these acquired characteristics. Darwin first conceived of variation as undirected and small with respect to selective conditions but later drifted toward Lamarck in thinking that as the organism responds to conditions, it furnishes the gametes with information enhancing the next generation's response. In

a 30-year period of confusion after Darwin, various evolutionists made internally driven phenotypic variation the creative factor in evolutionary change (e.g., orthogenesis and macromutation), even dismissing selection. The Modern Synthesis of the 1930s to 1950s dispelled such ideas about organism-directed phenotypic variation by combining Darwin's original hypothesis with new insights from transmission genetics, population genetics, and paleontology. Selection was restored to its central place.

Parallel to the Modern Synthesis, less known ideas did succeed in connecting long-term and short-term phenotypic variation without requiring an inheritance of acquired characteristics. Some of these premolecular ideas relate to recent proposals about the role of the organism in variation (Schlichting and Pigliucchi, 1998; Hallgrimsson and Hall, 2005) and to our proposals of facilitated variation. Baldwin in 1896 and 1902 reconciled aspects of Lamarck's and Darwin's proposals in what is now called the Baldwin effect (Baldwin, 1896, 1902). Accordingly, if an animal makes short-term physiological or behavioral adaptations to the environment, and then the conditions persist, these adaptations remain under selection, because at the adaptive limit they only provide marginal survival. They can become stabilized and extended by genetic change and hence become heritable traits. For Baldwin, adaptability of the animal's physiology and development is the source and path of evolutionary change.

Schmalhausen (1986) extended these ideas in the 1940s to include all nonlethal phenotypic changes of an organism that can be evoked by the environment, some adaptive to the evocative condition and others not (''morphoses'' he called them), some reversible (physiological) and others not (developmental). He called this enormous range of phenotypes, which are achievable without genetic change, the animal's ''norm of reaction'' to the environment. Once evoked, any of these traits could, under selective conditions, be stabilized and enhanced by genetic change, which he anticipated to be of a regulatory nature. Thereafter, the trait's expression in the new conditions would be heritable. For physiological and developmental adaptations, evocative and selective conditions were the same. For morphoses, a selective condition would fortuitously overlap the evocative condition.

Waddington independently developed similar ideas in the 1940s and 1950s under the name of ''genetic assimilation.'' He evoked phenotypic changes in *Drosophila* by ether, heat, or salt treatment and then, after 21 generations of treatment and selection, obtained flies that heritably exhibited new phenotypes without treatment (Waddington, 1953). Interestingly, the heritable fixation of the new traits was polygenic and arose only in genetically heterogeneous (non-inbred) populations, through repeated mating at the adaptive limit. Seemingly, the original population contained numerous variants of small effect, each too small for the full trait, and

then, as the marginal population mated for 21 generations, various small regulatory differences combined to the full trait (Sharloo, 1991). Recently, Rutherford, Lindquist, and colleagues (Rutherford and Lindquist, 1998) used heat, small-molecule inhibitors, and stop-codon suppressors to evoke a wide variety of new phenotypes (in interpretable ways) in *Drosophila*, plants, and yeast and recognized the latent variation of these phenotypic responses. Their phenotypes, too, could then be stabilized by genetic change under selective conditions, imposed by the experimentalist.

A major implication about phenotypic variation from these studies and ideas is that when novelty of some kinds is achieved in the course of variation and selection, rather little is really new; most components and regulatory linkages of the trait were already there. Novelty rests on small regulatory changes just stabilizing and enhancing an already extant physiological or developmental adaptation or evocable aberration. These were early attempts to restore the organism's present phenotype to the variation-generating process while still requiring genetic change. They also emphasize phenotypic plasticity (robustness, adaptability) as providing favorable sources and paths of evolutionary change, requiring few genetic changes. Such interpretations seem particularly suited to directional selections on physiological functions.

Recent advances in cell and developmental biology raise other possibilities for sources and paths of phenotypic variation. As noted previously, cellular adaptations occur repeatedly during development. Embryonic cells usually possess two or more developmental options under the control of a switch-like circuit. Via weak regulatory linkage, they respond to signals from neighboring cells, choosing one or the other option. At different times and places in the embryo, cells have different response options. If the adaptive states of embryonic cells are enumerated (states of gene expression, proliferation, secretion, shape, and signaling), the number is enormous. We suggest that this developmental cellular plasticity, which is based on ensembles of core processes already linked in various regulatory ways, is a major cryptic source of evolutionary novelty by regulatory stabilization. Such plasticity is, we think, rarely evocable by environmental conditions and hence would be omitted from the Baldwin effect.

Neural crest cells of vertebrates are a compelling example. These originate at the edge of the neural plate in early vertebrate development and migrate ventrally in the embryonic body, exploring numerous settlement sites having different regulatory signals. The cells possess many differentiation options (states), nearly unlimited powers of proliferation, and wide receptivity to local signals. Just within the head, they account for teeth, skull bones, the elephant's trunk, the narwhal's unicorn-like tooth, deer antlers, and probably the head shield of ceratopsian dinosaurs. These may all be but minor regulatory perturbations on neural crest cell

adaptability, provided at the settlement site (time, place, amount of local signals). Sewell Wright was prescient, we think, when he noted in 1931, "The older writers on evolution were often staggered by the seeming necessity of accounting for the evolution of fine details . . . for example, the fine structure of all of the bones . . . structure is never inherited as such, but merely types of adaptive cell behavior which lead to particular types of structure under particular conditions."

Although we concur that externally directed phenotypic plasticities are a rich source of variations for regulatory stabilization, we add to it the richer source of internally directed cellular developmental adaptations. The latter class would not be evoked by the environment and then stabilized, but stabilized directly by regulatory change driven by genetic variation.

COMPARTMENTATION

Thus far we have discussed how conserved core processes facilitate regulatory change, but we should also discuss how various regulatory processes, evolved in pre-Cambrian animals, have facilitated the use of core processes in different combinations, amounts, and states, while decreasing their chances of interference (pleiotropy). Spatial compartmentation of transcriptional regulation and cell–cell signaling is one of these.

In bilateral metazoa, the body of the mid-stage embryo, sometimes called the phylotypic stage of development, becomes divided into a regulatory grid or map of small compartments, each uniquely defined by its expression of one or a few selector genes encoding transcription factors or signaling molecules. The insect embryo at this stage contains ≈100 contiguous compartments, and the vertebrate embryo contains perhaps 200. The map is highly conserved within a phylum, and the stage is called phylotypic because embryos of all classes of the phylum then look most similar. Thereafter, selector genes of a compartment specify the anatomy and physiology to be developed within it; they "select" other genes, some encoding regulators and some encoding core process components, to be expressed or repressed in their compartment, thereby combining and customizing core processes for local usage. Different combinations, amounts, and states of core processes can be engaged in parallel in numerous regions of the embryo (Schlosser and Wagner, 2004; Carroll, 2005a). Conflicting processes such as cell death and proliferation can be run separately without interference.

One example of compartmentation is found in developing vertebrae, all of which contain bone-forming cells. In thoracic vertebrae they also form ribs, whereas in the cervical vertebrae they do not. Despite their equivalence as bone-forming cells, they differ, as shown by transplan-

tation experiments (Kieny *et al.,* 1972), solely because they arose along the dorsal midline in different compartments expressing different Hox genes. Similarly, *Drosophila* has a single developmental process for forming appendages; in the thorax it produces a leg, but in the head it produces biting mouthparts, because of different regulators introduced by different selector genes (Carroll *et al.,* 1995; Lovegrove *et al.,* 2006). Likewise, the forelimbs and hind limbs of vertebrates differ because of compartment-specific regulatory differences (Hox and Tbx genes).

Compartmentation facilitates the generation of phenotypic variation; that in one compartment does not constrain that in another (Schlosser and Wagner, 2004). Regulatory specification occurs independently and in parallel in different compartments. Also, we think that the compartment map deconstrains development preceding the phylotypic stage, when it first appears. The single-celled egg, we suggest, develops the compartment map by a robust adaptable process requiring little regulatory input. Thereby, the egg is freed to evolve fitness-enhancing diversifications of size, shape, nutrient provision, and gastrulation, as happened repeatedly in chordates and arthropods. After the phylotypic stage, as noted previously, members of different classes and families diversify their anatomies and physiologies, depending on which processes and regulation each compartment selects. The location of a conserved process (the compartment map) between diversified processes has been called the ''bowtie effect'' by Csete and Doyle (2002), who discuss its design benefits.

Other forms of regulatory compartmentation also facilitate diverse combinatorial uses of the gene repertoire while reducing pleiotropic interference. Each of the several hundred differentiated cell types of vertebrates is probably controlled by a few transcription factors and signaling proteins encoded by master regulatory genes, which select the expression of other regulatory genes and core processes of that cell's phenotype. In the temporal dimension, developmental stages such as the embryo, larva, and adult are sometimes compartmentalized by expressed heterochronic genes (Abbott *et al.,* 2005) that select stage-specific target genes, and in sexual dimorphism, target genes are selectively expressed in each sex.

EXPERIMENTAL EVIDENCE FOR FACILITATED VARIATION

To summarize, we argue that robustness, adaptability, modularity, capacity for weak regulatory linkage, exploratory behavior, and state selection of the conserved core processes, as well as the regulatory compartmentation of the conserved core processes, are key properties of the animal's phenotype that facilitate the generation of anatomical and physiological variation by regulatory change, which ultimately requires genetic change to be heritable. These special properties reduce the number of

genetic changes needed for phenotypic change, increase the number of targets for regulatory change, reduce lethality, and increase genetic variation retained in the population. Although the core processes are constrained in their own change of function, they deconstrain regulatory change.

Is this a testable hypothesis or merely a post hoc rationalization? To begin with, we should say that the theory emphasizes the targets of change and their consequences for phenotype, not the paths of change, although we especially like the plasticity-based paths because of what they say about targets and reuse of components. Basically, we accept any kind of regulatory change, arising by any path of genetic change, as long as it affects the combinations, amounts, states, times, and places of conserved core processes. Included would be the neo-Darwinian possibility of a rare, favorable, nonlethal, penetrant mutation that is selected to fixation of a new phenotype, and also included would be the Baldwin possibility of physiological adaptation at first without genetic change (in response to environmental change), followed by regulatory changes (via new allele combinations) enhancing and fixing a new phenotype. In both cases, genetic change results in regulatory change, which modifies the use of the conserved core processes.

The theory predicts that developmental biologists will continue to find (*i*) more examples of core processes used in diverse developmental and physiological traits in different combinations, amounts, and states, and (*ii*) in each new case a few small regulatory changes sufficing to redeploy core processes, which are themselves robust and adaptable. When introduced experimentally, such regulatory changes should significantly alter the phenotype, and other processes should accommodate to the directly altered ones, giving viable outcomes. Furthermore, it predicts that, as comparative experimental studies uncover the history of evolutionary innovation in animals, regulatory types of changes will predominate. Indeed, as is already clear, altered cis-regulation of gene expression and altered production of secreted signals lie behind specific phenotypic changes in stickleback fish and *Drosophila* (Sucena *et al.,* 2003; Crickmore and Mann, 2006; Shapiro *et al.,* 2006).

A recent example of bone morphogenetic protein (Bmp) and calmodulin signaling supports facilitated variation via robust adaptable processes. As described in the Introduction, Darwin noted the rapid divergence of beak morphologies by Galapagos finches. If we think mostly about selection and not phenotypic variation, we might imagine that selection acted repeatedly on many small changes occurring independently in the upper and lower beaks, adjacent skull, and head muscles to coordinate and order them into viable intermediate beaks throughout divergence. Many regulatory changes and many selections would be needed for this detailed coevolution of parts. Recent results, however, make a different

impression. Tabin's group has compared two Galapagos finches, one with a large nutcracker-like beak and another with a small forceps-like beak (Abzhanov *et al.,* 2004). In beak development, neural crest cells migrate from the neural plate to five primordia around the mouth. The primordia of the large-beaked finch express Bmp earlier and at higher levels than do those of the small-beaked finch. To test the importance of this difference, they introduced Bmp protein into the primordia of a chicken embryo, which normally develops a small pointed beak. The experimental chick developed a deep, broad beak, like the large-beaked finch. The beak was not monstrous; its parts fit together and properly adjoined the head. Recently the same group demonstrated that elevated levels of calmodulin, a ubiquitous calcium signaling protein, correlate with increased beak length, and experimental increases of this protein in the developing chick beak caused coordinated increases in beak length (Abzhanov *et al.,* 2006). Thus, two highly conserved factors quantitatively control much of the overall anatomy of the beak and adjacent head, producing a functional structure. Much coordination of parts is inherent in beak development; selection need not direct a detailed coevolution of parts; larger beak variations may be viable and selectable. Similarly the exuberant radiation of jaws in cichlid fishes of Lake Malawi is now attributed to changes at a small number of quantitative trait loci (Albertson and Kocher, 2006), including for Bmp. These results imply quantitative adjustments on robust, adaptable processes due to a few regulatory changes rather than many small independent changes coordinated by repeated selections.

A final feature deserves mention. Regulatory changes of the level of Bmp in the finch beak are in principle achievable in many ways, not only through altered transcription of the *bmp* gene (i.e., cis-regulation), or translation of the mRNA, or secretion, posttranslational modification, proteolytic processing, and breakdown of the protein. The levels of Bmp receptors could also be altered, as could the levels of any of several agonists and antagonists. Regulatory targets are many, yet all change Bmp signaling strength. Regulatory modification of the strength of Bmp or calmodulin signaling within one spatial compartment may have sufficed to achieve functional selectable changes in beak shape in a few steps. Other conserved processes also have multiple targets for regulatory change.

FACILITATED VARIATION AND EVOLUTION

Although recent insights in developmental biology and physiology deepen the understanding of variation, they do not undermine evolutionary theory. Laws of variation begin to emerge, such as regulatory change as the main target of genetic change, the means to minimize the number and complexity of regulatory changes, and the regulatory redeployment

of conserved components and processes to give phenotypic variations and selected traits. Regulatory change acts on the repertoire of unchanging core processes to select subsets, which are then externally selected upon. The burden of creativity in evolution, down to minute details, does not rest on selection alone. Through its ancient repertoire of core processes, the current phenotype of the animal determines the kind, amount, and viability of phenotypic variation the animal can produce in response to regulatory change. Thanks to the nature of the processes, the range of possible anatomical and physiological variations is enormous, and many are likely nonlethal, in part simply because the processes have been providing "useful" function since pre-Cambrian times. Phenotypic plasticities, both those evokable by environmental change and those developmental adaptabilities not evocable, are rich sources and favored paths of variation requiring little regulatory change.

These views are not at all Lamarckian, nor does facilitated phenotypic variation require selection for future good. Such facilitation arose, we think, as a by-product of the evolution of the special properties of the core processes, namely, of their robustness, adaptability, modularity, exploratory behavior, and capacity for weak regulatory linkage. These properties were probably selected at the level of the individual, simply for their capacity to make core processes work effectively under fluctuating external and internal conditions (Gerhart and Kirschner, 1997; Kirschner and Gerhart, 2005). In this way, the same molecular features that facilitate physiological and developmental change in an organism's lifetime also facilitate evolutionary change in the long run, as regulatory changes become genetically fixed.

4

Between "Design" and "Bricolage": Genetic Networks, Levels of Selection, and Adaptive Evolution

ADAM S. WILKINS

The extent to which "developmental constraints" in complex organisms restrict evolutionary directions remains contentious. Yet, other forms of internal constraint, which have received less attention, may also exist. It will be argued here that a set of partial constraints below the level of phenotypes, those involving genes and molecules, influences and channels the set of possible evolutionary trajectories. At the top-most organizational level there are the genetic network modules, whose operations directly underlie complex morphological traits. The properties of these network modules, however, have themselves been set by the evolutionary history of the component genes and their interactions. Characterization of the components, structures, and operational dynamics of specific genetic networks should lead to a better understanding not only of the morphological traits they underlie but of the biases that influence the directions of evolutionary change. Furthermore, such knowledge may permit assessment of the relative degrees of probability of short evolutionary trajectories, those on the microevolutionary scale. In effect, a "network perspective" may help transform evolutionary biology into a scientific enterprise with greater predictive capability than it has hitherto possessed.

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The analysis of complex molecular networks has become widespread in biology in recent years. The initiating event for this development was the publication of some landmark papers that argued for the ubiquity of so called "scale-free" networks in numerous biological systems and human communications networks (Barabasi and Albert, 1999; Jeong *et al.,* 2000). Subsequent analysis (Doyle *et al.,* 2005) has undermined the case for the near-universality of scale-free networks, but the general importance of networks as organizational devices is undisputed. In biology, an understanding of the structure and dynamics of genetic networks, in particular, is now widely viewed as crucial to understanding phenomena as diverse as metabolic systems, phage developmental switches, protein interaction systems, transcriptional controls, and complex developmental traits. Indeed, the study of molecular and genetic networks is central to the new field of systems biology (Strogatz, 2003).

The fundamental concept of genetic networks, however, is hardly new. A hint of the intricacy of the genetic architecture that underlies complex morphological traits, and of the complexity of the genetic interactions involved, can be found in a seminal paper on the gene by H. J. Muller (1922). Furthermore, the concept of genetic networks was implicit in much of C. H. Waddington's work (1940, 1957), although he did not use the term. Most importantly, however, the structure of genetic networks (in particular, one class of genetic networks, those underlying the biochemistry of mammalian coat colors) was central to the work of S. Wright (1968, 1980). Beyond these three great pioneers of 20th century biology, S. Kauffman added a molecular dimension to network-thinking with an early, if necessarily rather abstract, exploration of the generic structural properties of regulatory genetic networks (Kauffman, 1971). Not least, R. H. Britten and E. Davidson produced some thought-provoking schemes of how transcriptional networks might operate (Britten and Davidson, 1969, 1971). These specific hypotheses have not held up, but the basic thinking was prescient. It was, however, only the confluence, more than two decades later, of advances in genetics and molecular techniques in the 1990s with the then-new graph theory analyses of various networks (Barabasi and Albert, 1999; Jeong *et al.,* 2000) that launched the current wave of interest amongst biologists, in networks generally and, more specifically, in genetic networks (reviewed in Wilkins, 2007a).

An important part of this recent scientific development has been a focus on the evolutionary dynamics of networks (Dorogovtsev and Mendes, 2003; Barabasi and Oltvai, 2004; Berg *et al.,* 2004; Manke *et al.,* 2006). In principle, this theoretical work should provide a significant bridge between systems biology and evolutionary biology. In reality, however, there has remained a gap between the theoretical work on genetic network evolution and its application to understanding organismal evolution.

There seem to be two major reasons for this persisting "disconnect" between systems biology and evolutionary biology. First, the actual analysis of the kind of genetic networks that underlie complex morphological traits is more difficult, at both the conceptual and technical levels, than the analysis of some of the other kinds of networks (metabolic, protein interactomes, etc.) (Wilkins, 2007b). Second, a great deal of highly productive work in understanding the genetic basis of adaptive trait evolution is possible without reference to networks, simply through the traditional focus on individual genes and their immediate interacting partners, as will be discussed below. In effect, it seems that the importance of genetic networks for development and evolution can be fully accepted at the theoretical level while being essentially ignored in the experimental exploration of evolutionary change.

In this article, however, I will present the case that a genuine integration of network-thinking into evolutionary genetics can greatly enrich our understanding of evolutionary events. In particular, in the final part, I will present an argument that a deeper understanding of particular genetic networks, in conjunction with an appreciation of the generic properties of such networks, can, in principle, permit a larger predictive (or "retrodictive") element in evolutionary biology than has hitherto been possible.

EVOLUTIONARY METAPHORS AND THINKING ABOUT GENETIC NETWORKS

Each kind of network has its own distinctive kind of components and its own dynamics of behavior. Hence, each category requires a distinct analytical approach to identify its elements, dynamics, and evolutionary potentialities. However, in general, identifying the elements and boundaries of developmental genetic networks, which underlie morphological traits, is especially difficult (Wilkins, 2007b). These practical difficulties compound the interpretative problems in understanding the evolution of these networks. In the light of those vagaries, it becomes pertinent to ask what general perspective one should adopt for thinking about genetic networks and organismal evolution. The pioneering work of S. Wright (1968) on biochemical networks treated their genetic foundations as fixed entities, in both composition and connectivity, whose alternative behaviors were solely a function of the allelic properties of particular components. Developmental genetic networks, however, are evolutionarily more fluid entities, with a much wider range of capabilities for genetic alteration in both composition and behavior, as will be explained below. It becomes important, therefore, to be clear about the conceptual framework these networks require before proceeding to their detailed analysis or the

exploration of their evolution. Inevitably, that perspective will be influenced by the general evolutionary metaphor that one subscribes to.

Judging from their ubiquity, metaphors seem to be a universal aid to thinking, both in normal life and in science. For instance, the large number of creation myths to explain the origins of the world may differ greatly between different cultures, but nearly all are extended metaphors of either construction or birth. As these myths illustrate, the general appeal of metaphors is that they concretize otherwise mysterious processes, events, or objects and make them more accessible. At the same time, if used repetitively, or taken too literally, they can constrict thinking and obscure deeper understanding, as exemplified by the hold that the various creation myths have in various religious and ethnic subcultures.

In evolutionary biology, perhaps more than in any other branch of biology, the use of metaphors is especially marked. Evolutionary biology, in fact, was founded on a metaphor, ''natural selection,'' which was Darwin's explanation of the evolutionary process by means of a term that leaned on the ways that breeders artificially select and develop new varieties of animals and plants. Other, and later, evolutionary metaphors include such figures of speech as ''the adaptive landscape,'' ''the Red Queen hypothesis,'' ''the molecular clock,'' and ''Muller's ratchet.'' Two of the most prominent metaphors, however, are those of ''design'' and ''bricolage.'' Design, in fact, figures in the title of the Colloquium from which this article results, and, as Francisco Ayala discusses in this volume (Chapter 1), the challenge for evolutionary biologists is to explain how seemingly well designed features of organisms, where the fit of function to biological structure and organization often seems superb, is achieved without a sentient Designer. In reality, although organisms often seem designed efficiently for one trait, much is clearly suboptimal and many morphological/anatomical traits are baroque in their construction, defying the simplest notions of what constitutes good design. Furthermore, even the optimality of the well designed features is often at the slight expense of other traits, the phenomenon of ''tradeoffs'' (Sibly, 2002).

In contrast, the alternative metaphor of evolution as a tinkerer, engaged in piecemeal construction or bricolage, seems at first to be more apposite than that of design. Evolution clearly works by adapting preexisting structures to new purposes, many in ways that no sensible human engineer would have used. The evolution of certain reptilian jaw joint bones into two of the middle ear bones of the mammalian ear is a classic example of such evolutionary tinkering (Hopson and Crompton, 1969). Although the general concept of makeshift evolutionary construction from preexisting features was inherent in Darwin's thinking (Darwin, 1886), it was given prominence in contemporary evolutionary biology by Francois Jacob (1977). His special insight was that evolutionary tinkering takes

place not only at the morphological level but at the genetic level as well, with the use of "old" genes for new purposes. This latter process is now referred to as either "gene co-option" (Raff, 1996; True and Carroll, 2002) or "gene recruitment" (Wilkins, 2002), and its ubiquity and importance in evolution have been amply documented since Jacob's landmark paper (Duboule and Wilkins, 1998; True and Carroll, 2002).

MOLECULAR CONSTRAINTS ON EVOLUTIONARY TINKERING

Although the metaphor of bricolage seems far more appropriate for describing the evolutionary process than design, it too is somewhat misleading. It implies a higher degree of freedom about the elements that are borrowed and used in new evolutionary ways than is probably the case. The verb in French, "bricoler," often connotes an almost haphazard throwing of things together to see what happens and what works rather than the slightly more methodical procedure one associates with the term "tinkerer." Although there are no experimental studies yet of the constraints involved in gene recruitment, consideration of the basic properties of the properties of molecules and of the requirements of the process suggest that there are two kinds of general constraint that must operate. The first is the set of preexisting properties of the recruited molecule, permitting its adoption for new roles. When a transcription factor (TF) is evolutionarily recruited for a new activity (Duboule and Wilkins, 1998; True and Carroll, 2002), it must possess certain properties that confer capability for the new function, properties not shared with many other TFs. In effect, not any TF has equal potential for turning on, or repressing, a new gene or set of genes; the gain-of-function activity that a TF gene recruitment event comprises is determined by both the structure of the TF and the TF-binding sites in the enhancers of the target gene(s) (Davidson, 2001; Wray *et al.,* 2003). Second, the recruited gene must be already expressed in the cell/tissue where its new function initially takes place, or the mutation that leads to the recruitment event is one that prompts the *de novo* expression of the recruited TF, making it available for a new use. In the latter cases, presumably additional mutations would be required to optimize the expression or the function (or both) of the recruited molecule. There is, as yet, no direct evidence for such optimization after recruitment, but it seems an unavoidable conclusion from what is known about structure–function relationships in regulatory macromolecules. There is, however, good comparative evidence from bacteria for the converse situation, namely the relationship of structural properties to the probability of evolutionary loss of function. Evolutionary loss or substitution of TFs is influenced both by the kind of activity possessed by the factor (whether

positive, negative, or both) and the chromatin structure in which such factors operate (Hershberg and Margalit, 2006).

Hence, the tinkering/borrowing process at the molecular level is somewhat more channeled, thus restricted, than the metaphor of bricolage might suggest. In addition, however, it has become equally clear over the past decade that the recruitment process is often not a gene at a time but a functional grouping of genes, a network "module" (Davidson, 2006). This is most obviously relevant in the cases where a preexisting signal transduction pathway becomes recruited, via an enabling mutation, to a new use. But it almost certainly also involves certain functional groupings of TFs. The *Six-Dach-eya* functional ensemble of TFs was first identified as a key component in the fruit fly eye development network (reviewed in Kumar, 2001) but later found in muscle development (Heanue *et al.,* 1999) and then in still other developmental processes (Li *et al.,* 2003). In principle, the initial mutational event may elicit only a single activity, but that single recruited gene then induces or activates other members of the network modules. The distinction is that between "primary" and "secondary" recruitment (Wilkins, 2002). The induction of eye development in nonstandard sites by ectopic expression of *Pax6* in *Drosophila* (Halder *et al.,* 1995) is currently one of the best pieces of evidence that network module recruitment can take place in this way. The crucial point is that the recruitment of modules is made possible by the prior evolutionary-selective history that constructed and optimized performance of the network module.

The relevance of these points to organismal evolution is that it is the particular combination of network modules used that determines the composition of the entire genetic network governing a trait (Fig. 4.1) (Davidson, 2006; Wilkins, 2007b). Each module, however, is not, in itself, a rigidly delimited gene set, in either evolution or development, particularly in its downstream ("output") target genes. The set of target genes is almost certainly evolutionarily labile (Davidson, 2006) while it seems inevitable that the overall molecular machinery, with its plethora of regulatory devices (from alternative splicing to a host of posttranslational modifications), will affect the particular degree of activity of various members of the output target gene set differentially in different cellular contexts (within and between organisms).

The network module, however, is only one kind of modular unit in the genetic and regulatory machinery that influences the outcomes of genetic recruitment events. The fact that most complex proteins are made up of distinct domains, with different kinds of domains shared between members of the same or even distantly related gene families (Alberts *et al.,* 1989), constitutes a further layer of modular complexity and one that influences the behavior of those gene products that have this structure.

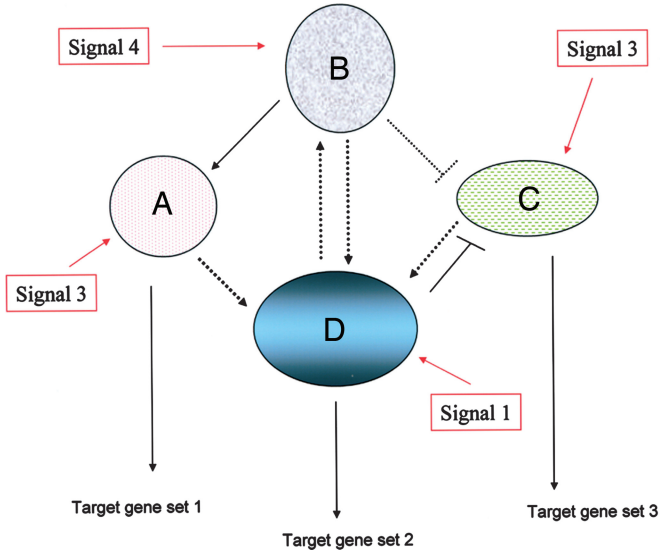

FIGURE 4.1 A schematic diagram of a simple genetic network, consisting of four component modules, signified by ovals and circles (with the component, interacting genes not indicated). Each module's expression is evoked by an external signal that can be one derived external to the cell (environmental, cell matrix-derived, hormonal, etc.) or by an internal gene product from another genetic module. Three of the modules depicted here have outputs in the form of target gene sets including cytodifferentiation gene products (*A*, *D*, and *C*) whereas one module (*B*) is purely regulatory. (Adapted from Wilkins, 2007b.)

Finally, there is the modular nature of the enhancer and silencer units that control whether and where and how long a particular gene product is expressed (reviewed in Davidson, 2001, 2006; see also Prud'homme *et al.*, 2007, Chapter 6, this volume).

Mutational events that affect either the structure or the presence/absence of particular domains within the encoded gene product or that affect the transcriptional modular units must influence how the respective genes are used in particular recruitment events. An example is the use of alternative v domains in the adhesion protein CD44 (Ruiz *et al.*, 1995), which can dramatically alter specific cellular capabilities of the expressing cells. The ubiquity of alternative splicing as a source of functionally altered proteins (Xing and Lee, 2006), however, serves as a general indicator of the importance of alterations in domain structure and composition as an input to regulatory change (Alonso and Wilkins, 2005). With respect to

transcription, the detailed structural organization of enhancer/silencer modules clearly establishes their activity as on/off switches (Davidson, 2001). In effect, mutational events at both the protein domain and transcription module levels must exert influences "upward" in the sequence of molecular interactions while selection must influence which ones are preserved and then amplified within the population in which those mutational events first occur. The relationships between these modular levels and the screening activity of natural selection is diagrammed in Fig. 4.2. If this point of view is validated by further analysis and experimental findings, it must be taken into account in the continuing debate about "levels of selection." Thus, below the level of the individual organism (the primary Darwinian "unit of selection") there are not just genes (as in the traditional levels-of-selection argument) but additional levels of genetic–molecular organization, namely genetic networks and their modules, that natural selection actively screens.

To sum up: if one accepts this view of a molecular sequence of upward interactions, with the effects of mutational events feeding through from the DNA sequence level, at the lowest modular levels, to that of network modules and networks, and selection screening downward through this

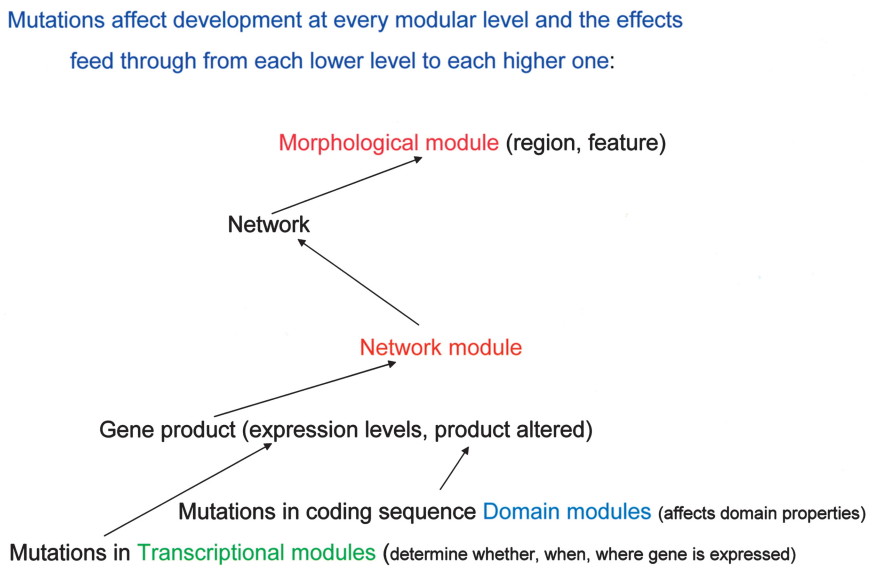

FIGURE 4.2 A diagrammatic representation of the effects of mutations at each modular level and the ways these transmit upward, within the developing organism, to affect the next modular level of organization.

hierarchy, then the nature of the gene tinkering process is seen to be much less haphazard than the process connoted by the term "bricolage," having more built-in molecular constraints yet, at the same time, lacking the goal-directed nature of a process that is implied by the term "design."

BUT IS A NETWORK PERSPECTIVE TRULY NECESSARY TO UNDERSTAND ADAPTIVE EVOLUTIONARY CHANGES?

All of the above statements can be accepted, however, without embracing the idea that the network perspective is needed for experimental research in evolution. A case can be made that a sufficient understanding of the genetic basis of adaptive evolutionary changes emerges from classic quantitative trait locus and molecular single gene-based experimental perspectives and that neither the concept of networks nor detailed knowledge of particular networks is needed for actual progress. This position is seemingly bolstered by recent success in understanding the genetic foundations of several adaptive traits, work that has underlined the key importance of a small number of specific genes. These cases involve finch beak dimensions in Darwin's finches, characteristics related directly to specific feeding adaptations (Abzhanov *et al.,* 2004, 2006); the adaptive evolution of bat wings for flight (Sears *et al.,* 2006; Weatherbee *et al.,* 2006); and the adaptive loss of pelvic armature in freshwater sticklebacks (Shapiro *et al.,* 2004; Colosimo *et al.,* 2004).

The adaptive radiation of Darwin's finches, with their different kinds of beaks suitable for different feeding adaptations, is one of the classic instances of evolutionary divergence due to natural selection (Lack, 1947). Abzhanov *et al.* (2004) have identified two key gene activities that are associated, respectively, with beak depth and width, on one hand and beak length on the other. The first characteristic, beak depth and width, is correlated with and evidently determined by an elevated level of activity of bone morphogenetic protein 4 (BMP4) during a critical phase of beak development. In contrast, finch beak length is evidently linked to a specific elevation of calmodulin activity during beak development (Abzhanov *et al.,* 2006).

The analysis of the genetic basis of bat wing evolution bears some similarity. A key component in the evolution of the distinctive wings of bats is the elevation in activity during a key phase of forelimb development in the embryo of another TGF-β activity, also a member of the BMP family, BMP2, which promotes the selective growth of the metacarpals to extend the key digits (Sears *et al.,* 2006). Making a bat wing, however, involves more than just exaggerated digit length; it also involves suppression of the waves of apoptosis that eliminate interdigital material in tetrapods with distinct digits. In the case of bats, the maintenance of

interdigital webbing, however, is due to the specific inhibition of at least three BMP activities in the post-outgrowth phase of the interdigital regions (Weatherbee *et al.,* 2006).

The finch and bat examples involve the control of differential growth and differential apoptosis (in the case of the interdigital webbing in bats) at key phases by regulation of expression of members of the TGF-β family, specifically of the BMP subfamily of this superfamily. In contrast, the loss of stickleback pelvic armature in at least three independent speciation events involves the transcriptional down-regulation of a specific key TF, namely *Pitx1*. The genetic analysis indicates that this loss of armature, which appears to be adaptive as an energy-saving measure in lakes that are essentially predator-free zones, also involves several minor loci (as determined by quantitative trait locus analysis), but the key major locus is *Pitx1*, and the loss of pelvic regional expression of this TF is due, apparently, to mutations in cis-linked enhancer modules that normally boost its specific regional activity (Shapiro *et al.,* 2004).

As informative and important as the findings of these impressive studies are, they provide only the first stage of an understanding of the respective cases. This is most obvious in the differential growth stories of finch beaks and bat wings. In these cases, the pinpointed key molecules whose changes in amount are essential for the developmental process are well known components of ubiquitously used signal transduction pathways. Those pathways are used in a host of different developmental processes, with a wide range of different phenotypic outcomes, both within and amongst the different animal systems in which they are used. Such ubiquitously used regulatory modules have been termed ''plug-ins'' by Eric Davidson (2006). It follows that identifying neither the particular plug-in module nor, even less, a particular rate-limiting component (e.g., BMP2, calmodulin, BMP4) can fully explain the developmental change that lies at the heart of the respective evolutionary change. The still missing parts of the explanation, in all of these instances, involve the genetic network of which the respective identified molecule is a part and how that network then regulates selective cell proliferation (and apoptosis in the case of the bat's interdigital webbing) in the relevant developing primordium.

At first glance, however, the loss of pelvic armature in sticklebacks seems to present quite a different situation. Ignoring, for the moment, the relatively small contributions from the minor loci that contribute to the phenotype, the key element in explaining the phenotypic change is a change in expression in one gene, namely the TF gene *Pitx1*. One does not need to understand in detail what genes *Pitx1* regulates, in specifying the development of the pelvic armature, to understand how down-regulation of its expression in selected sites leads to the loss of that structure, with

the consequent adaptive benefit in predator-free environments. If, however, one inspects the earlier part of stickleback evolutionary history and inquires about the initial gain of the pelvic armature in the stickleback lineage, one is immediately confronted with the question of how the genetic network that specifies the pelvic armature evolved. One hypothetical scenario is given in Fig. 4.3. Although the details of this scheme may well prove wrong and the picture is, at best, highly schematic, it illustrates the fact that this evolutionary change must have been a multistep process of network construction, involving several (possibly many) mutations and, presumably, either multiple or continuing selection pressures (from predation).

Although three traits in three organisms create far too small a sample from which to draw firm general conclusions, one can offer a few tentative generalizations. For new or modified evolutionary traits (e.g., finch beaks, bat wings), knowing the genetic network, with its component modules, is essential for understanding in depth what the phenotypic change actually involves. Single-gene stories, however informative in themselves, cannot provide comparable understanding. For loss of traits, however, as in the stickleback example, knowing the genetic networks responsible for those traits may be unnecessary. It is sufficient to know which genes

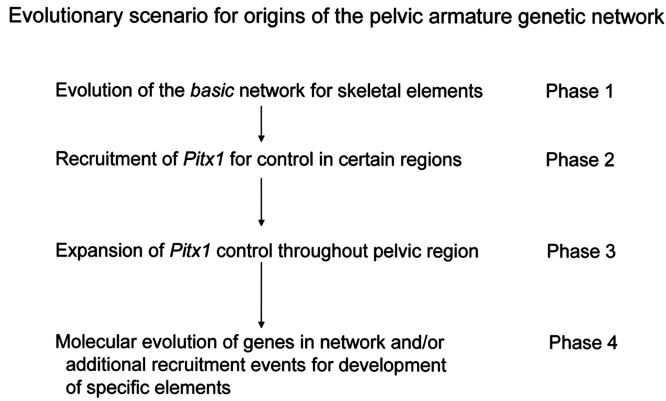

FIGURE 4.3 A tentative evolutionary scenario for the evolution of the genetic network underlying the pelvic armature of sticklebacks. The essential general feature is that it involves a stepwise (gradualistic mode) process of construction of the network. A particular aspect to note is that *Pitx1* is seen as having been recruited at an intermediary step, after evolution of the basic network/module structure for constructing skeletal elements, permitting it to have well defined regional control effects and its loss of expression to affect only that region.

in the network can be made rate-limiting for the trait to understand how their inactivation (partial or incomplete) can lead to loss of the trait. Such losses are genetically simple but can have large potential adaptive evolutionary significance. Nevertheless, loss-of-function evolutionary traits are, by definition, derivative traits. For most of the adaptive complex phenotypic traits that are of interest to evolutionists, the primary story lies in the acquisition of those traits. In turn, comprehending those evolutionary innovations requires understanding the underlying genetic networks.

USING GENETIC NETWORKS TO ASSESS RELATIVE PROBABILITIES OF MICROEVOLUTIONARY TRAJECTORIES

A network perspective, however, has further value for evolutionary biology. A detailed knowledge of genetic networks, which necessarily includes an understanding of their component modules, can provide even more: such knowledge provides a platform from which to assess the relative *a priori* probabilities of certain evolutionary trajectories. Such assessment would necessarily be approximate, but even that degree of understanding would be sufficient to allow the beginnings of a predictive approach to evolutionary trajectories, extending the potential range of hypothesis testing in evolutionary biology. It will be remembered that it was the apparent dearth of falsifiable hypotheses in evolution that led K. Popper initially to question whether evolutionary biology was truly science or simply a "metaphysical" framework of thought (Popper, 1972), although he later modified his stance (Popper, 1978).

The basis of using genetic network information to estimate relative probabilities of different evolutionary paths depends on understanding two generic properties that are shared by all developmental genetic networks. The first of these general properties is that each interactive step within a module, or between modules, is either an activation of the next gene activity (+) or an inhibition (–). This generalization is independent of the molecular mechanism involved in each such interaction and the stringency of any quantitative requirements. Thus, in a wild-type genetic network each step functions as either an activation (+) or a repression (–). In principle, therefore, one can encode each sequence of steps in a network, or network module, from the first triggering input signals, as a sequence of pluses and minuses.

The second general property concerns the structure of networks. Every network can be analytically decomposed into three kinds of elements (Wilkins, 2007b), which might be termed "functional connectivity motifs." This may not be immediately obvious from the existing genetic network diagrams, which show an initially bewilderingly complex array of lines, arrows, and bars. (See for instance the diagrams in Davidson *et*

al., 2002, and Stathopoulos and Levine, 2005.) Yet closer inspection of such diagrams validates the claim. The first of these functional connectivity patterns consists of linear sequences of action between genes, namely genetic pathways. In reality, each gene may (and most do) connect to more than one gene (both upstream and downstream), but if one follows each gene–gene link, from one to the next, a linear sequence of causal activation/inhibition steps is always found (although many gene activations, in particular, require multiple inputs from several gene products). The second set of structural elements are the functional links between the linear segments, the pathways. Again, the connecting links function as either + or – steps. The third class of element is that of feedback loops. These are either positive (+) feed-forward steps or inhibitory (–) negative feedback functions. For both sign types, such feedback loops can either involve a gene product acting on itself (or the encoding gene) or interact with other genes/gene products either upstream or downstream in the sequence.

These two generic properties of networks, namely, the +/– choice at each step and the decomposability into three structural motifs, ensures that if one knows all of the potentially rate-limiting (nonredundant) members of a network/network module, plus all of the relevant inputs (and which ones are being used in a particular developmental process), and, not least, the specific functional relationships (whether + or –) between each pair of interacting genes, one can determine whether a particular set of inputs will trigger a particular set of outputs of the whole functional unit. This principle was first noted by Kauffman (1971), who used the term "forcing structure" to denote this deterministic property of networks, but it has most recently been discussed by Davidson (2006).

This property is most easily illustrated in the abstract for the case of simple, linear pathways. Such sequences always consist of all activating (+) steps, all negative (–) steps (although this group is undoubtedly a minority class), or a mixture of + and – steps (which is almost certainly the most common category of pathways). These are illustrated in Fig. 4.4 for the putative wild-type situation in each case. In addition, the figure shows the effect of a complete loss-of-function mutation in an early ("upstream") gene of each kind of pathway. For all three pathway types, the effect of such a mutation is the complete r*eversal of sign* of all of the following ("downstream") gene activities. Thus, not only does the pathway structure allow one to predict outcomes in the wild-type case if one knows which inputs have been applied in any instance, but it also allows you to predict the effect of loss-of-function mutations on pathway/module output. In contrast, the effects of gain-of-function mutations are less predictable, at least where it is a + step that is affected in a pathway that has already been triggered. If, however, the activation step caused by a constitutive gain-

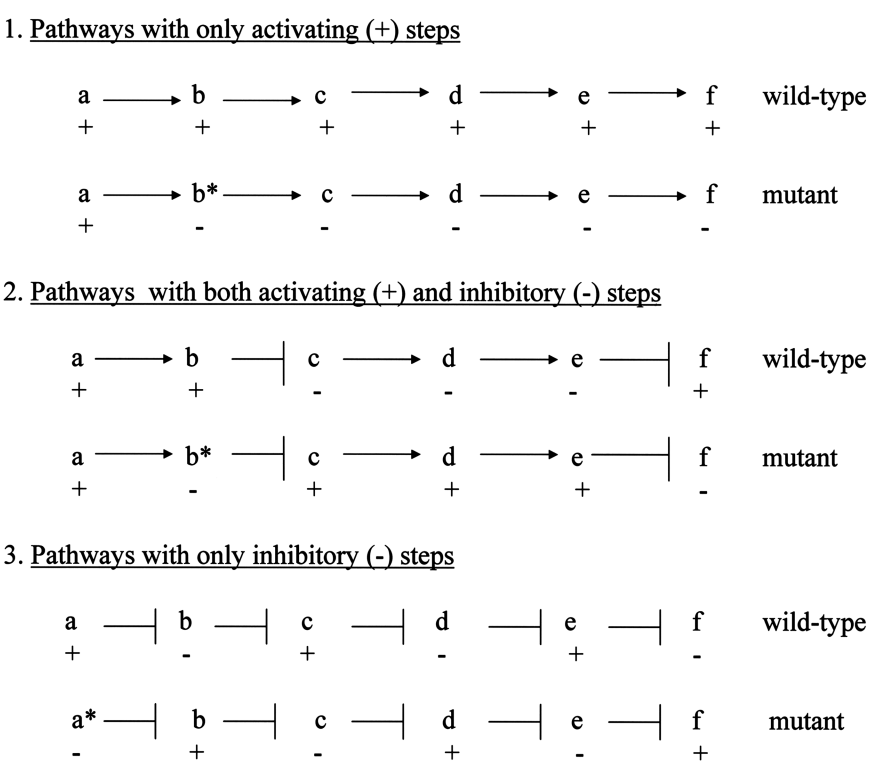

FIGURE 4.4 Three kinds of genetic pathways and the effects on their outputs from upstream loss-of-function mutations. In pathway 1, all of the steps in the pathway are activation (+) events; in pathway 2, the causal chain of gene expression events involves both activation and inhibition (–) steps; in pathway 3, all of the steps are inhibitory (–). For all three kinds of pathway, the effect of an upstream loss-of-function mutation is to change the sign of activity for all successive downstream activities, from + to – or from – to +. [Reproduced with permission from Wilkins (2007b) (Copyright 2007, Novartis).]

of-function mutation occurs in a gene in a previously inactive pathway, such gain-of-function mutations will, in principle, lead to ectopic activations of all steps downstream of the affected gene. (Whether this happens will depend, in part, on what other kinds of regulatory mechanisms are in operation in those cells and how they functionally link to the activated downstream functions.)

Pathways are, indeed, relatively simple structures, but similar reasoning can be used to predict outputs in functionally linked pathway

situations as well, such cross-linked pathways constituting the simplest kind of network situation. For instance, a loss-of-function mutation in a pathway upstream of a cross-inhibitory step should, in principle, activate the linked pathway below the point of functional linkage in the second pathway (Wilkins, 2007b).

This possibility, of predicting phenotypic outcomes from mutations in well characterized pathways, is, of course, only a potential for future work. At present, there is insufficient knowledge of any network, and of few network modules, to allow this kind of analysis. Furthermore, knowing the probability of a developmental outcome is only the beginning of estimating the chances of a particular evolutionary trajectory, which will be influenced at many steps by selective opportunities, genetic drift, the occurrence of rare external disasters (e.g., mass extinctions), or other chance events. Yet, knowing which phenotypic outcomes are more likely than others would provide a first step toward assessing the likelihood of certain trajectories vs. others. That there are certain propensities toward certain evolutionary trajectories is shown by the numerous instances of parallel evolution in related lineages that evolutionists have found. Although the traditional emphasis to explain this phenomenon is similarity of selective pressures, there must also be some inherent biases built into the genetics and development of the branching lineages that display it (Wilkins, 2004). Even instances of convergent evolution, e.g., the independent origination of shearing teeth in carnivorous marsupials and in the placental carnivores, may well involve the independent activation of highly conserved network modules within similar cellular developmental contexts in the unrelated lineages.

Clearly, evolutionary biology will never have the capacity for exact, flawless "retrodiction" (explaining why past events occurred in a certain way), with consequent capacity for formulating falsifiable hypotheses, on which a strict Popperian might insist. A network perspective, however, when coupled with the kind of detailed knowledge of network modules and networks, can, in principle, move the science of evolution toward a somewhat more predictive capability. Without such, it is difficult to see how the kinds of studies focusing on single genes or small numbers of genes can ever have the kind of full explanatory capability that, ideally, evolutionary biology should aim for.

Given the plethora of networks and morphologies, however, that there are to explore and the costs (in money, time, and sheer hard work) of characterizing developmental genetic networks (Wilkins, 2007b), the prospects sketched above might seem so distant as to be unachievable in the foreseeable future. Yet a simple consideration of microevolutionary morphological patterns suggests which networks and network modules might be most profitably explored. This number is considerably smaller

than the totality of possibilities. Specifically, when one looks at any group of animals, at the genus or family level, what strikes one is that the great majority of variations are in either growth properties (regional, appendage, or whole-organism level) or color patterns, or both. There are, of course, major structural novelties, which distinguish groups separated by large macroevolutionary distances and whose evolutionary origins demand an explanation (Muller and Newman, 2005). Yet, at the microevolutionary level, most speciation events, when reflected in or correlated with certain morphological characteristics, involve differential growth and/or color patterning. When one considers the high degree of functional conservation of basic patterning mechanisms in general (Carroll *et al.*, 2001; Wilkins, 2002), it seems not unreasonable that growth and pigmentation may also exhibit a degree of conservation in the immediately upstream genetic network modules that govern them.

The analysis of growth is, of course, a major subject area in biology, involving such disciplines as cell biology, traditional developmental biology, developmental genetics, and cancer biology. Yet there is still a relative dearth of information about the connections between developmental patterning mechanisms for specific structures and regions and the growth controls that directly regulate the rate and extent of cell proliferation in those regions. Nevertheless, progress is also being made in this area, and some of the relevant networks are beginning to be elucidated (Cho *et al.*, 2006). How widely conserved such networks might be and whether there are widely conserved network modules in the metazoa for evoking pigmentation patterns is not known, but these are at least possibilities and can be investigated. My principal suggestion here is that analyses of the networks or network modules that link developmental patterning mechanisms to growth and pigmentation patterns could have special importance in understanding the genetic basis of many microevolutionary-scale events. Furthermore, simple calculations involving gene size and mutation rate indicate that even relatively modest-sized genetic networks, found in organisms of moderately sized populations, should harbor significant standing variation of potential phenotype-changing capacity (Wilkins, 2007b). This sort of quantitative consideration further illustrates the value of, and need for, a network-based perspective on evolution.

CONCLUSIONS

This article has explored the ways that evolutionary trajectories are influenced by (*i*) the properties of gene products, (*ii*) the on/off switches that control transcription of individual genes, and (*iii*) the structured properties of the regulatory ensembles we know as genetic network modules. It would be overstating the case to call the biases created by these selection-

honed properties ''constraints,'' which connotes strong barriers, but it seems certain that these properties must exert some preferential influence or channeling effects at the start of every evolutionary departure. Such molecular attributes make possible the kinds of ''facilitated variation'' described by Gerhart and Kirschner (Chapter 3, this volume). Their perspective is both fully consistent with and complementary to the one sketched here.

The central point of this chapter, however, is that a knowledge of the network modules that constitute particular genetic networks, underlying specific developmental processes in particular organisms (hence, their morphological traits), can greatly enrich understanding of the ways in which particular genetic changes promote particular developmental changes. Furthermore, an appreciation of the generic properties of networks and the ways that they transmit effects along functional linear pathways can, when the knowledge of the composition of a network and its inputs and outputs is reliable, lead to predictions about the effects of mutations within network modules on eventual phenotypes. With this sort of analytical framework in place, evolutionary biology will possess a greater degree of predictive capability and potential for the falsification of hypotheses than has hitherto been possible.

5

The Frailty of Adaptive Hypotheses for the Origins of Organismal Complexity

MICHAEL LYNCH

The vast majority of biologists engaged in evolutionary studies interpret virtually every aspect of biodiversity in adaptive terms. This narrow view of evolution has become untenable in light of recent observations from genomic sequencing and population-genetic theory. Numerous aspects of genomic architecture, gene structure, and developmental pathways are difficult to explain without invoking the nonadaptive forces of genetic drift and mutation. In addition, emergent biological features such as complexity, modularity, and evolvability, all of which are current targets of considerable speculation, may be nothing more than indirect by-products of processes operating at lower levels of organization. These issues are examined in the context of the view that the origins of many aspects of biological diversity, from gene-structural embellishments to novelties at the phenotypic level, have roots in nonadaptive processes, with the population-genetic environment imposing strong directionality on the paths that are open to evolutionary exploitation.

Although biologists have always been concerned with complex phenotypes, the matter has recently become the subject of heightened speculation, as a broad array of academics, from nearly every branch of science other than evolutionary biology itself, claim to be in

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possession of novel insights into the evolution of complexity. The claims are often spectacular. For example, Kirschner and Gerhart (2005) argue that evolutionary biology has been "woefully inadequate" with respect to understanding the origins of complexity and promise "an original solution to the long-standing puzzle of how small random genetic change can be converted into complex, useful innovations." However, this book and many others like it (e.g., Depew and Weber, 1985; Kauffman, 1993; Carroll, 2005a; Davidson, 2006) provide few references to work done by evolutionary biologists, making it difficult to understand the perceived areas of inadequacy, and many of the ideas promoted are known to be wrong, making it difficult to appreciate the novelty. Have evolutionary biologists developed a giant blind spot; are scientists from outside of the field reinventing a lot of bad wheels; or both?

Evolutionary biology is treated unlike any science by both academics and the general public. For the average person, evolution is equivalent to natural selection, and because the concept of selection is easy to grasp, a reasonable understanding of comparative biology is often taken to be a license for evolutionary speculation. It has long been known that natural selection is just one of several mechanisms of evolutionary change, but the myth that all of evolution can be explained by adaptation continues to be perpetuated by our continued homage to Darwin's treatise (1859b) in the popular literature. For example, Dawkins' (1976, 1986, 1996) agenda to spread the word on the awesome power of natural selection has been quite successful, but it has come at the expense of reference to any other mechanisms, a view that is in some ways profoundly misleading. There is, of course, a substantial difference between the popular literature and the knowledge base that has grown from a century of evolutionary research, but this distinction is often missed by nonevolutionary biologists.

The goal here is to dispel a number of myths regarding the evolution of organismal complexity (Table 5.1). Given that life originated from inorganic matter, it is clear that there has been an increase in phenotypic complexity over the past 3.5 billion years, although long-term stasis has been the predominant pattern in most lineages. What is in question is whether natural selection is a necessary or sufficient force to explain the emergence of the genomic and cellular features central to the building of complex organisms.

NOTHING IN EVOLUTION MAKES SENSE EXCEPT IN LIGHT OF POPULATION GENETICS

Although the basic theoretical foundation for understanding the mechanisms of evolution, the field of population genetics, has long been in place, the central significance of this framework is still occasionally

TABLE 5.1 A Summary of Some Common Misconceptions About Evolution and Complexity, and Contrasting Views

Myth	Reality
1. Evolution is natural selection.	Natural selection is just one of four primary evolutionary forces.
2. Characterization of interspecific differences at the molecular and/or cellular levels is tantamount to identifying the mechanisms of evolution.	The resources deployed in evolutionary change reside at the molecular level, but whereas the cataloging of such differences at the interspecific level identifies the end products of evolution, it does not reveal the population-genetic processes that promoted such change.
3. Microevolutionary theory based on gene-frequency change is incapable of explaining the evolution of complex phenotypes.	No principle of population genetics has been overturned by an observation in molecular, cellular, or developmental biology, nor has any novel mechanism of evolution been revealed by such fields.
4. Natural selection promotes the evolution of organismal complexity.	There is no evidence at any level of biological organization that natural selection is a directional force encouraging complexity. In contrast, substantial evidence exists that a reduction in the efficiency of selection drives the evolution of genomic complexity.
5. Natural selection is the only force capable of promoting directional evolution.	Both mutation and gene conversion are nonrandom processes that can drive the patterning of genomic evolution in populations with sufficiently small effective sizes (common in multicellular lineages).
6. Genetic drift is a random process that leads to noise in the evolutionary process, but otherwise leaves expected evolutionary trajectories unaltered.	By reducing the efficiency of selection, random genetic drift imposes a high degree of directionality on evolution by increasing the likelihood of fixation of deleterious mutations and decreasing that of beneficial mutations.
7. Mutation merely creates variation, whereas natural selection promotes specific mutant alleles on the basis of their phenotypic effects.	Mutation operates as a weak selective force by differentially eliminating alleles with structural features that magnify mutational target sizes.

continued

TABLE 5.1 Continued

Myth	Reality
8. Phenotypic and genetic modularity are direct products of natural selection.	There is no evidence that the modular structure of gene regulatory regions or genetic networks is directly advanced by selective mechanisms. However, the processes of duplication, degenerative mutation, and random genetic drift can lead to the passive emergence of modularity in populations with genetic effective sizes of the magnitude found in multicellular species.
9. Natural selection promotes the ability to evolve.	There is no evidence that phylogenetic variation in the pathways open to evolutionary exploration is anything more than a by-product of physical processes that passively arise with expansions in genome size and generation length. There are no abrupt transitions in aspects of genomic architecture or gene structure between unicellular and multicellular species, nor between viruses, prokaryotes, and eukaryotes.

questioned, as exemplified in this quote from Carroll (2005a), "Since the Modern Synthesis, most expositions of the evolutionary process have focused on microevolutionary mechanisms. Millions of biology students have been taught the view (from population genetics) that 'evolution is change in gene frequencies.' Isn't that an inspiring theme? This view forces the explanation toward mathematics and abstract descriptions of genes, and away from butterflies and zebras. . . . The evolution of form is the main drama of life's story, both as found in the fossil record and in the diversity of living species. So, let's teach that story. Instead of 'change in gene frequencies,' let's try 'evolution of form is change in development'." Even ignoring the fact that most species are unicellular and differentiated mainly by metabolic features, this statement illustrates two fundamental misunderstandings. Evolutionary biology is not a story-telling exercise, and the goal of population genetics is not to be inspiring, but to be explanatory. The roots of this contention are fourfold.

First, evolution is a population-genetic process governed by four fundamental forces. Darwin (1859b) articulated one of those forces, the process of natural selection, for which an elaborate theory in terms of genotype frequencies now exists (Crow and Kimura, 1970; Bürger, 2000). The remaining three evolutionary forces are nonadaptive in the sense that they are not a function of the fitness properties of individuals: mutation is the ultimate source of variation on which natural selection acts, recombi-

nation assorts variation within and among chromosomes, and genetic drift ensures that gene frequencies will deviate a bit from generation to generation independent of other forces. Given the century of work devoted to the study of evolution, it is reasonable to conclude that these four broad classes encompass all of the fundamental forces of evolution.

Second, all four major forces play a substantial role in genomic evolution. It is impossible to understand evolution purely in terms of natural selection, and many aspects of genomic, cellular, and developmental evolution can only be understood by invoking a negligible level of adaptive involvement (Kimura, 1983; Lynch, 2007). Because all three nonadaptive forces of evolution are stochastic in nature, this conclusion raises some significant technical challenges. It is tempting to think that stochastic processes have no implications for the direction of evolution. However, the effects of mutation and recombination are nonrandom, and by magnifying the role of chance, genetic drift indirectly imposes directionality on evolution by encouraging the fixation of mildly deleterious mutations and discouraging the promotion of beneficial mutations.

Third, the field of population genetics is now so well supported at the empirical level that the litmus test for any evolutionary hypothesis must be its consistency with fundamental population-genetic principles. Grounded in basic Mendelian processes and sampling theory, many of these principles were laid down before the elucidation of the structure of DNA. Shortly after the genetic code was cracked, a series of technological breakthroughs advanced our ability to reveal molecular variation: protein sequencing in the 1950s, surveys of protein variants in the 1960s, ribosomal RNA sequencing in the 1970s, gene sequencing in the 1980s, and whole-genome sequencing in the 1990s. Each of these episodes brought the need for new methods for analysis and interpretation, and in each case the framework was drawn largely from preexisting population-genetic theory. Thus, although we do not yet fully understand the connections between evolution at the molecular and phenotypic levels, we can be confident that the machinery to do so is in place.

Fourth, some attempts to marginalize the contributions of population genetics to our understanding of evolution have pointed to the "bean bag" genetics debate that occurred in the middle of the last century (see Felsenstein, 1975). However, this is a misunderstanding, as the tensions during this period were not about the population-genetic basis of evolutionary change, but about the need to incorporate epistasis into the existing framework, something that population geneticists have now invested heavily in (Wolf *et al.,* 2000; Carter *et al.,* 2005). From the standpoint of its phenotypic products, evolution is more than a change in gene frequencies. Organisms are more than the sum of their parts, just as genes are more than the sum of their functional components. But if we are concerned with

the process of evolutionary change, then evolution is indeed a change in genotype frequencies.

In summary, population genetics provides an essential framework for understanding how evolution occurs, grounding us in reality by clarifying the pathways that are open to evolutionary exploitation. To quote Carroll (2005a) again, "Simplification may indeed be necessary for news articles, but it can distort the more complex and subtle realities of evolutionary patterns and mechanisms."

INTERNAL VERSUS EXTERNAL EVOLUTIONARY FORCES

The literature is permeated with dogmatic statements that natural selection is the only guiding force of evolution, with mutation creating variation but never controlling the ultimate direction of evolutionary change (for a review, see Stoltzfus, 2006a). This view derives from two types of arguments. First, hundreds of artificial selection experiments have generated changes in mean phenotypes well beyond the observed range in the base population in just a few dozen generations (Falconer and Mackay, 1996), inspiring the view that quantitative variation is distributed over an effectively infinite number of loci with minuscule effects (Kimura, 1965; Lande, 1975; Bulmer, 1980). Second, much of the earliest work in theoretical population genetics downplayed the ability of mutation to overcome the force of selection (Fisher, 1930; Haldane, 1932). Both arguments ignore significant complications that arise in finite populations, and it is now known that genome composition is governed by biases in mutation and gene conversion, some of which (e.g., mobile-element proliferation) operate via internal drive-like mechanisms (Lynch, 2007).

The notion that mutation pressure can be a driving force in evolution is not new (Darwin, 1859b, 1866; Morgan, 1925; Dover, 1982; Nei, 1987, 2005; Cavalier-Smith, 1997; Yampolsky and Stoltzfus, 2001; Stoltzfus, 2006b), and the conditions that must be fulfilled if mutation is to alter the direction of evolution relative to adaptive expectations are readily derived. Consider two alternative states at a locus, **A** and **a**, with the mutation rate of $\mathbf{a} \rightarrow \mathbf{A}$ being m times that of $\mathbf{A} \rightarrow \mathbf{a}$, but with type **A** having a fractional selective advantage s over type **a**. Further progress requires that we specify the effective number of gene copies per locus at the population level. This quantity, N_g, which is equivalent to the effective size of a haploid population and approximately twice that for an outcrossing diploid species, is influenced by many factors, including the breeding system, temporal fluctuations in population size, and the level of recombination (which influences the sensitivity of a locus to spurious hitch-hiking effects), and is generally orders of magnitude smaller than the absolute number of reproductive adults in a population (Lynch, 2007). With these definitions in

hand, standard theory (Kimura, 1983) shows that the fixation probability of a mutation to **A** is e^S times that for a mutation to **a**, where $S = 2\,N_g s$ is twice the ratio of the power of selection to the power of random genetic drift ($1/N_g$). Because the population-level rate of transition from one allelic type to another is equal to the product of the mutation rate and the fixation rate, the ratio of probabilities of being in states **A** versus **a** at selection-mutation-drift equilibrium is simply me^S (Fig. 5.1). This simple expression leads to two general conclusions: (*i*) regardless of the strength of selection, if $1/N_g >> |s|$, the population will be driven to a state expected under mutation pressure alone; and (*ii*) the equilibrium composition of a population depends not on the absolute power of mutation, but on the relative rates of forward and reverse mutations (the mutation bias).

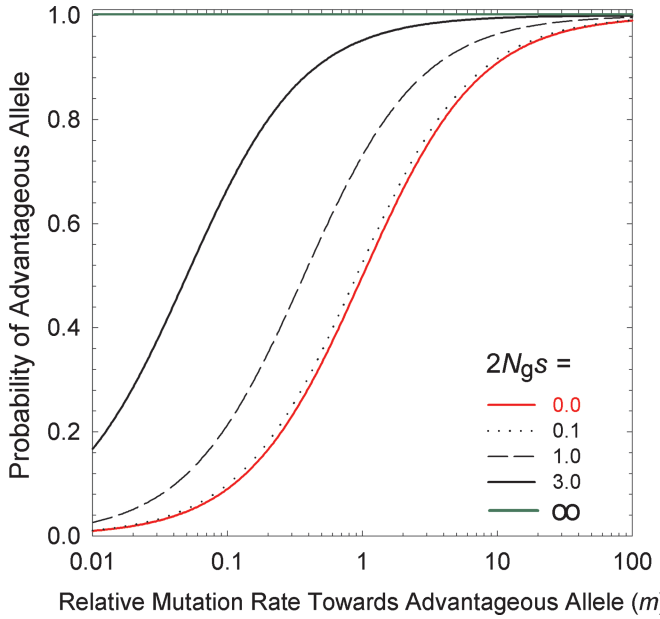


FIGURE 5.1 The long-term probability that an allele residing at a biallelic locus will be of the selectively advantageous type, given a selective advantage s, an effective population number of gene copies of N_g, and a mutation rate to the beneficial allele m times that in the reverse direction. The lowest curve ($2\,N_g s$ = 0.0) denotes neutrality, whereas the upper line ($2N_g s = \infty$) denotes an effectively infinite population, such that genetic drift is a negligible force.

THE PASSIVE EMERGENCE OF GENOME COMPLEXITY BY NONADAPTIVE PROCESSES

Most biologists are so convinced that all aspects of biodiversity arise from adaptive processes that virtually no attention is given to the null hypothesis of neutral evolution, despite the availability of methods to do so (Lande, 1976; Lynch and Hill, 1986; Lynch, 1994). Such religious adherence to the adaptationist paradigm has been criticized as being devoid of intellectual merit (Gould and Lewontin, 1979), although the field of molecular evolution has long been obsessed with potential for the "nearly neutral" accumulation of very slightly deleterious mutations (Ohta, 1972, 1973, 1974). The condition for near-neutrality is fulfilled when the ratio of the powers of selection and drift is substantially <1, i.e., $|2N_g s| << 1$.

This simple relationship has considerable utility in attempts to evaluate the potential role of nonadaptive mechanisms in the evolution of genomic architecture (Lynch and Conery, 2003; Lynch, 2006, 2007). Striking phylogenetic variation exists at the level of gene and genomic architecture. The genomes of multicellular eukaryotes are invariably packed with mobile elements, and individual genes are generally subdivided by multiple introns, harbor multiple transcription-factor binding sites, and are transcribed into units containing substantial untranslated flanking sequences. In contrast, prokaryotic genomes are usually nearly completely devoid of mobile elements and introns and have genes with very simple regulatory structures, often transcribed into polycistronic units (operons) with negligible leader and trailer sequences. Most unicellular eukaryotic genomes exhibit structural features on a continuum between these two extremes.

Understanding the origins of eukaryotic genome complexity in adaptive terms is rendered difficult by the fact that each embellishment added to a gene magnifies its vulnerability to mutational inactivation, thereby encouraging its elimination from the population (Lynch 2006, 2007). If a particular embellishment requires that n nucleotides be conserved for proper gene function, the mutational disadvantage is nu, where u is the mutation rate per nucleotide site. Each intron added to a protein-coding gene requires that $n \approx 30$ nucleotide sites within the intron and adjacent exons be reserved for proper spliceosomal recognition (Lynch, 2002). Transcription-factor binding sites are ≈10 bp in length (e.g., Gasch *et al.,* 2004), so the addition of each such site to a gene increases the degenerative mutation rate by $\approx 10u$. The average eukaryotic 5′ UTR increases the degenerative mutation rate by $\approx 4u$ by providing substrate for mutations to premature translation-initiation codons (Lynch *et al.,* 2005). Even completely nonfunctional intergenic DNA is a mutational hazard because it serves as a substrate for inappropriate gain-of-function mutations (Hahn *et al.,* 2003).

Numerous observations suggest that the amount of regulatory DNA associated with the average gene in a multicellular species is at least as great as the length of the coding region, and such genes typically also contain ~5–7 introns (Lynch, 2007). Thus, the average mutational target sizes of genes in multicellular eukaryotes are more than two to three times those for prokaryotes. To become established, the modifications that led to such mutational hazards must have either had a substantial immediate selective advantage or arisen in populations with effective sizes sufficiently small to render them immune to selection. Letting $s = nu$, the latter condition requires that $2N_g nu < 1$, and because the mutational cost of individual modifications is small, generally $<30u$, it is difficult to reject the hypothesis that incremental expansions of eukaryotic gene complexity were largely driven by nonadaptive processes.

More formal justification for this claim derives from molecular population-genetic data. Assuming the silent sites of protein-coding genes to be effectively neutral, the average number of silent-site substitutions between randomly sampled nucleotide sites within a population is a function of the rates of input of new variation by mutation ($2u$) and loss by genetic drift ($1/N_g$). At mutation-drift equilibrium, the expected level of silent-site divergence within a population is equal to the ratio of these rates, $2N_g u$. Average estimates of $2N_g u$ are 0.104 for prokaryotes, 0.057 for unicellular eukaryotes, 0.026 for invertebrates, 0.015 for land plants, and 0.004 for vertebrates (Lynch, 2006). Because some weak selective forces may operate on silent sites, the average estimates of $2N_g u$ for unicellular species (with high N_g) are almost certainly downwardly biased. Thus, rearranging the criterion for effective neutrality to $2N_g u << 1/n$, an embellishment that increases the mutational target size of a vertebrate gene by $n < 250$ will be largely immune from selection, and hence free to drift to fixation, whereas the critical value of n for a prokaryote is $<<10$.

These observations indicate that the paths open to evolutionary exploration are fundamentally different between unicellular and multicellular species for reasons completely unassociated with organismal complexity. Because of their relatively small N_g, multicellular species are expected to accumulate gratuitous gene-structural changes without any direct selection for them and to become laden with other deleterious genomic features (Lynch and Conery, 2003; Lynch, 2007). In contrast, unicellular lineages are expected to maintain streamlined genomes, not necessarily for metabolic reasons, but because of the exceptionally high efficiency of selection opposing the accumulation of mutationally hazardous DNA.

The hypothesis that expansions in the complexity of genomic architecture are largely driven by nonadaptive evolutionary forces is capable of explaining a wide range of previously disconnected observations (Lynch, 2006, 2007) (Table 5.2). This theory may be viewed as overly simplistic.

TABLE 5.2 Aspects of Gene and Genomic Architectural Evolution That Appear to Be Explainable Only After Accounting for Variation in the Relative Power of Nonadaptive Evolutionary Forces

Genomic streamlining in microbial species versus genome bloating in multicellular lineages.
Nucleotide composition variation within and among genomes: genomewide A/T composition, strand asymmetry, isochores, and codon-usage bias in unicellular species.
Differential proliferation of mobile elements in unicellular versus multicellular species.
Gene number: preservation of duplicate genes by degenerative mutations (subfunctionalization).
Origin of the spliceosome by subfunctionalization and proliferation of introns in lineages of multicellular species.
Expansion of UTRs of the messenger RNAs of eukaryotes.
Origin of modular regulatory regions in eukaryotic genes.
Demise of operons in eukaryotes.
Variation in organelle genome architecture: lean in animals; bloated in land plants.
Messenger RNA editing in plant organelle genomes.
Restriction of sex chromosomes to multicellular lineages.

However, simply making the counterclaim that natural selection is all powerful (without any direct evidence) is not much different from invoking an intelligent designer (without any direct evidence). If a successful adaptive counterargument is to be mounted, simpler nonadaptive models must be shown to be inadequate, and to accomplish that, something must be known about the expected pattern of evolution in the absence of selection. If nothing else, the ideas presented above provide the basis for a null model for genomic evolution. Certainly, many of the above-mentioned embellishments of eukaryotic genes have adaptive functions in today's multicellular species, but observations on current deployment may have little bearing on matters of initial origins. Most of the repatterning of the genomic real estate of eukaryotes occurred before the evolution of multicellularity (Lynch, 2007).

ARE THE ORIGINS OF ORGANISMAL COMPLEXITY ALSO ROOTED IN NONADAPTIVE PROCESSES?

Multicellularity is widely viewed as a unique attribute of eukaryotes, somehow made possible by the origin of a more complex cellular architecture and, without question, with the assistance of natural selection. However, it is difficult to defend this assertion in any formal way. Complex, multicellularity has only arisen twice, once in animals and once in vascular plants. One might add fungi to the list, although the number of fungal cell types is not large, and there is some question as to whether multicellularity was ancestral to the phylogenetic group that contains animals, fungi, and slime molds. In any event, the probability that two or three origins of multicellularity simply arose by chance within eukaryotes as opposed to prokaryotes is somewhere on the order of 1/4 to 1/2, well below the general standards of statistical validity. Of course, many other eukaryotes are capable of producing a few different cell types, but the same is true for prokaryotes, some of which produce radically different cell morphologies.

Nevertheless, King (2004) states that ''this historical predisposition of eukaryotes to the unicellular lifestyle begs the question of what selective advantages might have been conferred by the transition to multicellularity;'' and Jacob (1977) argues that ''it is natural selection that gives direction to changes, orients chance, and slowly, progressively produces more complex structures, new organs, and new species.'' The vast majority of biologists almost certainly agree with such statements. But where is the direct supportive evidence for the assumption that complexity is rooted in adaptive processes? No existing observations support such a claim, and given the massive global dominance of unicellular species over multicellular eukaryotes, both in terms of species richness and numbers of individuals, if there is an advantage of organismal complexity, one can only marvel at the inability of natural selection to promote it. Multicellular species experience reduced population sizes, reduced recombination rates, and increased deleterious mutation rates, all of which diminish the efficiency of selection (Lynch, 2007). It may be no coincidence that such species also have substantially higher extinction rates than do unicellular taxa (Raup, 1978; Stanley, 1985).

Although some aspects of the roots of the cellular interactions that constitute development are likely to reside in the resolution of adaptive conflicts between the advantages of cell–cell cooperation versus going it alone (e.g., Maynard Smith and Szathmáry, 1995; Michod, 1999), it need not follow that natural selection is a sufficient force for the exit from the unicellular world. Many developmental genes previously thought to have originated in the vertebrate lineage, owing to their absence in arthropods and nematodes, are now known to be present in basal lineages of animals

lacking mesoderm (the cnidarians), and by inference must have simply been lost from various invertebrate phyla (Technau *et al.,* 2005). Numerous examples of morphological simplification exist in animals (e.g., limb loss in lizards and salamanders, coelom loss in nematodes, and mouth and anal loss in hydrothermal-vent worms), and a plausible, albeit controversial, case has even been made that prokaryotic cell architecture is a simplified derivative of that of eukaryotes (Kurland et al., 2006).

Could nonadaptive processes have played a role in the evolution of something as intricate as cell architecture or developmental complexity? There are at least two ways by which such a cascade of events might be precipitated. First, intrinsically deleterious genome-level changes, such as those resulting from intron and mobile-element proliferation, must impose selection pressures for cellular defense mechanisms. It has been argued that by imposing a need to process mRNAs before their exposure to ribosomes, the establishment of spliceosomal introns provided the evolutionary pressure that led to the origin of the nuclear membrane (Lopez-Garcia and Moreira, 2006; Martin and Koonin, 2006), and Koonin (2006) has further suggested that the nonsense-mediated decay (NMD) and ubiquitin signaling pathways evolved as secondary mechanisms for minimizing the accumulation of aberrant transcripts and proteins resulting from splicing errors. This line of thinking could be taken in a number of additional directions. For example, the assembly of spliceosomal subunits occurs within intranuclear Cajal bodies (Stanek and Neugebauer, 2006), and aberrant transcripts flagged by the NMD pathway are degraded in cytoplasmic P bodies (Sheth and Parker, 2006). The nature of cause and effect in these relationships is difficult to resolve, as all hypothetical lines of defense against introns appear to have been present in the stem eukaryote (Lynch *et al.,* 2006), raising the possibility that the colonization of nuclear genes by introns followed the origin of permissive cellular features, rather than the other way around. Nevertheless, the idea that internal constraints played a role in cellular evolution is secure under either scenario.

Second, because cellular and developmental features reflect the transformation of gene-level information into gene expression, the opportunities for phenotypic evolution must ultimately be constrained by the physical resources existing at the genomic level, which as noted above are strongly influenced by nonadaptive aspects of the population-genetic environment. Reductions in N_g are expected to lead to increases in intron number and size, expansions in UTR lengths, losses of operons, the modularization of regulatory regions, and the preservation of duplicate genes by subfunctionalization (among other things; Table 5.2). Thus, as will be discussed more fully below, to the extent that an increase in gene-architectural complexity is a precondition for the emergence of greater complexity at the organismal level (including the hallmarks of multicellularity: multiple cell

types, complex patterns of gene expression, and mechanisms of cell signaling), a long-term synergism may exist between nonadaptive evolution at the DNA level and adaptive evolution at the phenotypic level. There is no need to abandon the idea that many of the external morphological and/or behavioral manifestations of multicellularity in today's organisms are adaptive. However, if the view promoted above is correct, the relatively simple phenotypes of the Earth's smallest organisms is not an inevitable outcome of the prokaryotic body plan nor a reflection of selection for metabolic efficiency, but an indirect consequence of the barrier to the passive emergence of genomic complexity imposed by high N_g.

In summary, the near-complete absence of the concept of nonadaptive processes from the lexicon of those concerned with cellular and developmental evolution does not reflect any formal demonstration of the negligible contribution of such mechanisms, and indeed, there is no fundamental reason why development should be uniquely immune to nonadaptive evolutionary forces. One could even argue that the stringency of natural selection is reduced in complex organisms with behavioral and/or growth-form flexibilities that allow individuals to match their phenotypic capabilities to the local environment. Some of these shortcomings have recently attracted attention, and a scaffold for connecting evolutionary genetics, genomics, and developmental biology is slowly beginning to emerge (Johnson and Porter, 2000, 2001; Stern, 2000; True and Haag, 2001; Delattre and Felix, 2002; Rockman and Wray, 2002; Wray *et al.,* 2003; Force *et al.,* 2005).

THE PASSIVE EMERGENCE OF MODULAR GENE INTERACTIONS

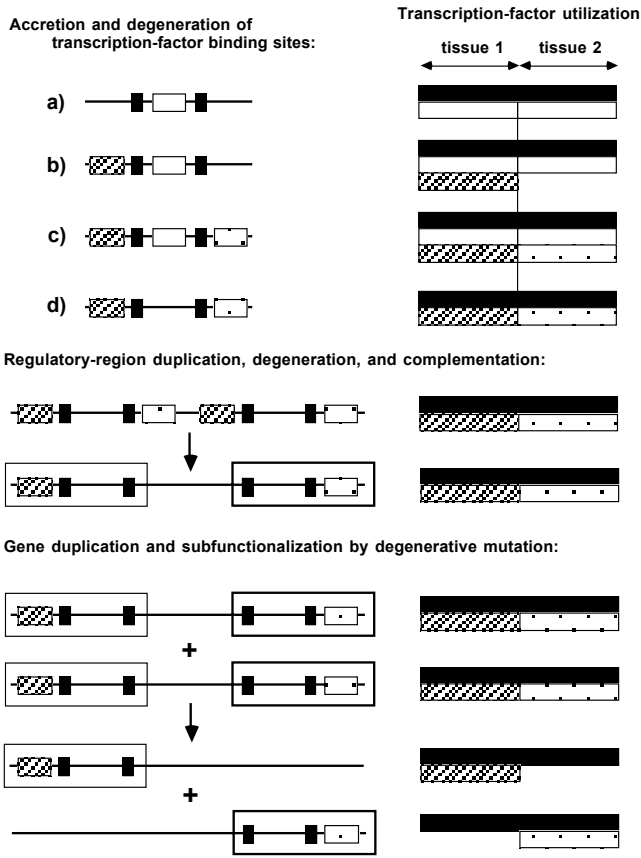
King and Wilson (1975) inspired the view that modularity and repatterning of regulatory-element utilization are the central determinants of the evolution of organismal complexity (Carroll, 2005a,b; Davidson, 2006). Although this view is not universally accepted even among developmental biologists (Alonso and Wilkins, 2005), because development always involves cross-talk between gene products, one must start with a consideration of the origins of the mechanisms that allow such transactions to take place. There is no evidence that gene-regulatory modules associated with complex functions arise as *de novo* integrated units, although some biologists seem to feel otherwise (Davidson and Erwin, 2006). Rather, like all aspects of evolution, the origins of changes in genetic pathways must be a function of descent with modification. Mutant alleles arise independently at individual loci, with features being defined by prior historical contingencies. Thus, although the idea that regulatory modules with functional significance in today's organisms can only have arisen via natural

selection is seductive, it remains to be determined how the stepwise alterations necessary for the construction of genetic pathways come about. The following is a preliminary outline of one approach to understanding the evolution of organismal complexity via the development of a formal population-genetic framework for the emergence of alternative forms of gene interactions.

Although compelling adaptive arguments are always accompanied by a formal rejection of simpler nonadaptive hypotheses, credible null hypotheses have rarely been pursued in evolutionary developmental biology. This concern is not trivial, as it has been shown that modular gene-regulatory structures (with unique transcription factors governing expression in different spatial/temporal contexts) can emerge passively, without any direct selection for modularity *per se*, starting from an initial state in which the entire expression breadth of the gene is under unified control (Force *et al.*, 2005). Under the scenario outlined in the top two tiers of Fig. 5.2, during the entire transition to a modular form of gene regulation, the spatial/temporal pattern of gene expression remains constant, with only the underlying molecular mechanisms for achieving this fixed pattern being modified. Thus, the new regulatory architecture emerges beneath a constant phenotype, without any bottleneck in fitness during the transitional phase of mixed genotypes. Such neutral transitions may help explain apparent cases of "developmental system drift," whereby closely related species achieved similar morphological structures by substantially different mechanisms (Sommer, 1997; Johnson and Porter, 2000; Ludwig *et al.*, 2000; True and Haag, 2001; Ruvinsky and Ruvkun, 2003; Tsong *et al.*, 2006).

The emergence of modular gene structure by the nonadaptive processes of duplication, degenerative mutation, and genetic drift is fully compatible with the known magnitudes of these forces in multicellular species (Lynch, 2007). For example, the rate of duplication of entire genes

FIGURE 5.2 The passive emergence of specialized gene functions via nonadaptive processes of duplication, degenerative mutation, and random genetic drift. (*Left*) Regulatory elements (transcription-factor binding sites) are depicted on the left, with each regulatory element coded according to the transcription factor that binds to it. (*Right*) Allele-specific utilizations of transcription factors are depicted. Transcription factors denoted by black and white are ubiquitously expressed, whereas those denoted by hatching and crosshatching are each expressed in single, non-overlapping tissues. For this particular gene, within their respective tissues, the hatched and crosshatched transcription factors are redundant with respect to the white factor, but the additional black factors are essential for complete expression. Three hypothetical phases of gene architectural modification



are shown. (*Top*) Accretion and degeneration of transcription-factor binding sites. The initial allele (a) is expressed in an identical manner in both tissues, but the regulatory region sequentially acquires the hatched and crosshatched elements. The redundant white element is then vulnerable to loss by degenerative mutation, yielding a descendant allele with a semi-independent mode of expression, as the black element is still essential to expression in both tissues. At this stage all four alleles (a–d) are interchangeable, as each of them achieves the same pattern of phenotypic expression. (*Middle*) Regulatory-region duplication, degeneration, and complementation. The entire enhancer region is tandemly duplicated, with each component then losing a complementary (hatched/crosshatched) element. The resultant allele has become modularized in the sense that it harbors two independently mutable subfunctions denoted by the hatched and crosshatched open boxes; a mutation in either region has effects confined to a single tissue. (*Bottom*) Gene duplication and subfunctionalization by degenerative mutation. The entire gene is duplicated, with each copy becoming silenced by degenerative mutation for a complementary subfunction. The expression of each copy is now confined to a single tissue.

is ~1% per gene per million years, and because small fragments of DNA are tandemly duplicated at much higher rates than entire genes (Katju and Lynch, 2003), variation in the regulatory modules of genes must arise at least as rapidly as single-nucleotide polymorphisms. However, because of the mutational cost of allelic complexity, the likelihood of completion of semineutral modularization processes becomes negligible once $1/N_g$ becomes smaller than the excess mutational burden (Force *et al.*, 2005). Thus, contrary to popular belief, natural selection may not only be an insufficient mechanism for the origin of genetic modularity, but population-genetic environments that maximize the efficiency of natural selection may actually promote the opposite situation, alleles under unified transcriptional control. Under this view, the reductions in N_g that likely accompanied both the origin of eukaryotes and the emergence of the animal and land-plant lineages may have played pivotal roles in the origin of modular gene architectures on which further developmental complexity was built.

Despite the initial invariance of phenotypic expression patterns during this type of gene-architectural repatterning, the emergence of independently mutable subfunctions in modularized alleles can contribute to adaptive evolution in significant ways. For example, if the ancestral allele under unified control was subject to pleiotropic constraints associated with shared regulatory regions, modularization may open up previously inaccessible evolutionary pathways. Relief from pleiotropy can be even further facilitated following the duplication of the entire gene (bottom of Fig. 5.2), as complementary degenerative mutations partition cellular tasks among paralogous copies (Force *et al.*, 1999). This process of subfunctionalization is known to be a frequent fate of duplicate genes in multicellular species (Prince and Pickett, 2002; Lynch, 2007), and theory suggests that it too is most likely to occur in populations with small N_g, again because of the mutational burden of distributing a fixed number of subfunctions over multiple genes (Lynch *et al.*, 2001). Thus, the joint operation of both processes (the emergence of gene subfunctions and their subsequent partitioning among paralogs) in the small to moderate population-size environment that exists in multicellular species provides a powerful mechanism for the passive remodeling of entire developmental genetic pathways (Lynch, 2007).

Another peculiar aspect of developmental pathways that has defied explanation is their seemingly baroque structure (Wilkins, 2002, 2005). It is common for linear pathways to consist of a series of genes whose products are essential to the activation/deactivation of the next downstream member, with only the expression of the final component in the series having an immediate phenotypic effect. For example, the product of gene D may be necessary to turn on gene C, whose product turns on gene B, whose

product finally turns on gene A, which carries out an essential organismal function. Pathways involving only inhibitory steps also exist, and these lead to an alternating series of high and low expression, depending on the state of the first gene in the pathway. It is often unclear whether such complexity has any advantages over the simple constitutive expression or self-regulation of the final member of the pathway.

In principle, pathway augmentation may be driven entirely by the nonadaptive processes of duplication, degeneration, and genetic drift. Consider the series of regulatory states for gene A in Fig. 5.3. In the simplest case, A carries out some function in a self-sufficient fashion, but in a series of steps, it can become completely reliant on upstream activation by transcription factor B. A scenario like this could unfold in the following way. Initially, A becomes sensitive to activation by B, either because gene A has acquired a binding site for factor B, or because factor B acquires a fortuitous mutation that causes it to serve as an activator of A. At this point, gene A has redundant activation pathways, and is therefore insensitive to loss of one of them. Should a degenerative mutation cause a redundantly regulated allele of A to lose the ability to self-regulate, B will have been established as an essential activator, i.e., the pathway will have been augmented by a step. In principle, this process could be repeated anew as B acquires sensitivity to a further upstream gene C and loses the ability to constitutively express.

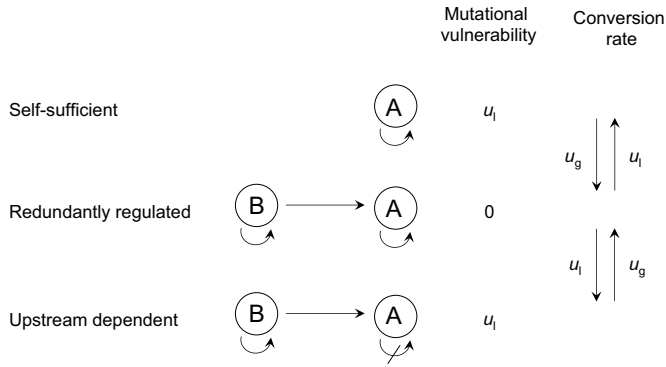


FIGURE 5.3 A series of allelic states for locus A, defined by the ability to self-express and/or be activated by an upstream transcription factor B. Mutational rates of gain and loss of regulatory abilities are denoted by u_g and u_l, here for simplicity assumed to be the same for both self-activation and upstream activation. The redundantly regulated allele is invulnerable to single-loss mutations.

As in the case of subfunction fission and duplicate-gene subfunctionalization, the probability of establishment of these types of changes will depend on N_g. This is because a redundantly regulated allele has a weak mutational advantage equal to the rate of loss of a regulatory site (u_l); one such mutation will result in the nonfunctionalization of either a self-regulated or an upstream-dependent allele, but will leave the function of a redundantly regulated allele unaltered. If $1/N_g > u_l$, such an advantage will be too small to be influenced by selection, and the population will evolve to an allelic state that simply depends on the relative rates of gain and loss of regulatory sites (u_g and u_l in Fig. 5.3). In contrast, if $1/N_g < u_l$, the accumulation of upstream-dependent alleles will be inhibited by their weak mutational burden and their lack of function in genetic backgrounds that fail to support A–B crosstalk. Thus, whereas small N_g may promote the passive elongation of genetic pathways, large N_g has the opposite effect. This does not mean that the augmentation of obligatory pathways cannot occur in very large populations, but if such changes are to occur, they must be of immediate selective advantage.

EVOLVABILITY

All replicating populations are capable of evolution, but it has recently been argued that some species are better at it than others, with natural selection directly advancing features of genomic architecture, genetic networks, and developmental pathways to promote the future ability of a species to adaptively evolve. Such speculation, which is almost entirely restricted to molecular and cell biologists and those who study digital organisms (e.g., Gerhart and Kirschner, 1997; Kirschner and Gerhart, 1998, 2005; Rutherford and Lindquist, 1998; True and Lindquist, 2000; Caporale, 2003; Earl and Deem, 2004; Bloom *et al.,* 2006; Federici and Downing, 2006), has been subject to considerable criticism by evolutionary biologists (e.g., Williams, 1966; Dickinson and Seger, 1999; Partridge and Barton, 2000; Brookfield, 2001; Sniegowski and Murphy, 2006). The term evolvability has long been in use in quantitative genetics, where it has a precise definition closely related to the concept of heritability, i.e., the relative amount of standing variation that is subject to a response to natural selection (Houle, 1992; Lynch and Walsh, 1998). However, the above-mentioned authors use the word in a rather different way, loosely defining evolvability to be the ability of a lineage to generate useful adaptive variation via mutational flexibility. Regardless of the definition, the idea that variation in evolvability exists among species is secure, as it has long been known that organisms and classes of traits vary in their propensities to respond to natural selection (Falconer and Mackay, 1996). Less secure is the idea that the ability to evolve itself is actively promoted by directional selection. Four reasons for skepticism follow.

First, evolution is a population-level feature. Thus, if an organismal feature that modifies the ability to evolve is to be advanced directly by adaptive mechanisms, selection must operate efficiently at a higher level of organization than the individual. This requires a significantly subdivided population structure, with levels of evolvability being positively correlated with population longevity and/or productivity. Because populations survive longer than individuals, such group selection is expected to be a much weaker force than individual selection, and necessarily operates on much longer time scales. If evolvability is to be subject to selective advancement, at least three stringent conditions must be met: (*i*) the group advantages of the genomic attribute that enhances evolvability must exceed any conflicting pressures operating at the individual level; (*ii*) the enhanced capacity for rapid evolutionary change must persist over the time scale of group selection; and (*iii*) while en route to fixation at the population level, the alleles that promote evolvability must remain tightly linked to the loci whose evolution is advantageous. Do such conditions ever exist in nature? The evidence for individual-level selection is overwhelming (Endler, 1986; Kingsolver *et al.,* 2001), but aside from the matter of kin selection in behavioral evolution (Hamilton, 1964a,b; Wilson, 1975), the evidence for the operation of group selection is weak, although some investigators remain more optimistic than others (Coyne *et al.,* 2000; Goodnight and Wade, 2000).

Second, it is by no means clear that an enhanced ability to evolve is generally advantageous. The dynamics of genetic variance for quantitative traits is complex, with selection modifying allele frequencies at epistatically interacting loci in ways that can either increase or decrease heritabilities, regardless of the advantage of the traits under selection (Carter *et al.,* 2005). In addition, one can just as easily point to a long list of pathologies that can arise from an overly rapid proliferation of a new phenotype, and such scenarios have motivated a completely alternative, and equally speculative, view, that selection can favor mechanisms that suppress evolvability (Altenberg, 2005). Furthermore, theoretical studies have shown that the kinds of complexities that are often focused on by those enamored with evolvability (e.g., increased dimensionality and modularity) can actually inhibit the rate of adaptive evolution (Orr, 2000; Welch and Waxman, 2003; Haygood, 2006). Although the arguments are technical, they are no more abstract than the verbal reasoning of the evolvability school.

Third, there is no evidence that phylogenetic variation in evolutionary features reflects anything more than diversity in variation-generating factors that exist for purely physical reasons. For example, the biological features most likely to influence the ability to evolve, recombination and mutation rates, vary by orders of magnitude among species, with no

sudden discontinuities in the lineages imagined to be most evolvable (animals and land plants) (Lynch, 2006, 2007). Such variation appears to be a simple by-product of alterations in chromosome lengths and numbers of germ-line cell divisions: because chromosome number is independent of genome size, and there is typically one meiotic cross-over event per chromosome, a doubling in genome size generally results in a 50% reduction in the recombination rate per physical distance; and because a large fraction of mutations are generated during replication, a doubling in the number of germ-line cell divisions doubles the per-generation mutation rate.

Fourth, comparative genomics provides no support for the idea that genome architectural changes have been promoted in multicellular lineages so as to enhance their ability to evolve (Lynch, 2007). Indeed, other than the appearance of spliceosomal introns, some forms of mobile elements, and organelles in the stem eukaryote, there are no discontinuities in the basic features of genomes across the entire domain of cellular life. Moreover, as noted above, the additional genomic complexities of multicellular eukaryotes appear not to have arisen by positive selection but instead to have emerged passively in population-genetic environments where the efficiency of selection is relaxed, quite contrary to the view espoused by the evolvability school. Many unicellular species are excluded from certain evolutionary pathways that are open to multicellular species, and vice versa, but this is simply an indirect consequence of the altered power of nonadaptive evolutionary forces in these different contexts, not a direct outcome of natural selection for the ability to engage in particular evolutionary pursuits.

CLOSING COMMENTS

Because it deals with observations on historical outcomes, frequently in the face of incomplete information, the field of evolution attracts significantly more speculation than the average area of science. Nevertheless, a substantial body of well tested theory provides the basis for understanding the pathways that are open to evolutionary exploration in various population-genetic contexts. Four of the major buzzwords in biology today are complexity, modularity, evolvability, and robustness, and it is often claimed that ill-defined mechanisms not previously appreciated by evolutionary biologists must be invoked to explain the existence of emergent properties that putatively enhance the long-term success of extant taxa. This stance is not very different from the intelligent-design philosophy of invoking unknown mechanisms to explain biodiversity. Although those who promote the concept of the adaptive evolution of the above features are by no means intelligent-design advocates, the burden of evidence for invoking an all-powerful guiding hand of natural selection

should be no less stringent than one would demand of a creationist. If evolutionary science is to move forward, the standards of the field should be set no lower than in any other area of inquiry.

The field of population genetics is technically demanding, and it is well known that most biologists abhor all things mathematical. However, the details do matter in the field of evolutionary biology. As discussed above, many aspects of biology that superficially appear to have adaptive roots almost certainly owe their existence in part to nonadaptive processes. Such conclusions would be difficult to reach without a formal population-genetic framework, but they equally rely on observations from molecular, genomic, and cell biology. Such conclusions also raise significant challenges. If complexity, modularity, evolvability, and/or robustness are entirely products of adaptive processes, then where is the evidence? What are the expected patterns of evolution of such properties in the absence of selection, and what types of observations would be acceptable as a falsification of a null, nonadaptive hypothesis?

This tone of dissent is not meant to be disrespectful. The development of a mature field of evolutionary biology requires the participation of not just population geneticists, but molecular, cell, and developmental biologists. However, the integration of these fields needs to be a two-way street. Because the forces of mutation, recombination, and genetic drift are now readily quantifiable in multiple species, there is no longer any justification for blindly launching suppositions about adaptive scenarios without an evaluation of the likelihood of nonadaptive alternatives. Moreover, if the conclusion that nonadaptive processes have played a central role in driving evolutionary patterns is correct, the origins of biological complexity should no longer be viewed as extraordinarily low-probability outcomes of unobservable adaptive challenges, but expected derivatives of the special population-genetic features of DNA-based genomes. A similar point has been made previously by Kauffman (1993), although his conclusions were derived from models far removed from mainstream population genetics.

ACKNOWLEDGMENTS

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Part III

FROM INDIVIDUAL ONTOGENY TO SYMBIOSIS: A HIERARCHY OF COMPLEXITY

Biological complexity is displayed at many hierarchical levels, from molecular and cellular operations within an organism to species' interactions in ecological communities. At any level, biological entities are enmeshed in interactive networks that typically involve potential conflicts as well as collaborations. For example, a multicellular organism can be viewed as a social collective of cells whose genes must not only collaborate to generate a viable individual but also compete for inclusion in gametes that will form the next generation. Chapters in Part III deal with some of the complex interactions that characterize biological systems at the levels of ontogeny, multicellularity, eusociality, and symbiosis.

During ontogeny, suites of genes (and the RNA and protein molecules they encode) direct the molecular dances of development that produce a functional multicellular organism. The ontogenetic choreographies themselves evolve, as evidenced by the great diversity of body plans and other phenotypes in different organismal lineages. What kinds of genetic mechanisms underlie ontogenetic shifts and the emergence of novel morphologies? Most researchers suspect that evolutionary changes in gene regulation are especially important, and that such alterations often involve the cooption of preexisting genes and proteins into new functions. In Chapter 6, Benjamin Prud'homme, Nicolas Gompel, and Sean Carroll illustrate how such cooptions can occur via shifts in the deployment of cis-regulatory elements and their associated transcription factors. They argue that this specific kind of architectural change in regulatory networks

offers a key to understanding how morphological evolution is linked to molecular ontogenetic processes.

Multicellularity itself is a complex trait, yet the phenomenon has arisen independently on numerous occasions. Each evolutionary transition from unicellularity to multicellularity likely proceeds through a succession of stages: initial aggregation of cells, increased cooperation within the group, the evolution of policing mechanisms against cheater cells, increases in group size, and the spatial and functional specialization of cell types. The process is remarkable because it entails, in effect, the emergence of reproductive altruism, wherein most cells forego personal reproduction in favor of working on the colony's behalf, a situation that undoubtedly necessitates high within-colony kinship (Maynard Smith and Szathmáry, 1995). In Chapter 7, Rick Michod discusses these topics with special reference to living volvocine green algae, which collectively display several stages along the unicellularity to multicellularity continuum. Michod contends that multicellularity is not irreducibly complex in an evolutionary sense, but rather can be understood in terms of evolutionary trade-offs and fitness advantages that can attend various intermediate stages in the evolutionary transitions between one kind of individual and another.

Eusociality is perhaps the epitome of complex social behavior and apparent reproductive selflessness. In eusocial colonies, such as those of many hymenopteran insects, individuals show striking reproductive divisions of labor, with sterile workers striving to maintain and defend a colony whose offspring are produced by the reproductive elites. Eusociality has long intrigued biologists. A key insight came from Hamilton (1964a,b) who proposed that the evolution of extreme reproductive altruism by workers was facilitated by the altered genetic relationships among various colony members stemming from haplodiploid sex determination. In Chapter 8, Joan Strassmann and David Queller review current thought about the evolution of eusociality, including the important point that kin selection predicts a degree of cross-purpose and conflict (as well as extensive cooperation and common purpose) in eusocial insect colonies. They conclude that kin-selection theory, by making specific testable predictions about behavioral phenomena in eusocial colonies, nicely exemplifies the power of scientific explanation for complex biological phenomena.

Genomic evolution was traditionally thought to proceed independently in different lineages, but a growing body of literature has revealed numerous exceptions. For example, horizontal gene transfer events have proved to be rather common in various prokaryotic groups, sometimes affording the recipient with novel metabolic capabilities. Another evolutionary route by which lineages may acquire functional innovations involves the establishment of stable (and sometimes heritable) symbiotic associations. In Chapter 9, Nancy Moran interprets various symbioses

among microorganisms, and between microorganisms and their multicellular hosts, as important (and previously underappreciated) evolutionary sources of phenotypic novelty. Using compelling examples from insects and other organisms, Moran shows how obligate symbiosis can yield complex evolutionary outcomes, ranging from the emergence of specialized cell types and organs to various developmental mechanisms that regulate the intergenerational continuance of the symbiotic association.

6

Emerging Principles of Regulatory Evolution

BENJAMIN PRUD'HOMME,*‡ NICOLAS GOMPEL,†‡ and SEAN B. CARROLL*

Understanding the genetic and molecular mechanisms governing the evolution of morphology is a major challenge in biology. Because most animals share a conserved repertoire of body-building and -patterning genes, morphological diversity appears to evolve primarily through changes in the deployment of these genes during development. The complex expression patterns of developmentally regulated genes are typically controlled by numerous independent cis-regulatory elements (CREs). It has been proposed that morphological evolution relies predominantly on changes in the architecture of gene regulatory networks and in particular on functional changes within CREs. Here, we discuss recent experimental studies that support this hypothesis and reveal some unanticipated features of how regulatory evolution occurs. From this growing body of evidence, we identify three key operating principles underlying regulatory evolution, that is, how regulatory evolution: (*i*) uses available genetic components in the form of preexisting and active transcription factors and CREs to generate novelty; (*ii*) minimizes the penalty to overall fitness by introducing discrete changes in gene expression; and (*iii*) allows

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interactions to arise among any transcription factor and downstream CRE. These principles endow regulatory evolution with a vast creative potential that accounts for both relatively modest morphological differences among closely related species and more profound anatomical divergences among groups at higher taxonomical levels.

It has long been understood that morphological evolution occurs through alterations of embryonic development (Gould, 1977; Raff and Kaufman, 1983). The key catalyst to the molecular study of morphological evolution has been the identification and functional characterization of developmental genes in animal model systems beginning in the 1980s. The development of specific body parts and organs was revealed to be orchestrated by networks of patterning genes that encode mostly transcription factors and cell-signaling molecules. It was then gradually realized that the formation of similar body parts and functionally equivalent organs in widely divergent animals is controlled by remarkably similar sets of orthologous pattern-regulating genes that have been conserved over hundreds of million years of evolution (Duboule and Dolle, 1989; Graham *et al.,* 1989; Quiring *et al.,* 1994; De Robertis and Sasai, 1996; Panganiban *et al.,* 1997; Bodmer and Venkatesh, 1998; Carroll *et al.,* 2004). However, the unexpected widespread genetic similarities presented a new paradox: if all animals are built by using similar genetic tools, how did their seemingly endless morphological diversity arise?

A vast body of comparative studies has revealed that morphological differences among taxa are correlated with differences in developmental gene expression patterns, which has supported the proposal that evolutionary modifications of gene expression (i.e., ''regulatory evolution'') are the basis of morphological diversification (King and Wilson, 1975; Carroll, 1995). The question of morphological evolution then turned to how such spatial differences in gene expression arise. In principle, gene expression may evolve through changes in either the activity or the deployment of the proteins (primarily transcription factors) that govern gene expression, or in the regulatory sequences that modulate the expression of individual genes (at the DNA or RNA level).

Two clues to the general resolution of these alternatives were emerging from molecular developmental biology by the early 1990s. The first was the structural conservation and functional equivalence of key transcription factors, such as Hox proteins, which indicated that their biochemical activities were not diverging much, if at all (McGinnis *et al.,* 1990; Halder *et al.,* 1995). The second was the discovery of the unexpectedly complex and modular organization of the cis-regulatory regions of pattern-regulating genes (Stanojevic *et al.,* 1991; Davidson, 2001). Most loci encoding pattern-

regulating proteins were found to include multiple individual cis-regulatory elements (CREs), with each CRE typically comprising binding sites for multiple distinct transcription factors and controlling gene expression within a discrete spatial domain in a developing animal. The realization that the total expression pattern of a gene was the sum of many parts, each directed by distinct CREs, marked a profound change in concepts of gene regulation. The modular arrangement of CREs also had clear implications for evolutionary genetics, because it suggested a mechanism for how selective changes in gene expression and morphology could evolve in one part of the body, independent of other parts (Carroll, 1995). The conservation of the biochemical activity of regulatory proteins, the divergence of their expression patterns across taxa, and the modular organization of CREs provided the basis for the general proposal that gene expression evolution, and therefore morphological evolution, would occur primarily through changes in cis-regulatory sequences controlling gene transcription (Carroll, 1995).

However, the evolutionary significance of the properties of CREs was not widely recognized at the time and, in our view, may still not be fully appreciated. We think there are several possible reasons for this (Carroll, 2005b). First, there is a much longer history of the analysis of coding sequences in evolutionary and population genetics. Second, the role of gene duplication has also long figured prominently in ideas about evolutionary novelty (Ohno, 1970). In contrast, the recognition of the complexity and evolutionary potential of CREs is more recent and has emerged primarily from molecular developmental genetics, outside of the primary literature of evolutionary genetics. And finally, there have been few detailed functional studies of CRE evolution. Most studies have focused on the functional conservation of CREs (Ludwig *et al.,* 2000, 2005; Wratten *et al.,* 2006). Until very recently, there have been very few direct empirical examples linking CRE evolution to morphological evolution (Belting *et al.,* 1998; Wang and Chamberlin, 2002). As a result, beyond the growing acceptance of why regulatory evolution plays a role in morphological evolution, our understanding of how regulatory evolution occurs has been limited.

The elucidation of the mechanisms of CRE evolution in morphological diversification has required the identification of appropriate experimental systems. Because coding sequences are usually sufficiently conserved to identify orthologous sequences among different phyla, it was naïvely assumed initially that the same would hold true for CREs, and that functional comparison of divergent CREs from distantly related taxa would be possible. However, it was progressively realized that the turnover rate of noncoding DNA is much higher than for coding sequences, largely because of looser functional constraints, making orthologous sequence

identification and comparison much more difficult (Erives and Levine, 2004; Richards *et al.*, 2005; Wittkopp, 2006) and often impossible, especially among higher arthropod taxa. To circumvent this difficulty, an alternative strategy has been to focus on rapidly evolving traits among closely related species or populations. The advantages of this approach are 2-fold: first, because the organisms under comparison diverged recently, it is expected that the number of genetic changes responsible for morphological divergence will be relatively modest and more readily distinguished from other changes not involved in morphological divergence. Second, in some cases, the relevant genetic changes can be investigated in their native ecological context and related to the potential adaptive role, if any, of morphological evolution.

Following this approach, recent studies have provided direct evidence of the role of CRE evolution in morphological evolution (Gompel *et al.*, 2005; Jeong *et al.*, 2006; Prud'homme *et al.*, 2006). More importantly, these detailed functional analyses have revealed some surprising and previously unanticipated features of how gene regulation evolves at the molecular level that, we suggest, reflect general principles. The goal of this article is to articulate these emerging principles, namely how regulatory evolution: (*i*) proceeds using available preexisting genetic components, (*ii*) introduces discrete changes in gene expression thus minimizing deleterious effects and fitness penalties, and (*iii*) allows the association between any transcription factor and any downstream gene and thereby provides immense potential for evolutionary novelty. These principles explain both how and why regulatory sequence evolution is a pervasive, although not the exclusive, mechanism underlying morphological diversification.

PIGMENTATION PATTERNS AND GENE EXPRESSION AS MODELS OF REGULATORY EVOLUTION

Because morphological evolution is the product of the modification of the expression patterns of underlying genes, to understand how morphological changes arise, we must understand how changes in gene expression pattern arise. Pigmentation patterns in insects have been particularly amenable for these purposes for two reasons: first, many genes governing their formation have been characterized; and second, patterns are highly variable among closely related species, giving very different appearances to otherwise identical body parts (Wittkopp *et al.*, 2003). For instance, the wings of some higher Diptera are largely identical with respect to their overall shape and venation patterns. Yet the various pigmentation patterns superimposed onto the common wing plan, ranging from a simple line, dot, or blotch to complex compound patterns make each species wing pattern largely different from the others (Fig. 6.1).

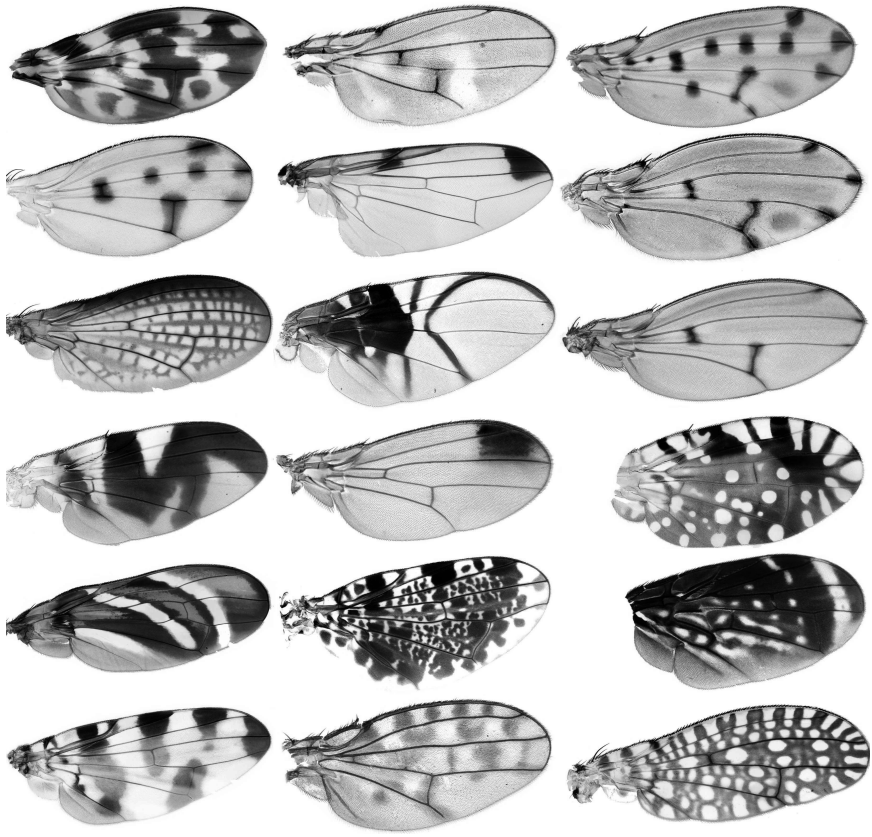

FIGURE 6.1 Wing pigmentation pattern diversity across higher Diptera. This plate illustrates the diversity of wing pigmentation patterns in the Acalyptratae, a large group of higher Diptera (Cyclorrhapha), which contrasts with the remarkable conservation of shape, dimension ratios, and venation patterns of these wings after >70 million years of evolution.

Pigmentation patterns result from the local conversion of precursor metabolites into pigment deposits by several enzymes (Wittkopp *et al.,* 2003). The expression patterns of these enzymes, generally specified at an advanced developmental stage when the overall morphology closely resembles the adult layout, are the blueprints of the visible pigmentation patterns (Wittkopp *et al.,* 2002a). Therefore, understanding how the expression patterns of the genes encoding these enzymes are established and change among species is key to understanding the formation and diversification of pigmentation patterns.

In principle, pigmentation patterns could evolve by changing the activity or spatial deployment of transcription factors that regulate pigmentation genes and/or by changes in the CREs of pigmentation genes themselves. Furthermore, such changes in CREs could entail either the modification of existing CREs or the *de novo* evolution of a CRE. Six cases of the gain or loss of pigmentation gene expression in fruit fly species have now been traced to the evolution of pigmentation gene CREs (Gompel *et al.,* 2005; Jeong *et al.,* 2006; Prud'homme *et al.,* 2006). The frequency and details of CRE sequence evolution and the identity of the transcription factors involved in regulating these CREs and other case studies of morphological divergence among closely related species or populations (Belting *et al.,* 1998; Sucena and Stern, 2000; Wang and Chamberlin, 2002; Sucena *et al.,* 2003; Shapiro *et al.,* 2004; Colosimo *et al.,* 2005; Marcellini and Simpson, 2006) illustrate what we submit are general insights into the process of evolution by gene regulation.

USING AVAILABLE GENETIC COMPONENTS TO GENERATE NOVELTY

In some members of the *Drosophila melanogaster* species group, the males bear dark spots at the anterior tips of their wings, whereas in most other species, they do not (Kopp and True, 2002; Prud'homme *et al.,* 2006). The evolution of the male wing spot thus presents a simple example of a novel pattern and poses a simple question: what changed between unspotted and spotted species? The difference in pigmentation patterns is reflected by differences in pigmentation gene expression. In particular, the product of the *yellow* (*y*) gene, which is critical for the production of black pigment (Walter *et al.,* 1991), is expressed uniformly at low levels in the developing wing blade in unspotted species and in spotted species; it is also expressed at high levels where the spot will appear.

How did *yellow* expression evolve? The evolutionary divergence in Yellow expression results from functional changes in a CRE controlling *y* expression in the developing wing (the *wing* CRE). In unspotted species, this CRE, which is ≈1 kb long, drives a uniform expression pattern throughout the wing (Wittkopp *et al.,* 2002b). In spotted species, the regulatory activity of this element has changed to also drive high levels of *yellow* in the spot area (Gompel *et al.,* 2005). Therefore, in this instance, an ancestral CRE has been coopted and functionally modified to become a *wing + spot* CRE and to generate a novel pattern.

In theory, a *spot* CRE with a full complement of transcription factor-binding sites necessary to drive a wing spot pattern could have evolved anywhere in the *yellow* locus. However, a functional CRE usually requires a substantial number of inputs to generate a spatially restricted expression

pattern (Davidson, 2001). If a functional CRE were to evolve from naïve DNA, the evolutionary path to acquire all of the necessary transcription factor-binding sites, in a functional arrangement, would be relatively long, and it is difficult to see how selection might favor the intermediates. In contrast, a CRE that is functional in a given tissue already contains some of the sites necessary to direct gene expression in that tissue, and therefore it represents a more likely template to accommodate a new expression pattern in that tissue, because a relatively shorter evolutionary path would lead to functional novelty. Consequently, it seems more probable that a novel gene expression pattern in a tissue will arise from random mutations creating binding sites in the vicinity of an existing CRE driving expression in that tissue than from mutations in non-functional DNA.

Which trans-acting factor-binding sites have evolved in the *wing* CRE to create the spot pattern? In principle, this element could have evolved binding sites for a single transcriptional activator, which, in turn, had evolved to be expressed in a spot pattern. In fact, however, the formation of the *yellow* spot pattern entailed the evolution of binding sites for both activators and repressors involved in the building of the wing. In particular, the transcription factor Engrailed, present in cells in the posterior part of the wing, directly represses *yellow* expression, confining elevated *yellow* expression and, therefore, the formation of the pigmentation spot to the anterior region (Gompel *et al.*, 2005).

The key point regarding the identity of Engrailed is that it is not a transcription factor specifically dedicated to pigmentation. Engrailed is a deeply conserved component of arthropod segmentation and appendage development, and its expression in the posterior compartment long preceded its involvement in the patterning of the pigmentation spot (Patel *et al.*, 1989). Nevertheless, in this particular context, the evolutionary process took advantage of its presence and established a direct regulatory connection between Engrailed and a pigmentation gene, thus sculpting the contour of the pigmentation spot. In this instance, Engrailed has been recruited for a new function, without any change occurring in its activity, protein sequence, or expression.

The evolution of the wing spot illuminates a general mechanism by which a novel pigmentation pattern can be generated (Fig. 6.2). The development of the wing or any body part or organ is a sequential process controlled by an array of regulatory proteins (Carroll *et al.*, 2004). As development proceeds, the expression of these proteins progressively delineates the wing layout, position of the veins, sensory organs, and so on. Collectively, the expression profiles of all wing-building transcription factors compose a complex mosaic of superimposed patterns or ''trans-regulatory landscape'' (Fig. 6.3). If and when combinations of binding sites for members of the trans-regulatory landscape evolve in the CRE of

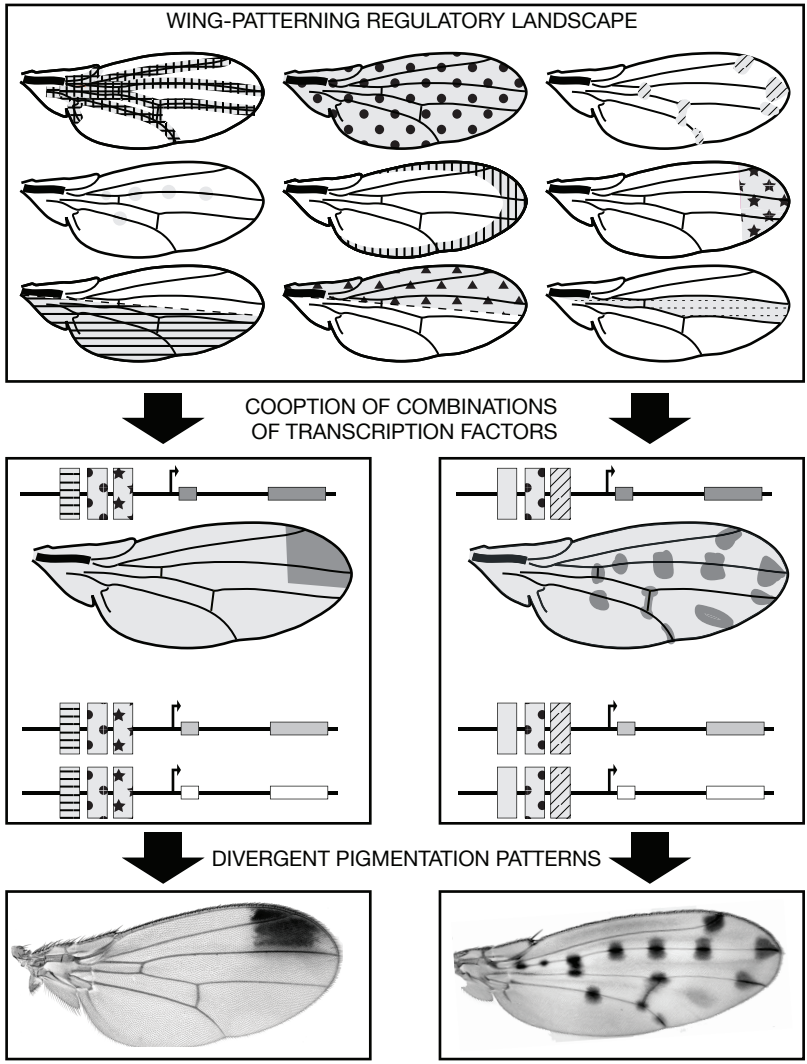

FIGURE 6.2 Regulatory evolution and wing pigmentation pattern diversity. A suite of transcription factors control the development of the fly wing, each one being expressed in a particular pattern. Altogether, these expression patterns constitute a wing trans-regulatory landscape, conserved among *Drosophila* species (*Top*). The recruitment of a subset of the trans-regulatory landscape components by pigmentation genes results in the corresponding redeployment of these genes (*Middle*) and ultimately in a novel wing pigmentation pattern (*Bottom*). The recruitment of different combinations of trans-acting factors in different fly species yields distinct pigmentation patterns. In *Middle*, rectangles with motif represent binding sites for different trans-regulatory landscape components.

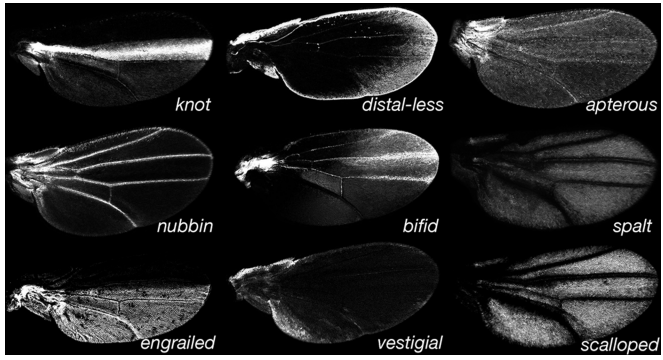

FIGURE 6.3 A glimpse of the actual wing trans-regulatory landscape. The expression of GFP reports the expression patterns of various wing transcription factors at the time pigmentation genes are being expressed.

a pigmentation gene, then the expression profile of this gene may change. Because the formation of pigmentation patterns requires the coincident deployment of multiple pigmentation genes, it can be anticipated that these genes will fall under the control of a common suite of trans-acting factors.

In this view, diverse pigmentation patterns can arise from the evolution of regulatory connections among pigmentation gene CREs and different combinations of transcription factors (Fig. 6.2). In particular, more elaborate patterns can evolve through the progressive accumulation of regulatory links between components of the trans-landscape and pigmentation genes CREs. This gradual elaboration of complex patterns is reflected in the graded series of pigmentation patterns found in several fly lineages, where new elements are added in more derived species (e.g., the top three wings in the right column in Fig. 6.1). Hence, the complexity of the wing trans-regulatory landscape and the combinatorial nature of gene expression regulation are sufficient to account for the spectacular diversity of wing pigmentation patterns.

More generally, the evolution of wing patterns illustrates a fundamental principle of regulatory evolution: novel patterns arise more readily from the recruitment of available components, CREs, and transcription factors into new regulatory interactions rather than from the *de novo* creation of genes or CREs. Indeed, all of the diversity of wing pigmentation patterns illustrated in Fig. 6.1 may be accounted for by regulatory changes and would not require, in principle, any coding sequence changes among species.

CIS-REGULATORY EVOLUTION MINIMIZES FITNESS PENALTIES

Two processes shape the course of evolution: first, genetic mechanisms generate variations in different individuals of a population, regardless of their biological outcome and ecological consequences. Second, these variations are sorted out either by a selective process based on their relative consequences to reproductive success (fitness) or by random population sampling. Although we are mainly concerned with the genetic and molecular mechanisms of evolutionary innovations, the existence of selective pressures constantly sifting through the spectrum of emerging variations must be considered, because they constrain the scope of genetic changes permitted under natural selection.

The genetic changes contributing to morphological evolution can affect protein function through mutations in gene coding sequences or, instead, gene regulation, mainly through CRE evolution. What circumstances influence which of these changes is more likely to be tolerated under natural selection? The study of pigmentation pattern evolution has also proven insightful to address this question. During the course of *Drosophila* evolution, discrete pigmentation patterns have been gained by the ancestors of identified groups of flies, preserved in many descendant species, and occasionally lost in others.

In theory, the loss of a particular pigmentation pattern could occur by the loss of pigmentation gene expression or the disruption of pigmentation protein functions through mutations in their coding sequences. However, the latter kinds of genetic changes would have substantial collateral effects, affecting all pigmentation patterns and other processes in which these proteins are involved. Many fly pigmentation proteins are also involved in cuticle formation and the metabolism of dopamine, an essential neurotransmitter, and *D. melanogaster yellow* mutants are notorious for their poor mating success (Bastock, 1956; Chia *et al.,* 1986; Walter *et al.,* 1991; Drapeau *et al.,* 2006). Hence, losses of pigmentation through changes in the coding sequences of pigmentation genes are unlikely to be tolerated by natural selection, because their fitness cost is too high.

Supporting this idea, three cases of loss of pigmentation patterns have involved the selective functional inactivation of a CRE of the *yellow* locus (Fig. 6.4). Both the male wing spots discussed above and male abdominal pigmentation on segments A5–A6 (see below) have been lost repeatedly in distinct lineages. In two independent cases examined, the loss of the wing spot involved the inactivation of the *yellow spot* element (Prud'homme *et al.,* 2006). Similarly, the loss of *yellow* expression in A5–A6 results from the disruption of a specific CRE (Jeong *et al.,* 2006). In each case, mutations altered the spatial distribution of the gene product in only one domain of the body, leaving the rest of the expression pattern and the protein activity intact. These examples illustrate that disruption of a dedicated CRE mini-

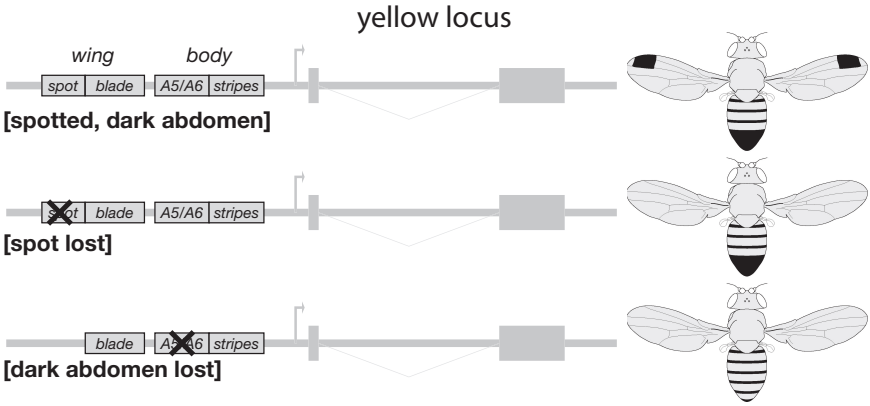

FIGURE 6.4 Cis-regulatory evolution circumvents pleiotropic effects. The *yellow* locus contains a series of CREs controlling the spatiotemporal expression of the gene. Four of them are represented on these schematics, driving expression in the wing blade, wing spots, segmental abdominal stripes, and male posterior abdominal segments. Some species have lost the wing spots or male abdominal pigmentation (*Middle* and *Bottom*, respectively) through the inactivation of the corresponding CRE. The selective disruption of a CRE does not affect other aspects of the expression pattern or the gene activity.

mizes the fitness penalties by affecting only one specific aspect of a gene's function while leaving the other functions undisturbed.

Additional examples of the selective loss of gene expression are inferred to be associated with CRE evolution, including the loss of larval hairs in *Drosophila* (Sucena and Stern, 2000; Sucena *et al.*, 2003) and pelvic reduction (Shapiro *et al.*, 2004) and bony armor loss (Colosimo *et al.*, 2005) in stickleback fishes. In all of these examples, there is one common denominator: the evolutionary changes involve mutations in a pleiotropic gene, i.e., a gene with multiple functions. A clear principle is emerging from the increasing number of case studies: pleiotropy imposes a genetic constraint on the type of changes that can be accommodated in morphological evolution. Highly pleiotropic genes (including most developmentally regulated genes) are more likely to contribute to morphological evolution through cis-regulatory changes than through coding sequence alterations. In contrast, known examples of pigmentation evolution resulting from the alteration of coding sequences affect genes involved in a single process, such as the overall body color in fish, mammals, or birds (Ritland *et al.*, 2001; Theron *et al.*, 2001; Eizirik *et al.*, 2003; Nachman *et al.*, 2003; Mundy *et al.*, 2004; Hoekstra *et al.*, 2006; Protas *et al.*, 2006). Coding sequence changes

appear to be better tolerated in minimally pleiotropic genes. This principle of minimizing fitness penalties delimits the scope of what changes are permissible under natural selection and explains why CRE evolution is a pervasive mechanism underlying morphological diversification (Carroll, 2005b).

INTERACTION MAY EVOLVE BETWEEN ANY TRANSCRIPTION FACTOR AND DOWNSTREAM CRE

We have advocated that novelty in gene expression arises primarily through new regulatory interactions between existing CREs and transcription factors. But is it the case that any transcription factor may be coopted into regulating a CRE, or are there constraints on regulatory evolution imposed, for example, by the position components occupy in developmental gene networks? Development is often described as a hierarchical process governed by cascades of regulatory genes. The formation of body parts and organs typically begins with the expression of a particular combination of transcription factors in a small set of precursor cells. These proteins then direct the organization of the developing structure by regulating other pattern-regulating genes, the expression of which define smaller territories and progenitors of the multiple parts constituting the final structure. Ultimately, batteries of structural genes, including pigmentation genes, establish the terminal differentiation of the various cell types.

Intuitively, it may have been thought that proteins at a particular tier in the hierarchy primarily regulate genes in the next tier and so on, such that evolutionary modifications in regulatory connections occur mainly between two consecutive levels. However, studies of pigmentation pattern evolution in flies have revealed that regulatory evolution takes advantage of transcription factors throughout genetic hierarchies in an opportunistic way to generate new regulatory connections.

This notion is clearly illustrated by the evolution of abdominal pigmentation in the relatives of *D. melanogaster*. In many species, abdominal segments are pale with a stripe of black pigment. However, darkening of the entire terminal segments A5 and A6 has evolved in males of an ancestor of the *melanogaster* species group (Jeong *et al.,* 2006). This pattern has been preserved in most species of the group but secondarily lost in others. As in the wing spot case, Yellow distribution prefigures the actual pigmentation pattern and is specifically expressed at high levels in the A5–A6 segments in species that are fully pigmented (Fig. 6.5).

How was the strong expression of *yellow* gained, and subsequently lost, selectively in A5–A6 segments? Dimorphic abdominal pigmentation in *D. melanogaster* is controlled by a genetic regulatory circuit that includes

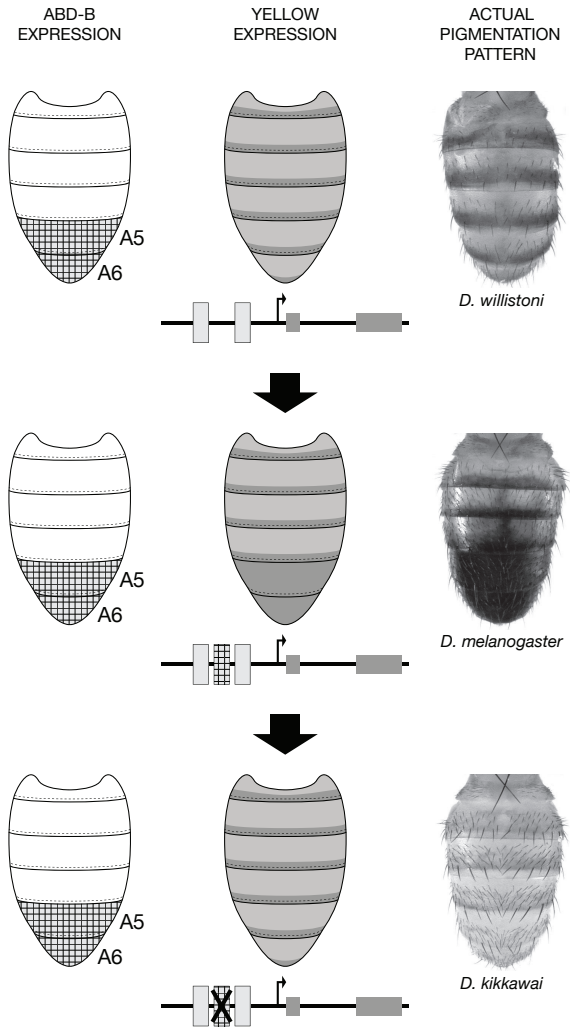

FIGURE 6.5 Regulatory changes underlying male abdominal pigmentation pattern evolution. In the *D. melanogaster* species group, the male abdominal pigmentation pattern is variable (*Right*). In the ancestral situation, here illustrated with *Drosophila willistoni*, both sexes carry an identical segmental stripe pattern. *D. melanogaster* males have evolved fully pigmented posterior segments (A5 and A6). This pattern has been secondarily lost in *Drosophila kikkawai*. These transitions result in part from changes in the regulation of the pigmentation gene *yellow* (*Center*). The gain and the loss of binding sites for the Hox protein Abd-B, a transcription factor expressed in posterior segments in *Drosophila* (*Left*), was involved in the gain and loss of expression of Yellow in the posterior abdomen, respectively.

the Hox protein Abdominal-B (Abd-B), which is expressed in terminal segments (Kopp *et al.,* 2000). Initially, it was expected that Abd-B controls the formation of pigmentation pattern indirectly, through the regulation of another transcription factor (Kopp *et al.,* 2000). Surprisingly, however, analysis of a *yellow* CRE revealed that expression in A5–A6 is directly controlled by Abd-B (Fig. 6.5) (Jeong *et al.,* 2006). Evolution of Abd-B-binding sites in this CRE correlates with the evolution of pigmentation in A5–A6 and mutational inactivation of some of these Abd-B-binding sites is associated with the loss of *yellow* expression from A5–A6 and the loss of posterior pigmentation in one lineage (Fig. 6.5).

In the same way the evolution of the wing spot entailed the recruitment of the wing-building protein Engrailed, posterior abdominal pigmentation arose through the evolution of a direct regulatory link between a major body plan architect (Abd-B), lying at the top tier of the genetic hierarchy, and a far downstream structural gene (*yellow*). In these examples, deeply conserved body plan- and body-part-building transcription factors contributed to morphological evolution of closely related species through changes in the regulation of their sets of target genes.

The evolution of pigmentation patterns through coopting body-plan regulatory proteins illustrates a third principle of regulatory evolution: association between any transcription factor and a downstream CRE may evolve, irrespective of positions of these components in genetic hierarchies. This opportunistic nature of regulatory interactions contributes to the vast evolutionary potential of CREs. The three principles of regulatory evolution we have described explain the mechanisms through and circumstances in which regulatory changes are more likely to contribute to morphological evolution. These rules also have implications for understanding overall patterns of morphological change. Here we will expand our discussion to issues concerning the direction (i.e., gain versus loss) and magnitude of trait evolution over larger evolutionary time scales.

LOSSES ARE EASY, GAINS ARE HARDER

The evolution of form occurs in part through the gain and loss of morphological traits. However, it should be emphasized that the frequency of occurrence of gains and losses is very different. The pattern of trait turnover shows that the frequency of losses is generally much larger than that of gains. This is because a trait arising once in the common ancestor of a group of species is offered as many opportunities to be lost as there are descendant species in the group. The male wing spot and abdominal pigmentation pattern discussed above, for instance, have been lost independently at least five and three times, respectively (Jeong *et al.,* 2006; Prud'homme *et al.,* 2006). Furthermore, the loss of a trait could potentially

occur by the functional alteration of any of the loci involved in its formation. In the case of abdominal pigmentation losses, three different mutational paths, affecting distinct genes, have been followed during these evolutionary transitions (Jeong *et al.,* 2006). For these reasons, losses of morphological traits are expected to be frequent and relatively "easy," i.e., they have a simple genetic basis and may even occur in a single step.

In contrast, the gain of genetically complex traits appears harder, in that it requires the deployment of multiple gene products in a coordinated spatial and temporal manner. Obviously, this is unlikely to happen in a single step, because it requires potentially numerous changes at multiple loci.

The contrast between the paths of trait gain and loss is also manifested at the level of CRE evolution. The functional inactivation of a CRE can result from a few mutations or perhaps even a single point mutation, as exemplified by the disruption of the *yellow spot* element in a species that has recently lost its wing spot (Prud'homme *et al.,* 2006). In comparison, the evolution of a new regulatory function, even through the cooption of an existing CRE, appears to require a relatively longer mutational path involving the acquisition of multiple transcription factor-binding sites. Furthermore, in documented cases where a new regulatory activity has evolved from cooption of an existing CRE (Jeong *et al.,* 2006; Prud'homme *et al.,* 2006), we observe that the two cis-regulatory activities reside in physically separable regions of DNA. These observations suggest that subfunctionalization of the ancestral CRE has occurred through additional changes that fine-tune gene expression in the domains governed by the now-separate elements (as discussed in Carroll *et al.,* 2004, p. 222).

CONNECTING THE DOTS FROM PIGMENTATION PATTERNS TO BODY PLAN DIVERSIFICATION: THE COMPOUNDING OF REGULATORY CHANGES OVER EONS

A long-standing question in evolutionary biology has been whether the genetic and molecular mechanisms underlying morphological changes within populations (so-called ''microevolution'') are sufficient to account for the differences in body patterns between species and at higher taxonomic levels (so-called ''macroevolution'') (Huxley, 1942; Arthur, 2000; Leroi, 2000; Stern, 2000). We submit that an expanding body of evidence, including the examples described in the previous sections, is affirming that macroevolution is a matter of the very same genetic and molecular changes ongoing in populations, compounded over longer periods of time and large numbers of cladogenetic events.

The morphological differences among closely related species we have discussed above evolved by functional changes in CREs. The genetic

modifications underlying these changes are ordinary mutations of the same kind as those arising in natural populations in every generation (Rockman and Wray, 2002; Balhoff and Wray, 2005) and not rare genomic rearrangements or duplication events. Therefore, it appears the genetic changes generating morphological differences among species are of the same nature as the ones that arise within populations. One may wonder whether it is also the case for morphological divergences at higher taxonomic levels, or whether these rather large-scale morphological differences require distinct genetic mechanisms.

A large body of work has documented associations between major morphological differences, such as body-plan differences, with those in the expression pattern of Hox proteins or their downstream target genes (Averof and Akam, 1995; Burke *et al.,* 1995; Averof and Patel, 1997; Belting *et al.,* 1998; Cohn and Tickle, 1999; Carroll *et al.,* 2004). One illuminating example where the mechanisms have been addressed in depth is the evolution of the two-winged dipteran body plan from four-winged ancestors by reduction of the hindwings. Many winged insects bear two pairs of wings attached to their second (T2) and third thoracic segments (T3). However, in Diptera, the hindwings have been modified into small balancing organs, the halteres. In *Drosophila*, the Hox protein Ubx, which is expressed in the T3 segment and appendages, controls the differentiation between wing and haltere (Weatherbee *et al.,* 1998; Crickmore and Mann, 2006). Because Ubx is also expressed in the developing hindwings of four-winged insects (Warren *et al.,* 1994), the evolutionary reduction of the hindwing in Diptera occurred under the control of the Ubx protein and did not result from a shift of the expression of Ubx.

In the *Drosophila* haltere, Ubx directly represses the expression of a set of wing-patterning genes (Galant *et al.,* 2002; Hersh and Carroll, 2005). In contrast, in four-winged butterflies, Ubx does not repress those genes (Weatherbee *et al.,* 1999). Hence, during dipteran evolution, this set of wing-patterning genes became Ubx-regulated. These genes became Ubx-responsive in the haltere by evolving Ubx-binding sites in CREs that direct gene expression in fore- and hindwings (Galant *et al.,* 2002; Hersh and Carroll, 2005). However, Ubx is not a ''wing repressor'' protein per se, because in other non-dipteran insects, Ubx presumably regulates different sets of target genes in the hindwings (Warren *et al.,* 1994; Tomoyasu *et al.,* 2005). Therefore, the reduction of hindwings in Diptera results from changes in the regulatory connections between Ubx and downstream target genes that evolved changes in their wing CREs (Fig. 6.6).

Importantly, the evolutionary mechanisms of hindwing reduction comply with the regulatory principles we have described. The repression of wing-patterning genes in halteres exploits available CREs and transcription factors (Ubx). Because Ubx is involved in many develop-

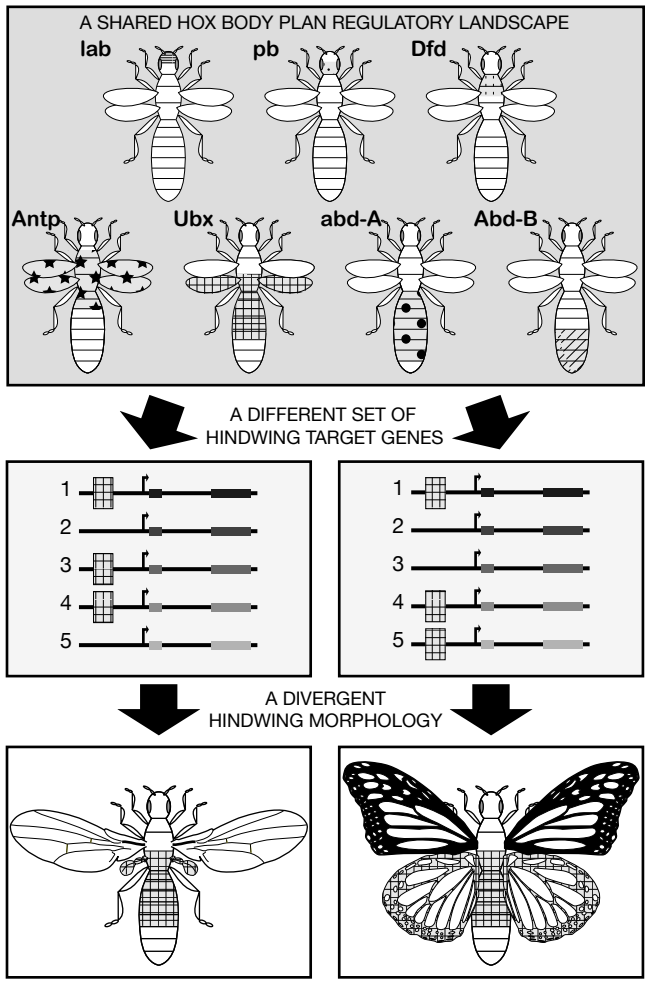

FIGURE 6.6 Body plan evolution by compounding regulatory changes. Hindwing reduction in Diptera results from changes in the regulatory connections between the Hox protein Ubx and downstream target genes (1 to 5). In Diptera, a suite of wing-patterning genes have evolved Ubx-binding sites in their CREs and, as a result, are repressed during hindwing development. In contrast, in four-winged butterflies, Ubx regulates a distinct set of target genes in the hindwing.

mental processes other than wing development, evolution of downstream wing-specific CREs enabled the selective changes in gene expression in the haltere while preserving other functions. Finally, multiple genes, at different levels of the wing genetic regulatory hierarchy, evolved Ubx regulation (Weatherbee *et al.,* 1998; Hersh *et al.,* 2007), suggesting that any gene of the wing developmental program can fall under the regulation of Ubx.

Thus, in the same way that abdominal pigmentation pattern evolved by changes in the regulatory connections between Abd-B and downstream pigmentation genes, the two-winged dipteran body plan evolved by changes in the regulatory interactions between Ubx and downstream wing-patterning genes. In this light, we see that the differences between the evolution of modest morphological traits, such as pigmentation patterns, and changes of larger magnitude, such as body-plan modifications, are not in the nature of the genetic changes but rather in their degree. Indeed, because several genes have evolved Ubx regulation, it is inescapable that hindwing reduction evolved progressively in a multistep process. More generally, it is reasonable to infer that large-scale morphological differences must typically arise by regulatory sequence mutations, presumably of small individual phenotypic effect, accumulating over time. Therefore, we submit that the same kind of genetic and molecular mechanisms are sufficient to account for both simple morphological changes and more profound body plan differences and that the principles of regulatory evolution we have delineated are general principles underlying morphological evolution.

Comparisons over large taxonomic distances have documented multiple examples of developmental gene duplications and coding sequence changes, and there is no doubt that these types of changes play a role in morphological evolution. However, what must be appreciated is the relative contribution of the different types of mechanisms to morphological diversity. It is well established that changes in gene number and regulatory protein motifs have been relatively few and far between during the >500-million-year span of animal evolution. In contrast, regulatory evolution, through regulatory sequence changes, is pervasive and constitutes the primary fuel of the continuous morphological diversification of lineages and traits in the ''far between.''

CONCLUSION

A growing number of case studies exploring the mechanisms of morphological changes have provided direct evidence that CRE evolution plays a major role. From these examples, we have identified general rules regarding regulatory evolution, namely how regulatory evolution exploits available genetic components, irrespective of their hierarchical position

in gene networks to generate novelty, and minimizes fitness penalties. These rules offer a rationale explaining why regulatory changes are more commonly favored over other kinds of genetic changes in the process of morphological evolution, from the simplest traits diverging within or among species to body-plan differences at higher taxonomic levels.

Although progress has been made in understanding CRE evolution in a single gene, there are important outstanding issues that need to be addressed for a fuller picture of the origins of morphological diversity. In particular, two areas that have been largely unexplored seem now to be within reach. First, we need a dynamic picture of CRE evolution within populations. This entails, on the one hand, elucidating the contribution of mutation and recombination to the origin of variation in gene expression, and on the other hand, a sense of how genetic drift and selection shape the fixation of these variations over time (Rockman *et al.,* 2005). Ultimately, such a dynamic picture of CRE evolution will help to reconstruct the mutational paths that lead to the origin of novel gene expression patterns.

Second, for complex genetic traits, we need to focus our attention not just on individual genes but on the complete set of genes involved in the formation, variation, and evolutionary divergence of the traits. Such a perspective is critical to understanding how sets of genes assemble into functional networks through the evolution of regulatory interactions (Tsong *et al.,* 2003; Ihmels *et al.,* 2005; Tsong *et al.,* 2006) and thus shape morphological diversity and novelty.

ACKNOWLEDGMENTS

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7

Evolution of Individuality During the Transition from Unicellular to Multicellular Life

RICHARD E. MICHOD

Individuality is a complex trait, yet a series of stages each advantageous in itself can be shown to exist allowing evolution to get from unicellular individuals to multicellular individuals. We consider several of the key stages involved in this transition: the initial advantage of group formation, the origin of reproductive altruism within the group, and the further specialization of cell types as groups increase in size. How do groups become individuals? This is the central question we address. Our hypothesis is that fitness tradeoffs drive the transition of a cell group into a multicellular individual through the evolution of cells specialized at reproductive and vegetative functions of the group. We have modeled this hypothesis and have tested our models in two ways. We have studied the origin of the genetic basis for reproductive altruism (somatic cells specialized at vegetative functions) in the multicellular *Volvox carteri* by showing how an altruistic gene may have originated through cooption of a life-history tradeoff gene present in a unicellular ancestor. Second, we ask why reproductive altruism and individuality arise only in the larger members of the volvocine group (recognizing that high levels of kinship are present in all volvocine algae groups). Our answer is that the selective pressures leading to reproductive altruism stem from the increasing cost of reproduction with increasing group size. Concepts from population genetics and evolutionary biology

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appear to be sufficient to explain complexity, at least as it relates to the problem of the major transitions between the different kinds of evolutionary individuals.

The theme of this article, which could well be the theme of this Colloquium, is that evolutionary biology can explain complexity. I will consider the problem of explaining the "major transitions" between the different kinds of evolutionary individuals that make up the familiar hierarchy of life: genes, bacteria-like cells, cells-in-cells (eukaryotic cells), multicellular organisms, and societies (Maynard Smith and Szathmáry, 1995). Evolutionary individuals are integrated and indivisible wholes with the property of heritable variation in fitness so that they may evolve adaptations at their level of organization. Being wholes, evolutionary individuals may be thought to be irreducibly complex, but this has not been the case during evolutionary history; a series of stages, each advantageous in itself, may be shown to exist allowing evolution to get from one kind of individual to another. The evolutionary concepts we use to understand evolutionary transitions in individuality involve fitness and its reorganization, fitness tradeoffs (especially the cost of reproduction to survival) and their roles in life-history evolution, and kin selection and altruism and their roles in social evolution. We focus on the transition from unicellular to multicellular life, but the points made apply more generally to the other transitions (Michod, 1999).

Our understanding of life is being transformed by the realization that evolution occurs not only through the standard processes operating within populations, but also during evolutionary transitions in individuality, when groups of individuals become so integrated that they evolve into new higher-level individuals. Indeed, the major landmarks in the diversification of life and the hierarchical organization of the living world are consequences of a series of evolutionary transitions: from genes to gene networks to the first cell, from prokaryotic to eukaryotic cells, from cells to multicellular organisms, from asexual to sexual populations, and from solitary to social organisms. Such transitions require the reorganization of fitness, by which we mean the transfer of fitness from the old lower-level individual to the new higher level, and the specialization of lower-level units in fitness components of the new higher-level individual. It is a major challenge to understand why (environmental selective pressures) and how (underlying genetics, population structure, physiology, and development) the basic features of an evolutionary individual, such as fitness heritability, indivisibility, and evolvability, shift their reference from the old level to the new level.

The evolution of multicellular organisms is the premier example of the integration of lower-level individuals (cells) into a new higher-level

individual. How does a cell group evolve into a multicellular individual? This is the central question asked in this article. Although kinship has long been appreciated as a necessary precondition for the transition to multicellularity (Maynard Smith, 1988, 1991; Maynard Smith and Szathmáry, 1995; Michod, 1999), there are colonial species with high degrees of kinship that have not evolved true individuality (based on specialization of cells at reproductive and vegetative functions). For example, in all colonial members of the volvocine green algae (Fig. 7.1), all cells in the colony are clonally derived from a single cell, often by just a few cell divisions, yet true individuality based on specialization of reproductive and somatic functions emerges only in the larger colonies. What additional factors are required for the evolution of reproductive altruism, that is, specialization at vegetative somatic functions? Specialization of reproductive and vegetative viability-enhancing functions, what we term germ soma

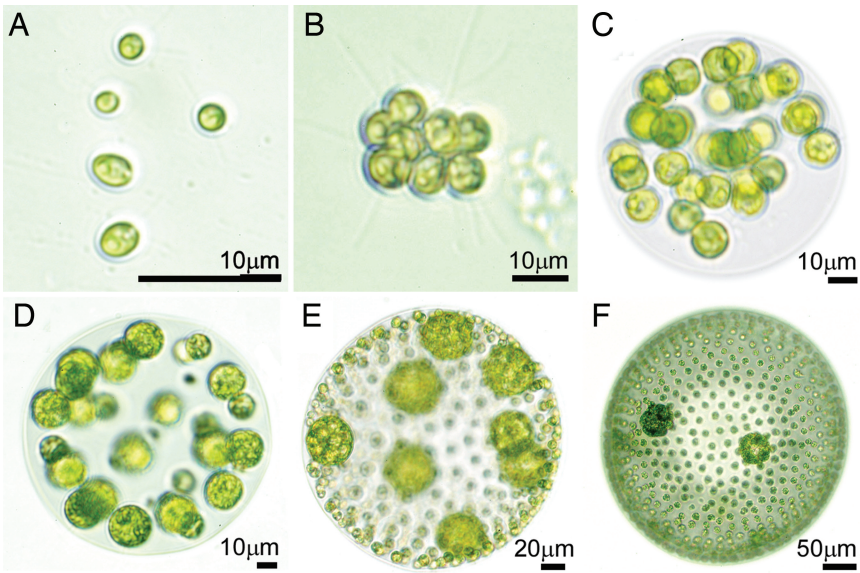

FIGURE 7.1 Examples of volvocine species varying in cell number, colony volume, degree of specialization, and proportion of somatic cells. (*A*) *Chlamydomonas reinhardtii*, a unicell. (*B*) *Gonium pectorale*, a flat or curved sheet of 8–32 undifferentiated cells. (*C*) *Eudorina elegans*, a spherical colony of 16–64 undifferentiated cells. (*D*) *Pleodorina californica*, a spherical colony with 30–50% somatic cells. (*E*) *Volvox carteri*. (*F*) *Volvox aureus*. Where two cell types are present (*D–F*), the smaller cells are somatic cells and the larger cells are reproductive cells. Photos were taken by C. Solari (University of Arizona).

specialization, is a major factor in the conversion of cell groups into true multicellular individuals. Once cells specialize in fitness components, they cannot survive and reproduce on their own: the group becomes indivisible and, hence, an individual.

The individuality of multicellular groups is a complex trait. Following Darwin and his approach in *The Origin of Species* to understanding an organ of such complexity as the human eye, we reduce the complexity to a set of evolutionary steps involving simpler traits, each advantageous by itself. In the case of the evolution of multicellular individuals, these stages might involve the formation of cell groups, the increase of cooperation within cell groups, the evolution of conflict mediators to protect the group against cheaters, the increase in group size, the specialization of cells in essential fitness components of the group, and the spatial organization of these specialized cell types.

Evidently this has happened many times. Multicellularity arose in the myxobacteria some 2,000 mya (Shimkets, 1990) and has evolved in several of the major eukaryotic groups. In the animals and plants, multicellularity evolved between 600 and 1,000 mya. Studying the factors involved in these ancient origins of multicellularity is difficult because the events are obscured by hundreds of millions of years of subsequent evolution. The protists provide a useful group for studying the stages identified above. The volvocine green algae, which by some estimates are between 38 and 70 million years old, present a nearly continuous array of differentiated stable forms representing each of the stages given above. There have been at least three independent origins of individuality based on specialization of reproductive and vegetative functions in this group (Herron and Michod, in prep.).

The volvocine green algae are flagellated, photosynthetic, facultatively sexual haploid eukaryotes with varying degrees of complexity stemming from differences in colony size, colony structure, and specialization of reproductive and vegetative cells (Fig. 7.1). This informal grouping includes the "colonial volvocines" (the families Tetrabaenaceae, Goniaceae, and Volvocaceae) and their close unicellular relatives in the genera *Chlamydomonas* (Fig. 7.1*A*) and *Vitreochlamys*. Colonial forms are generally small clumps or sheets of up to 32 cells such as *Gonium* (Fig. 7.1*B*) or spheres with cells arranged on the periphery, such as *Eudorina* (Fig. 7.1*C*), *Pleodorina* (Fig. 7.1*D*), and *Volvox* (Fig. 7.1 *E* and *F*).

The volvocine algae readily form groups by keeping the products of mitosis together through the use of extracellular materials. There are several adaptive reasons to form groups, and to increase in group size, such as to avoid predators, maintain greater homeostasis in the group, and/or to acquire new specialized cell functions. In addition, there may be a covariance effect described in Eq. 1 in which the fitness of the group

is augmented over the average fitness of member cells. This chapter takes for granted the advantages of larger group size and considers instead the associated costs of groups and how these costs may be ameliorated so as to enhance the benefits of group living. We wish to understand how groups become individuals. The central idea motivating our hypothesis is that by coping with the fitness tradeoffs and the challenges of group living, the group evolves into a new evolutionary individual.

There are several hypotheses for the evolution of cell specialization. The first involves the evolution of cooperation (versus defection). To cooperate, cells presumably must specialize at particular behaviors and functions. The evolution of costly forms of cooperation, altruism, is fundamental to evolutionary transitions, because altruism exports fitness from a lower level (the costs of altruism) to a higher level (the benefits of altruism). The evolution of cooperation sets the stage for defection, and this leads to a second kind of hypothesis for the evolution of specialized cells involving conflict mediation. If the opportunities for defectors can be mediated, enhanced cooperativity of cells will result in more harmonious functioning of the group. A variety of features of multicellular organisms can be understood as ''conflict mediators,'' that is, adaptations to reduce conflict and increase cooperation among cells (Michod, 2003): high kinship as a result of development from a single cell, lowered mutation rate as a result of a nucleus, self-policing of selfish cells by the immune system, parental control of cell phenotype, programmed cell death of cells depending on signals received by neighboring cells, determinate body size, and early germ soma separation. These different kinds of conflict mediators require different specialized cell types. The third hypothesis for specialization involves the advantages of division of labor and the synergism that may result when cells specialize in complementary behaviors and functions. The most basic division of labor in organisms is between reproductive and vegetative or survival-enhancing functions.

This chapter is primarily concerned with the division of labor and cooperation hypotheses. As a model system, we are considering volvocine algae cell groups that are of high kinship because they are formed clonally from a single cell. Hence, the opportunity for conflict should be low in these groups. Nevertheless, the opportunity for conflict can increase with the number of cell divisions and can depend on the type of development (e.g., rapid cell divisions, as in some volvocine algae, might not allow enough time for DNA repair). For these reasons, the conflict mediation hypothesis may help explain the early sequestration of the germ line in some volvocine lineages (Michod *et al.,* 2003).

Evolutionary individuals must have heritable variation in fitness-related traits. The fitness of any evolutionary unit can be understood in terms of its two basic components: fecundity (reproduction) and viability

(survival). As embodied in current theory, tradeoffs between fitness components drive the evolution of diverse life-history traits in extant organisms (Stearns, 1992; Roff, 2002). In the present chapter we are primarily concerned with the cost of reproduction to viability and how this cost scales with colony size. Fitness tradeoffs gain special significance during the transition from unicellular to multicellular life for several related reasons (Michod, 2006; Michod *et al.*, 2006): (*i*) fitness tradeoffs often create a covariance effect at the group level so that group fitness is augmented beyond the average fitness of component cells (see Eq. 1); (*ii*) fitness tradeoffs based on preexisting life-history variation provide a basis for the origin of altruistic interactions within the group (see *Origin of Reproductive Altruism*); and (*iii*) fitness tradeoffs between survival and reproduction, if of convex curvature, may select for cells specialized for reproductive and survival-related functions of the group (see *Cost of Reproduction and Covariance Effect*).

How do groups become individuals? Our hypothesis is that fitness tradeoffs drive the transition of a cell group into a multicellular individual through the evolution of cells specialized at reproductive and vegetative functions of the group. We have modeled this hypothesis (Michod, 2005, 2006; Michod *et al.*, 2006) and have tested our models in two ways. We first ask whether a life-history gene present in the unicellular ancestor was coopted to be an altruistic gene in the multicellular *Volvox carteri* (Fig. 7.1*E*) (Nedelcu and Michod, 2006). By answering this question we address *how* an altruistic gene may originate, that is, by cooption of an existing life-history tradeoff gene. Second, we ask *why* reproductive altruism arises only in the larger members of the volvocine group. Our answer is that the selective pressures leading to reproductive altruism stem from the increasing cost of reproduction with increasing group size (Solari *et al*., 2006a,b).

ORIGIN OF REPRODUCTIVE ALTRUISM

Altruism refers to a behavior or interaction that benefits other individuals at a cost to the individual exhibiting the behavior. Altruism is widely appreciated to be the central problem of social evolution. It is also central to the reorganization of fitness during evolutionary transitions, as already mentioned, because altruism trades fitness from the lower level, the costs of altruism, to the higher level, the benefits of altruism.

In the multicellular green alga *V. carteri*, reproductive altruism is a property of the small flagellated somatic cells. *V. carteri* consists of ≈2,000 permanently biflagellated somatic cells and up to 16 nonflagellated reproductive cells. Terminal differentiation of somatic cells in *V. carteri* involves the expression of *regA*, a master regulatory gene that encodes

a transcriptional repressor (Kirk *et al*., 1999) thought to suppress several nuclear genes coding for chloroplast proteins (Meissner *et al.,* 1999). Consequently, the cell growth (dependent on photosynthesis) and division (dependent on cell growth) of somatic cells are suppressed. Because they cannot divide, they do not participate directly in the offspring but contribute to the survival and reproduction of the colony through flagellar action (Short *et al.*, 2006; Solari *et al.,* 2006a,b). In other words, the somatic cells express an altruistic behavior, and *regA* [whose expression is necessary and sufficient for this behavior (Kirk *et al*., 1999)] is an altruistic gene. Which cells express *regA* and differentiate into somatic cells is determined early in development through a series of asymmetric cell divisions. The asymmetric divisions ensure that some cells (i.e., the germ-line precursors) remain above the threshold cell size associated with the expression of *regA* (Kirk, 1995). As with all forms of cooperation, this altruistic behavior is also susceptible to defection and selfish mutants; indeed, mutations in *regA* result in the somatic cells regaining reproductive abilities, which in turn results in them losing their flagellar capabilities (Kirk *et al.,* 1987). Because motility is important for these algae (flagellar activity is required to maintain themselves in the water column at an optimum position relative to sunlight intensity), the survival and reproduction of *V. carteri* individuals in which such mutant somatic cells occur are negatively affected (Solari *et al.,* 2006b).

How can an altruistic gene such as *regA* originate, and can its evolutionary origin be traced back to the unicellular ancestor of this group? The basic life cycle in *Chlamydomonas reinhardtii* (presumed to be similar to the unicellular ancestor of this group) involves a flagellated and motile vegetative stage, during which the cell grows in size, followed by absorption of the flagella and cell division to produce daughter cells. It seems reasonable to expect that life-history genes would exist in *C. reinhardtii* that would allocate effort to these different stages depending on environmental conditions and, in particular, allocate effort away from reproduction toward survival in conditions not promoting growth. Such a gene could become altruistic in the context of a cell group if it was turned on developmentally in some cells and if its vegetative functions also benefited the group.

Nedelcu and Michod (2006) showed that reproductive altruism (i.e., a sterile soma) in the multicellular green alga *V. carteri* (Fig. 7.1*D*) evolved via the cooption of a life-history gene whose expression in the unicellular ancestor was conditioned on an environmental cue (as an adaptive strategy to enhance survival at an immediate cost to reproduction) through shifting its expression from a temporal (environmentally induced) into a spatial (developmental) context as summarized in Fig. 7.2. The *regA*-like gene in *C. reinhardtii* (Fig. 7.1*A*) belongs to a diverged and structurally heterogeneous multigene family sharing a SAND-like domain (a DNA-

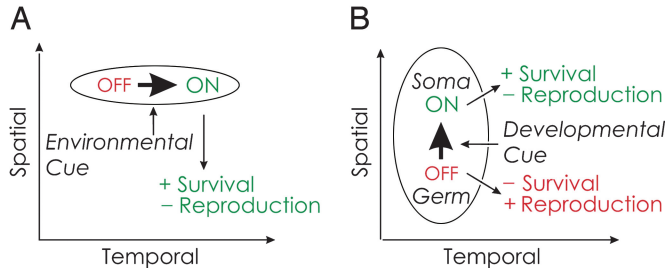

FIGURE 7.2 Change in expression of a life-history gene in space and in time. Expression of genes is indicated by the thick arrows. The effect on fitness is also specified when the gene is off and on. (*A*) In a unicellular individual, the gene is expressed in response to an environmental cue in a temporal context and has the effect of increasing survival while decreasing effort at reproduction. (*B*) This same gene is expressed in a spatial context within a multicellular individual in response to a developmental cue. The cells in which the gene is expressed increase their effort at survival and decrease their effort at reproduction. This figure was modified from Nedelcu and Michod (2006).

binding module involved in gene transcription regulation). This example is perhaps the only example of a social gene specifically associated with reproductive altruism, whose origin can be traced back to a solitary ancestor.

COST OF REPRODUCTION

Having considered *how* an altruistic gene might originate (by cooption of a life-history gene in a unicellular ancestor), we now ask *why* this happens, that is, what are the selective forces favoring soma and reproductive altruism. We wish to understand why it is that soma evolves only in the larger members of this lineage, given that in all species the groups are clonally derived from a single cell and hence of high genetic relatedness. We hypothesize that the selective pressure for soma stems from the increasing cost of reproduction to survival as colonies increase in size.

Flagellar action is an important component of survival. Volvocine algae are denser than water and need flagellar beating to avoid sinking and to find nutrients. These algae are found in quiet, standing waters of transient vernal puddles or in permanent lakes when thermal stirring stops and the lake becomes stratified (Reynolds, 1984; Kirk, 1998). For example, *Volvox* colonies migrate vertically several meters at night, presumably in search of higher phosphorous concentrations (Sommer and Giliwicz, 1986). In addition to motility, flagellar action provides for mixing

the surrounding medium to aid in uptake of metabolites and elimination of waste (Short *et al*., 2006; Solari *et al.*, 2006a).

The first factor that leads to a cost of reproduction to flagellar action is the so-called ''flagellation constraint'' (Koufopanou, 1994). The flagellation constraint refers to the fact that, because of their rigid cell wall, the basal bodies cannot take the position expected for centrioles during cell division while still remaining attached to the flagella (as they do in naked green flagellates). The flagellation constraint becomes critical at the 32-cell colony size, because a flagellum may beat for up to five cell divisions without the basal bodies attached. The second factor leading to a tradeoff between reproduction and motility is that the increasing mass of the reproductive cells and embryos during reproduction decreases motility by increasing drag (Solari *et al*., 2006b). This increasing mass is especially noticeable in the larger species.

Large germ cells are required to form large colonies because of the unusual and likely ancestral form of cell division found in most volvocine species, known as palintomy or multiple fission. Instead of growing to twice their initial size and dividing in two, reproductive cells in palintomic species grow to many times their initial size before undergoing up to ≈13 rounds of division in rapid succession, with little or no growth between divisions. For a reproductive cell to undergo d rounds of (symmetric) division without interspersed growth, it must begin mitosis at a minimum of 2^d times the initial size of the daughter cells.

Koufopanou (1994) argued for the volvocine green algae that soma evolved to keep larger colonies afloat and motile while reproductive cells divide and develop. She showed that the soma-to-reproductive-cell ratio increases with colony size and that the investment in somatic tissue increases twice as fast with colony size as does the investment in germ tissue. However, no direct evidence was given as to why a higher investment in somatic cells is needed for motility as colony size increases. Although the between-species trend is consistent with an increasing cost of reproduction with increasing group size, what selective factors operate within species?

We have modeled the hypothesis that life-history tradeoffs drive evolutionary transitions in individuality by selecting for cell specialization by considering how cells should change their allocation to reproduction and viability as colony size increases (Michod, 2006; Michod *et al*., 2006). Our theoretical results predict that in unicellular organisms the tradeoff curve between viability and fecundity should be concave, but as groups form and increase in size the curve should become increasingly convex (Fig. 7.3*A*) as a result of the increasing cost of reproduction to survival as colonies increase in size (Fig. 7.3 *B* and *C*). A central focus of Solari's hydrodynamic work (Solari, 2005; Solari *et al*., 2006b) is to quantify this hypothesized increasing cost of reproduction.

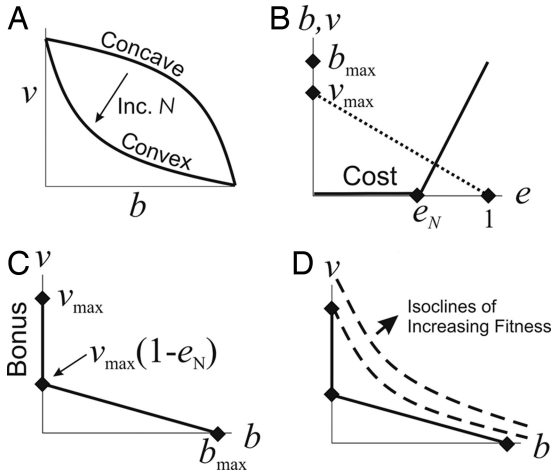

FIGURE 7.3 Fitness tradeoffs. Contribution to viability (v) on y axis and reproduction (b) on x axis. (A) A concave curve changes to a convex curve as group size increases. The piece-wise linear reproduction curve (solid line in B) with linear viability curve (dotted line in B) approximates a convex tradeoff curve (C) at the cell level. (D) Isoclines of group fitness are plotted with this convex tradeoff curve at the cell level. The reproductive effort e_N in B is the cost of reproduction, which increases with group size N, and in C $v_{\max} - v_{\max}(1 - e_N)$ is the "bonus" of soma specialization. This bonus can be obtained only by groups. Alternatively, the bonus of specialization in soma may be viewed as the initial cost of somatic cells dedifferentiating into reproductive cells.

To illustrate how an increased cost of reproduction creates convex curvature, we construct a convex tradeoff curve between viability v and fecundity b in a piecewise linear fashion as shown in Fig. 7.3 B and C. The cost of reproduction e_N is defined as the effort needed to produce an offspring colony of size N. In volvocine algae (Fig. 7.1) this effort depends on the time, energy, and resources needed to grow and divide the embryo so as to produce a daughter colony with N cells. In Fig. 7.3D the convex tradeoff curve from Fig. 7.3C is plotted with isoclines of the additional fitness to the group contributed by a newly added cell. The construction of Fig. 7.3D illustrates qualitatively a prediction of our model (Michod, 2006; Michod *et al*., 2006), which is that the greater the cost of reproduction (e_N), the more likely the isocline touches the tradeoff curve at $v_{\max}$ (meaning the new cell will be soma-specialized; $b = 0$) as opposed to touching at an intermediate value $0 < b < b_{\max}$. Soma-specialized cells get a bonus to viability by virtue of their not paying the cost of reproduction

indicated in Fig. 7.3 *B* and *C*. This bonus can be obtained only in groups and is the basis for the synergistic effects of specialization according to our hypothesis. Alternatively, the bonus of specialization in soma may be viewed as an initial cost when somatic cells dedifferentiate into reproductive cells. Below we present evidence for this cost in terms of decreased flagellar force in *regA* mutants in which somatic cells have flagella for a day before dedifferentiating into reproductive cells.

Solari and colleagues developed a hydrodynamics approach using videotaping of colonies to understand motility and its determinants in volvocine algae (Solari, 2005; Solari *et al.,* 2006b). The swimming force exerted by a single motile cell for the benefit of group motility can be calculated for different species and mutants by these techniques. Single gene mutations in life-history traits can be a powerful approach to understanding the cost of reproduction and tradeoffs between life-history traits (Reznick, 1985; Roff, 2000, 2002). In the *V. carteri regA* mutant, ≈235 cells change their phenotype from being somatic (S) with no reproductive function back to the ancestral state of having both somatic and reproductive functions (being flagellated first and then absorbing the flagella and reproducing). As a result of these changes in reproductive effort at the cell level, the size and motility capacities of the group change. The striking result is that as specialized somatic cells (cells with $b = 0$ in Fig. 7.3) prepare to exert reproductive effort (cells with $b > 0$), there is not only a large decrease in colony motility, but there is a large decrease in the motility force contributed by a single flagellated cell. For example, the average force exerted for group motility by a single motile cell is approximately half in the *regA*$^-$ mutant of what it is in wild type (4.9×10^{-8} dynes versus 8.0×10^{-8} dynes). The cost of reproduction to motility that underlies the convex nature of the fitness tradeoffs (Fig. 7.3) is real and directly measurable in these organisms and is attributable to a change in the effort exerted by single cells within the cell group. There is a caveat in that we do not know whether there are genetic differences (other than a mutation at the *regA* locus) between the *regA*$^-$ mutant strain we have obtained from the Culture Collection of Algae at the University of Texas (Austin, TX) and the wild-type strain.

In summary, comparative data indicate that reproductive effort increases with colony size and that as the investment in reproduction increases, motility declines. The regA mutant indicates that flagellar force declines if somatic cells are to dedifferentiate and start reproducing. In addition, during development, as reproductive cells increase in size, motility does not change for small species, but declines for the larger species (Solari, 2005; Solari *et al.,* 2006b). Apparently, because the length of the flagella increases as cells increase in size, this allows the smaller

species to maintain their motility as they increase in size during development (Solari, 2005; Solari *et al.*, 2006a).

COVARIANCE EFFECT

Tradeoffs among the contributions of cells to the fitness components of the group leads to the "covariance effect," whereby the fitness of the group, W, is greater than the average fitness of its members, $\overline{w}$, by the magnitude of the covariance among fitness components (Michod, 2006; Michod *et al*., 2006) as given in Eq. 1.

$$W = \overline{w} - Cov[v, b] \quad \textbf{[1]}$$

In Eq. 1, $Cov[v, b] < 0$ expresses a tradeoff, and $\overline{w} = \Sigma_i \, v_i b_i / N$. The viability and fecundity of cell i (or its contribution to group viability and fecundity) are v_i and b_i, respectively, and $i = 1, \ldots N$, where N is group size. We take fitness as the product of viability and fecundity, as is appropriate for organisms with discrete generations such as the volvocines. For groups to obtain the benefit of the covariance effect, cells must vary in their reproductive effort. As already mentioned, under a convex curvature of the tradeoff function, there is an advantage of cells specializing in different fitness components (Fig. 7.3).

Convexity or concavity of tradeoffs between fitness components is a basic issue in life-history theory (Levins, 1968; Schaffer, 1974; Michod, 1978; Reznick, 1985; Stearns, 1992; Benkman, 1993; Carriere and Roff, 1995; Takada and Nakajima, 1996; Benson and Stephens, 1996; Strohm and Linsenmair, 2000; Kisdi, 2001; Sato, 2002; Roff, 2002; Blows *et al.*, 2004; Rueffler *et al.*, 2004). For a convex function $v(b)$ the second derivative is positive, and for a concave function $v(b)$ the second derivative is negative, so if we take a particular point b^* and two points equidistant below and above b^*, b^- and b^+, then $v(b^-) + v(b^+) > [<]\ 2\, v(b^*)$. If b is reproduction and $v(b)$ is viability, then convexity of v implies there is an advantage to specializing in the two fitness components. Despite the central relevance of this issue to life-history theory, a recent review (Rueffler *et al.*, 2004) states, "Unfortunately, there is no study known to us which has revealed the details of this curvature for any life-history tradeoff in a specific organism. However, these curvatures are central in life-history theory which indicates a major gap between theory and empirical knowledge." We have addressed this difficult empirical problem by viewing a convex curve in a piece-wise linear fashion (Fig. 7.3) and quantifying the initial cost of reproduction to motility shown in Fig. 7.3*C* and as discussed in *Cost of Reproduction*.

The particular mathematical representation of the covariance effect given in Eq. 1 depends on additivity of fitness effects as described in

Michod *et al.* (2006). Additivity of fitness effects is the simplest assumption possible, and it corresponds to group selection of type 1 in the terminology of Damuth and Heisler (1988) and likely applies early in evolution as groups first start forming. For example, in the volvocine green algae, flagellar action is a main adaptive capacity underlying viability, and the forces contributed by cells to group motility are nearly additive as cells start forming groups (Roff, 2002; Michod *et al.*, 2006). Nevertheless, the assumption of additivity of the contributions of cells to the viability of the group may be relaxed, and the general point underlying the covariance effect still holds (Michod *et al.*, 2006).

As illustrated in Fig. 7.4, if one cell has a high reproductive effort (and hence a low viability and a low cell fitness), this may be compensated for by another cell with high viability (and hence a low fecundity and a low cell fitness) (Michod *et al.*, 2006). Consequently, even though each of these cells by itself would have a low fitness, together they can bring a high fitness to the group, especially under conditions of convexity of the tradeoff. This kind of joint effect, whereby multiple cells may contribute more to the group than could each alone, does not require additivity (Michod *et al.*, 2006). Also, this kind of joint effect would not be possible if group fitness were simply assumed to be the average of the cell fitnesses.

Concerning the transition from single cells to cell groups, the model predicts the following. Single cells must be generalists as far as their

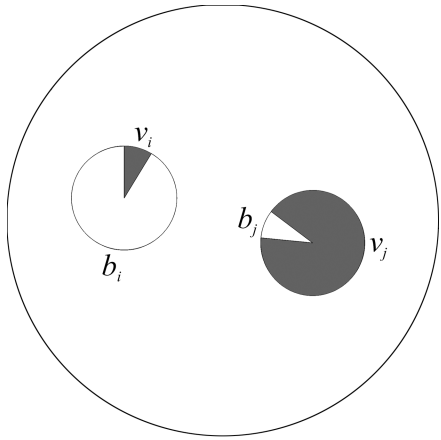

FIGURE 7.4 Two cells jointly specializing in reproduction and viability. Cell i specializes in reproductive effort, b_i, with less effort put into vegetative functions, v_i. Cell j does the reverse. Alone they would each have low fitness, but together in a group they may have high fitness if the tradeoff between reproduction and viability is convex.

components of fitness regardless of the curvature of the tradeoff curve. However, stability of the single-cell life habit to groups requires a concave tradeoff in unicells. In cell groups, if the tradeoff remains concave, cells will not specialize, and there will be no variance to speak of and no covariance effect. However, if the tradeoff becomes convex, as a result of, for example, an increasing cost of reproduction, then cells should start specializing in viability and fecundity leading to an increased group fitness according to the covariance effect.

HOW DOES A GROUP BECOME AN INDIVIDUAL?

Let us return to the basic question asked at the beginning of the chapter: How is it that a group becomes an individual? In answering this question we assume that there is a selective benefit for forming groups and for increasing group size. We also assume there is a means of forming groups, such as by cells sticking together after cell division. According to our hypothesis, as colonies increase in size, the costs of reproduction increase and the curvature of the tradeoff between reproduction and viability goes from concave to convex. This convexity of the tradeoff curve selects for specialization in reproductive and vegetative viability-enhancing functions (germ soma specialization). As cells specialize in these essential fitness components, the fitness of the cells declines while the fitness of the group increases. The covariance effect further enhances the fitness of the group. As a result of the specialization of the cells, fitness is transferred from the cell to group level and the group becomes indivisible and an individual.

Underlying this process is high kinship among the cells, which is fundamental to, but not sufficient for, the emergence of individuality (as the volvocine algae teach us). The evolution of altruism within groups trades fitness from the lower level to the higher level, and the evolution of conflict mediation further enhances cooperation while restricting the opportunity for defecting mutants. How does a gene become altruistic? The hypothesis we have tested in the volvocine algae is that life-history genes in unicells may be coopted for reproductive altruism in the group. What are the selective factors involved, and, in particular, why doesn't altruism originate in the smaller-sized groups? The hypothesis we have tested is that tradeoffs between reproduction and survival become increasingly convex with increasing size selecting for reproductive altruism, that is, soma. In the case of the volvocine algae, soma benefits the group both by enhancing motility and by mixing the surrounding medium allowing for more effective transport of nutrients and waste than would be possible by diffusion alone (Solari *et al.,* 2006a; Short *et al.,* 2006).

In this way, using the concepts of fitness, fitness reorganization, fitness tradeoffs, altruism, kin selection, life history evolution, and social evolution, we can explain a major evolutionary transition in individuality: the evolution of complex multicellular individuals from unicellular and colonial ancestors.

ACKNOWLEDGMENTS

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8

Insect Societies as Divided Organisms: The Complexities of Purpose and Cross-Purpose

JOAN E. STRASSMANN and DAVID C. QUELLER

Individual organisms are complex in a special way. The organization and function of their parts seem directed toward a purpose: the survival and reproduction of that individual. Groups of organisms are different. They may also be complex, but that is usually because their parts, the individual organisms, are working at cross-purposes. The most obvious exception to this rule is the social insects. Here, the individuals cooperate in complex ways toward the common goal of the success of the colony, even if it means that most of them do not reproduce. Kin selection theory explains how this can evolve. Nonreproductive individuals help in the reproduction of their kin, who share and transmit their genes. Such help is most favored when individuals can give more to their kin than they give up by not reproducing directly. For example, they can remain at their natal site and help defend a valuable resource ("fortress defenders"), or they can ensure that at least one adult survives to care for helpless young ("life insurers"). Although kin selection explains the extensive cooperation and common purpose of social insect colonies, it also predicts a certain amount of cross-purpose and conflict behavior. Kin selection has predicted how workers and queens disagree over sex ratios, how potential queens struggle to be the colony's head, how workers try to produce sons, and how other workers often prevent them. Kin

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selection analysis of cooperation and conflict in social insects is one of the outstanding achievements of evolutionary theory.

THE ROCK, THE CLOCK, AND ORGANISMAL COMPLEXITY

Darwin built his theory of descent with modifications from many quarters. He took uniformitarianism from the geologist Charles Lyell, the struggle for existence from the economist Thomas Malthus, and homology from a number of continental biologists. Perhaps most surprising is his debt to a theologian, William Paley. At university, Darwin had Paley's *Natural Theology* (Darwin, 1887b) almost by heart. Paley pointed to the complexity of organisms and claimed that such complexity required a supernatural intelligence. Darwin's chief achievement was to provide a scientific explanation for adaptive complexity.

Paley had famously built his argument from a rock and a clock (Paley, 1802). A stone, he argued, did not beg for any special explanation. It was simple, predictable, unchanging, devoid of obvious purpose. It might have been put there by some intelligence, but nothing about it begged for that explanation. A watch told a different story. The gears, levers, and springs work together in intricate harmony, causing the hands to move across the labeled face and measure time. Such complexity of design or purpose could not arise by chance. The watch must have had a designer, a watchmaker. Paley then applied the argument to organisms and their parts. The eye has a complex arrangement of parts that have a clear purpose, endowing its bearer with sight, and such complexity of purpose seemed to imply a designer and a maker. Throughout the rest of the book, Paley polishes the argument and applies it to other cases, including the sting of the worker honey bee, which he called a neutral bee.

Darwin won the argument with Paley long ago. Both had candidate explanations for complexity, but only Darwin also described a natural mechanism for adaptation and a natural explanation for the changes observed in fossils. Only Darwin explained aspects of biology that were nonadaptive consequences of history, from vestigial organs and other homologies to biogeographical patterns. Our understanding that organisms are a mix of historical constraint and adaptation by natural selection has led to many successful predictions about the natural world, whereas Paley's theory stands mute about the details. In other words, Darwin's theory is much richer than a simple explanation for design; it makes many further extensions and predictions. Some of these extensions and predictions were not fully appreciated in Darwin's time. The last several decades have seen increased attention to a further important question

about the apparent design of organisms. A good theory of design also ought to explain what kinds of entities are adapted and what kinds of complexity they show.

Organisms, together with man-made machines, seem to show a unique kind of complexity. We will call this the ''complexity of purpose.'' ''Purpose,'' as used here, is a metaphor, just as ''natural selection'' is a metaphor and has no real selector. This kind of complexity can even be used to define biological organisms. The organism is the consolidated unit of design or adaptation; almost everything in the organism seems built to further the individual's survival and reproduction (Queller, 1997). Few parts of the organism are organized to gain at the expense of other parts, and few parts of the organism are organized to benefit other organisms (the chief exception being offspring).

The same cannot generally be said about groups of organisms. How does a flock of birds compare with the rocks and clocks? The parts of a flock of birds, the individual birds themselves, do not generally appear organized to benefit the flock. To the contrary, the members compete for food and mates, sometimes by physical fights, and they hide behind each other as shields against predation. Groups of organisms, e.g., flocks, populations, species, and communities, are not themselves clock-like or organismal.

Neither are they like the rock, because they are far more complex. But in contrast to the complexity of purpose shown by organisms, these aggregates have what we call the ''complexity of cross-purpose.'' The behavior of flocks, populations, and communities is extraordinarily rich, but not in a predictable and unified manner like the meshing of gears in a watch. Instead, much of the complexity stems from indifference of the parts to other parts and the apparent striving of each part to further its own survival and reproduction, if necessary at the expense of other parts.

Evolutionary theory has been addressing this issue of what kinds of units are adapted, and as it has done so, an interesting puzzle has emerged. The entities that we recognize as individual organisms actually originated as groups of lower-level units (Buss, 1987; Maynard Smith and Szathmáry, 1995). Somehow, the first cell assembled a group of components sufficient to sustain replication. The eukaryotic cell began as an assemblage of several prokaryotic cells, with at least the mitochondria and chloroplasts having independent origins. Larger organisms are groupings of cells. If groups show cross-purpose, how did they combine and make the transition to the unity of purpose of a single organism?

Social insect groups can give us special insight into this question. We will argue that social insect colonies are much like organisms, and we will show how their unity of purpose can arise through kin selection. We will also show that some cross-purpose remains, that colonies are not perfectly coherent. These remaining conflicts might be viewed as

compromising the organismal nature of the colony. But a closer look at traditional individuals shows that they too have some internal conflicts (Burt and Trivers, 2006; Haig, 1996; Hurst *et al.,* 1996). For example, selfish genetic elements such as transposons can not only make up large parts of genomes, using expensive resources and extending replication times, but they can also interfere with the functioning of the individual (Burt and Trivers, 2006). The conflicts within cooperative social insect colonies have helped biologists to identify conflict in other cooperative entities, for example the conflicts between maternal and paternal genes mediated by genomic imprinting (Haig, 2000).

A HOUSE DIVIDED

A small stingless bee out in a tropical forest might seem like any other animal as it searches for food to survive and reproduce. Upon closer inspection, a more complex picture would emerge. The foraging bee is a member of a complex colony inside a tree hollow. Within, there is a citadel of wax with a smooth protective skin surrounding fat peripheral cells that contain honey and pollen, and central combs of smaller brood cells, all held together and supported by a lacy network of wiry wax struts. Small female worker bees are busy everywhere, bringing in food and propolis, adding to the structures, cleaning, and guarding. But the focus of their attention is a single female, the queen, with a greatly distended abdomen and worn, useless wings. A throng of workers surrounds the queen so closely as to slow her approach to an empty cell. At the empty cell, the queen antennates the inside, as if checking its construction. The workers dart in and out, at one time crouching before the queen, at other times rearing up before her. This agitated ballet ends with the queen stroking the workers, who then regurgitate larval food into the cell, one after the other, until it is full. Then the queen lays a single egg that floats on the provisions. When she leaves, the workers carefully bend the collar of wax over and close the cell. The egg will hatch and grow to adulthood undisturbed but benefiting from the workers' attention to climate control and defense (Zucchi, 1993).

This scene summarizes what is special about social insects: complex communication and integration of behavior, and individuals caring for the offspring of another. The colony as a whole appears to have the kind of integration and common purpose normally associated with individual organisms, with the parts subservient to the whole. The stingless bee colony is highly organized, both structurally and behaviorally. The provisioning and oviposition process seems to have an almost clock-like precision, with elaborate coordination between the queen who lays the egg and the workers who build and provision the cell. This process only

works as a whole. If any step in the provisioning and oviposition process is omitted, the whole operation may fail.

For societies with this level of organization, it is no wonder that the claim for organismal status of groups has sometimes been made (Seeley, 1989; Wheeler, 1911). If this claim stands up to scrutiny, it is extraordinary in two ways because we think of organisms as consolidated units in two senses: they are both physically contiguous and genetically uniform.

An organism is typically one solid, connected mass. If it is a single cell, it is bounded by a membrane; if it is multicellular, the cells abut one another and form a discrete larger unit. If a social insect colony is an organism, however, it is a divided one, with parts (the individuals) freely moving past each other and only occasionally coming into direct contact. Other organisms with separated parts are known. A lizard may detach its tail to save itself from a predator, and the tail continues to twitch, distracting the predator long after the main body of the lizard has escaped. Similarly, when a honey bee worker stings a foe, the barbed sting can easily detach from the honey bee's body, and when it does, the sting continues to dig into the victim's skin and the poison sac continues to contract and deliver more of its venom. But these detached organs act independently for only a brief time before expiring. Moreover, these parts are clearly secondary, in the sense that a joining of trunk and tail did not form the lizard. Instead, tails are normally attached parts of the organism, both in the lizard itself and in its relatives with nondetachable tails. A social insect superorganism, on the other hand, is built from the very beginning of detached parts. Physical attachment is rare and ephemeral, such as when army ant workers interlock to form a sheltering bivouac.

A typical organism is also genetically homogeneous. Again, social insect colonies differ from this standard. In the simplest colony structure, all members are offspring of a single queen and her mate, so they share many genes, but each receives its own unique combination of parental genes. In other species, this genetic distinctness is exacerbated by the presence of multiple queens or multiple mates. This genetic structure is utterly different from the clonal, mitotically derived set of cells that constitute a typical multicellular organism (Maynard Smith, 1988). Given that natural selection operates by favoring genes that pass copies into the next generation, it is little surprise that a clonal entity can evolve cooperation. If social insect colonies lack this unity of genotype, what gives them the unity of purpose that makes them an organism rather than a contentious flock?

HOW ARE COLONIES ORGANISMAL?

It is not hard to view a termite castle, an army ant bivouac, or a wasp colony as a single, coordinated organism. Each shows division of labor,

with specialization for reproduction, nutrition, communication, defense, and often thermoregulation. Seemingly autonomous individuals are actually workers whose function appears directed entirely to the whole, such as a worker who fans the colony to cool it or one who lives her life as a living honey storage pot (Seeley, 1989). The earliest analogies with multicellular organisms focused on these physiological processes and led to Wilson's physiologically oriented definition of a superorganism (Wheeler, 1911; Wilson, 1971; Hölldobler and Wilson, 1990; Bonner, 2006). But the analogy could not be pushed too far, perhaps because of fundamental differences between the physiology of a divided organism (with separately mobile individuals) and a multicellular organism. The mobility of individuals means information and resources can be walked throughout the colony with no need for specialized structures.

Mobility may therefore underlie the relatively small number of castes in social insects. Castes are in some ways analogous to cell types in multicellular organisms. Each caste or cell type specializes in certain tasks, with the division of labor aiding the whole. All social insects have functional reproductive and worker roles, but only some are morphologically differentiated into queen and worker castes. A fraction of these species have multiple worker castes, with the primary distinction being between small foragers and large soldiers (Wilson, 1971). Even highly specialized functions, such as being a honey storage vessel in honeypot ants or using one's head to block the colony entrance in *Colobopsis* ants, are usually performed by castes that also have more general functions.

Fig. 8.1 shows the complexity, measured as the number of types of subunits, of social insect colonies, compared with multicellular individuals. In one sense, of course, social insect colonies are more complex than multicellular individuals because the colonies include all of the complexity of their constituent individuals and then add more complexity at the colony level. But it is still interesting to compare the degree of complexity added by the specialization of parts in the two cases. Following Bonner (2006), as a measure of the complexity of specialization, we use cell-type number and caste number to represent the complexity of individuals and colonies, respectively. We are unable to use phylogenetically independent contrasts, but Fig. 8.1 well illustrates how depauperate in specialized castes social insects are compared with cell specialization in organisms, a pattern that is unlikely to disappear when analyses are performed with accurate phylogenies. Complexity increases with the number of units, the units being cells for organisms and individuals for colonies (Fig. 8.1). The lower complexity of colonies can be explained partly by size. On average, social insect colonies do not have as many units as multicellular animals; colonies rarely have more than a million individuals, whereas large organisms have billions of cells. But that is not the complete explanation. The

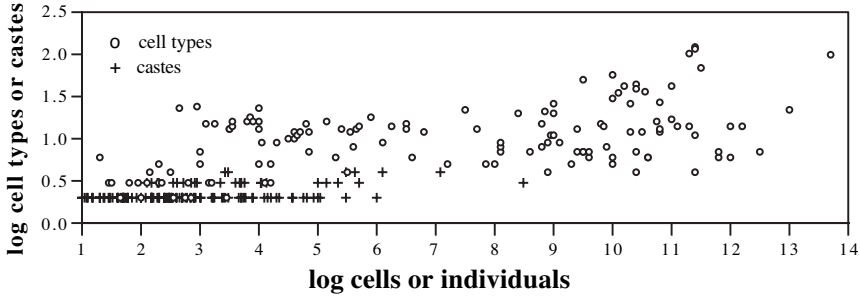

FIGURE 8.1 The number of specialized types (cell types or worker castes) as a function of the number of units (cells in an organism or number or individuals in a colony). Cell data (open circles) are from Bell and Mooers (1997). Caste data (crosses) are for ants and were compiled by Bonner (1993) from data in Hölldobbler and Wilson (1990) and from judgments of caste number by E. O. Wilson (Bonner, 1993). Mean colony sizes were used when available; when they were not available, we used the midpoint of the range.

specialization complexity of insect societies is lower for a given number of units than the complexity of multicellular organisms. This conclusion seems fairly clear despite the difficulties inherent in defining the number of castes or cell types, for which we have relied on the judgments of others (see Fig. 8.1). Social insects almost never have even as many as five castes, whereas many small multicellular organisms attain 10, or many more, cell types. We suggest that the mobility of the separate parts of a social insect colony reduces the need for specialized types at the level of the colony.

Despite the limitations of the physiological superorganism model, the superorganism view can be useful, not only for understanding divided organisms but also for stimulating new ways to view traditional organisms. Colonies may not have the same kinds of systems as animals and plants for fulfilling colony functions, just as in typical organisms the parts of a colony are subordinate to the whole. As Darwin himself noted, ". . . if on the whole the power of stinging be useful to the social community, it will fulfill all of the requirements of natural selection, although it may cause the death of some few members" (Darwin, 1872, p. 163). A colony can be viewed as an organism simply because it is highly adapted at the colony level. Of course, this logic does not apply to just any social community, so the social insects force the question of how some entities become organismal while others do not.

Although social insect colonies may not have physiologies that closely match those of multicellular organisms, they do have their own systems for

defense, nutrition, and reproduction. Close study of any social insect species would reveal examples of individual traits that have evolved for colony function, but the honey bee is the best-studied case. For example, honey bees use floral food sources, and they need to track this ever-changing resource. Their famous dance language allows workers to communicate the quality and location of a resource to nestmate workers. But they also have mechanisms to effectively allocate work among multiple food sources, even when each individual has quite limited information. Fidelity to a good food source is not absolute; a fraction of workers always seeks to discover new food sources. Returning foragers adjust the intensity of their waggle dancing (the number of waggle runs) according to the profitability of their trip. Foragers from better sources therefore recruit more followers, so the colony concentrates on the better food source (Seeley, 1997).

Foragers can also tune the intensity of their recruitment dances according to how much the colony needs food, but this requires coordination with the workers that specialize in nectar processing. If a colony needs more nectar, the nectar-processing bees that have this information crowd closer to the hive entrance. This means that a returning forager unloads her nectar quickly, which cues her to intensify her dancing to recruit more foragers (Seeley, 1997) and allay the shortage of nectar. On the other hand, there may sometimes be more nectar coming into the colony than the nectar processors can handle, resulting in inefficiently long unloading times. In this event, foragers perform the tremble dance, which is different from the waggle dance in that it stimulates other workers to become nectar processors (Seeley, 1997).

Such integrated behaviors of many workers in a honey bee colony allow the colony to find and exploit food efficiently, to alter group foraging based on individual information, and to adjust the number of foragers and nectar processors to meet changing needs. No individual is doing anything that by itself would be very useful; instead, each is performing a role in a process that only makes sense in terms of increasing colony function. Such a smooth coordination among workers in finding, harvesting, and processing food makes the argument for the colony as an organism compelling. Similar kinds of coordination are found wherever they are looked for in social insects, for example, in nest construction in *Polybia* wasps (Jeanne, 1986), in nest-finding in honey bees (Seeley and Visscher, 2004) and *Leptothorax* ants (Mallon *et al.,* 2001), and in the establishment of foraging trails by army ants (Franks *et al.,* 1991).

THE SUCCESS OF SOCIAL INSECTS

If colonies have found ways to be more efficient than separate individuals, one might expect social insects to be particularly successful. In

fact, they are fantastically successful, particularly the ants and termites, but also the bees and wasps (Wilson, 1987). Success can be measured as current ecological success, e.g., geographic diversity, species richness, and biomass. Ants are very speciose and are native to all terrestrial habitats except Antarctica, Iceland, Greenland, and a few remote islands (Wilson, 1987). At one thoroughly studied location in the Amazonian forest, social wasps, ants, bees, and termites make up four-fifths of all insect biomass and over a fifth of all animal biomass (Fittkau and Klinge, 1973).

Success can also be measured in evolutionary antiquity, staying power, and diversity. Social insects are represented in the fossil record by impressions of their bodies and their nests. The fossils of bodies are most useful for tying these insects to extant lineages, whereas the fossil nests demonstrate clear evidence of ancient sociality. Body fossils of presumably social termites, ants, bees, and wasps are found in the Cretaceous, whereas nest fossils are found for some lineages as early as the Triassic (Grimaldi *et al.,* 1997; Hasiotis, 2003; Bordy *et al.,* 2004). Only an origin before the breakup of Pangaea in the late Triassic is consistent with the worldwide extent of major social insect lineages. The dispersion of major social insect lineages was essentially complete before the high sea levels of the Late Cretaceous isolated many land masses 100 Mya (Hasiotis, 2003; Bordy *et al.,* 2004). Another indication of the evolutionary robustness of the social habit is the absence of clear evidence for major lineages of social insects that subsequently went extinct (Wilson, 1987).

AN INORDINATE FONDNESS FOR KINSHIP

Any theory of adaptation or design ought to explain why social insect groups are so well adapted while most groups of multicellular organisms are not adapted. Perhaps the most common feature of insect societies, aside from their cooperation, is that they are family groups. In some species, colonies are headed by one singly mated queen (or in termites, a queen and a king), and all other colony members are full siblings. In others, the degree of relatedness is lower, but it is nevertheless substantial in all but a few species of unicolonial insects that are recently derived from those with higher relatedness. The high relatedness within colonies is often enforced by overt kin recognition: nonrelatives are recognized and rejected. Haldane once quipped that nature suggests that the creator must have had an inordinate fondness for beetles. With respect to superorganisms, there also seems to be an inordinate fondness for kinship ties among cooperators.

Darwin had at least an inkling of this: "As with the varieties of the stock, so with social insects, selection has been applied to the family, and not to the individual, for the sake of gaining a serviceable end" (Darwin, 1872, p. 230). But the idea was not formalized until W. D. Hamilton's work

in the 1960s (Hamilton, 1964a,b). Taking a gene's-eye view, Hamilton reasoned that a gene could spread in future generations not only by contributing to the production of offspring, but also by aiding reproduction of other relatives who might share the gene. Genetic relatedness specifies the comparative values of different kin. Hamilton's rule predicts what behaviors will be favored by selection. A particularly useful form of Hamilton's rule, for behaviors that exert a fitness cost, c, on some relatives and give a fitness benefit, b, to others, is $r_b b > r_c c$. The two fitness effects are scaled by the relatedness of the actor to those benefited, r_b, and to those harmed, r_c. Crucially, individuals can be selected to give up their own offspring ($r_c = 1/2$) to help other relatives, provided the benefit b is sufficiently higher than the cost c. Genetic relatedness among individuals is essential, for without it no value of b, the benefit to cooperation, will favor giving up reproducing oneself.

THE HAPLODIPLOID HYPOTHESIS

Hamilton (1964b) also noticed a special feature of social ants, bees, and wasps: they share a haplodiploid sex determination mechanism in which haploid males arise from unfertilized eggs and diploid females arise from normal fertilized eggs (Normark, 2003). This is an ancestral trait in Hymenoptera that arose long before sociality (Hamilton, 1967), but it affects relatednesses in ways that could favor sociality. What makes it significant for sociality is that sisters are related by 3/4 because they share all their genes from their haploid father. Other things being equal, a sister would therefore pass on more genes by rearing sisters ($r = 3/4$) than by rearing her own progeny ($r = 1/2$), favoring daughters who remain with their mothers to rear additional sisters. Hamilton noted that this observation could potentially explain at a stroke at least two salient feature of social insects (Hamilton, 1964b). First, there have been many origins of sociality in the haplodiploid Hymenoptera and few elsewhere, termites being the most notable exception. Second, only females work in the Hymenoptera, whereas both sexes work in diploid termites.

However, this haplodiploid hypothesis is debatable, for a variety of reasons (Alexander *et al.,* 1991; Queller and Strassmann, 1998). First, most haplodiploids have not evolved sociality, whereas a few diploids have. Another issue is that relatedness is elevated only to full sisters and is lowered to brothers ($r = 1/4$) (Crozier, 1970). Thus, if a female helps to rear an equal mixture of sisters and brothers, the average relatedness (1/2) is exactly the same as to her own offspring and exactly the same as full siblings in diploids. Haplodiploidy can still help if workers can concentrate on rearing sisters (Hamilton, 1972), but the advantage is transitory and disappears at sex ratio equilibrium (Crozier and Pamilo, 1996).

Also, the special 3/4 relatedness applies only in colonies with a single, once-mated queen. Multiple queens and queen replacement reduce relatedness, as does multiple mating. Accurate estimates of relatedness among colony members are now available for hundreds of species, typically based on inherited variation in DNA microsatellite loci (Gadagkar, 1990b; Crozier and Pamilo, 1996; Peters *et al.*, 1999). Although relatedness among female colony members in many species is near the full-sister value of 3/4, in many other species it is lower.

Finally, other possible explanations, some of them noted by Hamilton himself (Hamilton, 1964b), have been proposed for the facts the haplodiploid hypothesis seems to explain. Specifically, the high incidence of sociality in the Hymenoptera and the all-female workforce may relate to preadaptations involving parental care (Alexander *et al.*, 1991). The solitary Hymenoptera have an unusually high level of parental care, meaning that adaptations for nest-building, prey capture, brood care, sanitation, and defense are already in place. It must be much easier to evolve alloparental care in groups that already have parental care. And because it is females that provide the parental care in solitary Hymenoptera, with special adaptations such as the sting, it is not surprising that females provide the care in social Hymenoptera (Alexander *et al.*, 1991).

KIN SELECTION AND SYNERGISM: LIFE INSURANCE AND FORTRESS DEFENSE

Kin selection has been so closely identified with the haplodiploid hypothesis that concerns with the latter have caused some to question kin selection in general. But of course Hamilton's rule does not require that relatedness to beneficiaries must be higher than relatedness to one's own offspring. If $r_b = r_c$, or even if $r_b < r_c$, Hamilton's rule can still favor altruism if the benefit is sufficiently greater than the cost ($b > c$) (West-Eberhard, 1975). The question then concerns how it is possible to rear more young by aiding the beneficiary than by reproducing independently. Synergies from division of labor between helpers and reproducers are easy to see after sociality has evolved, but this kind of specialization seems unlikely to be present at the beginning of sociality.

Two kinds of factors seem especially likely to provide the necessary advantage to helping: "fortress defense" and "life insurance" (Queller and Strassmann, 1998). Fortress defenders live in protected, expandable sites that generally include food (Andersson, 1984; Alexander *et al.*, 1991; Crespi, 1994), such as the wood galleries of termites and the plant galls of social aphids and thrips. An offspring can gain by remaining at the natal site, even if she has to rear less-related collateral relatives, because she avoids risking death by migrating to a new site (see Fig. 8.2*a*). Because

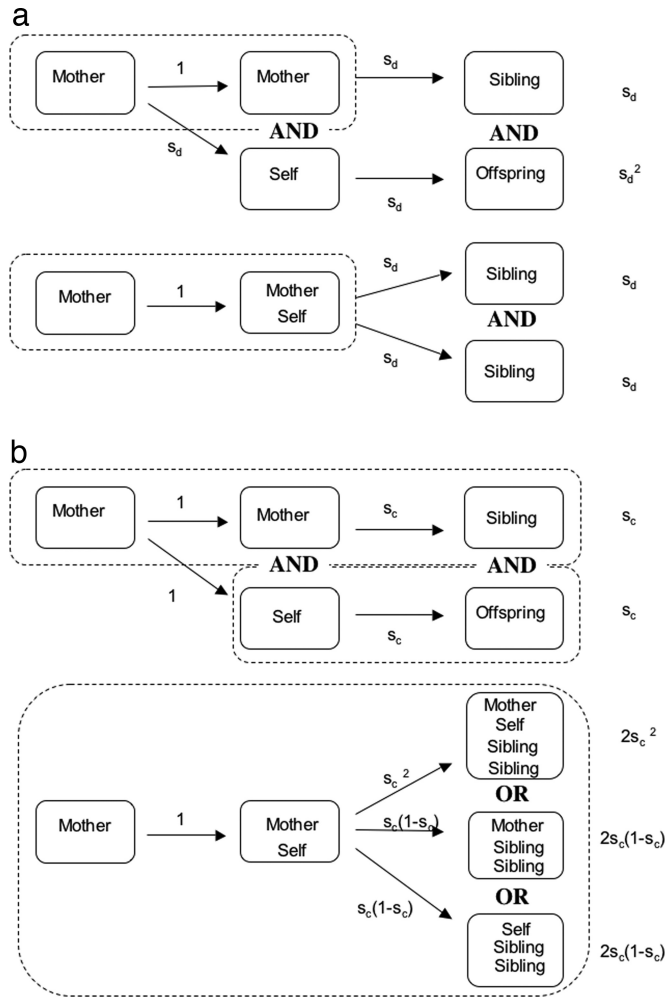

FIGURE 8.2 Fitness advantages of sociality when siblings and offspring are equally valuable. Arrows show transitions from one time period to another, and enclosure within dashed rectangles indicates the same nest. (*a*) Leaving vs. Fortress Defense. If an individual (self) leaves her natal site, she survives dispersal with probability s_d, whereas her mother survives in the safe natal site. Both then produce offspring. Alternatively, if self stays in the safe site with her mother, she avoids a bout of dispersal mortality and doubles her mother's output. (*b*) Leaving vs. Life Insurance. Here we assume that dispersal carries no cost but that a parent survives the period of parental care with probability s_c. If she dies during this period, her offspring also die. When self stays with her mother, there are three ways for the offspring to survive: if both adults survive, if only the mother survives, and if only self survives.

food is available at the home site, little feeding care is needed, and the first worker specialists are generally soldiers specialized for defense.

By contrast, life insurers cooperate to ensure that dependent young survive (Queller, 1989, 1994, 1996; Strassmann and Queller, 1989; Gadagkar, 1990a). They can live in a variety of sites, safe or unsafe, but generally have helpless young that need food and protection. The problem is that an adult must undertake dangerous foraging for her young, but if she dies during one of these trips, her still-dependent young also die. But when a daughter stays at the natal nest to help, and either the mother or the daughter dies, the survivor can take over feeding and protecting the young, giving rise to the synergistic advantage (Fig. 8.2*b*). Wasps, bees, and ants appear to fit this mold. The crucial assumption that dead individual's investments can be saved by its surviving colony mates has been experimentally confirmed in a stenogastrine wasp (Field *et al.,* 2000). Other advantages besides fortress defense and life insurance are also possible, and much work remains to be done on assessing their relative importance.

RELATEDNESS IS STILL IMPORTANT

Whatever the fitness advantages of altruism might be, they are selectively irrelevant unless they go to relatives. Hamilton's kin selection theory (Hamilton, 1964a,b) still provides the framework for understanding altruism, even if the altruism is not driven by extra-high relatedness. As noted above, the fact that social insect colonies consist of families, and that they exclude outsiders, shows that relatedness matters. But other studies have tested more specific predictions about the importance of relatedness.

A recent comparative study of wasps and bees (Wenseleers and Ratnieks, 2006) showed how workers modulate their altruism and selfishness according to relatedness in queenless colonies. Colonies with queens removed were used because (as we will see below) worker selfishness can be repressed in colonies with the queen present, either by the queen herself or by other workers. With the queen gone, some workers develop ovaries and lay unfertilized eggs that will develop into males. If all workers ceased working and took up laying eggs, the colony's production of males would presumably fall, because a certain number of workers are needed to feed the larvae. In fact, the queenless colonies never had more than 40% reproductive workers; at least 60% remained as helpers. Most interesting was the finding that fewer workers laid eggs from species in which relatedness among workers was high. In other words, more workers stayed in the altruistic helping mode when relatedness was high. Relatedness explained 62% of the variance in percentage of helpers (Wenseleers and Ratnieks, 2006). Variation in relatedness also predicts variation in helping behavior in birds and mammals (Griffin and West, 2003).

Unexpected and powerful evidence of the importance of relatedness has come from sex ratio studies. As noted above, haplodiploid female workers are related to full sisters by 3/4 and to brothers by 1/4. This implies that, other things being equal, these workers ought to prefer helping sisters (Hamilton, 1972). Specifically, Trivers and Hare (1976) showed that in a colony with a single once-mated queen, workers should prefer to invest three times as much in future queens as in males. They also showed that, in species of ants likely to have a single, once-mated queen, investment ratios are in fact closer to this 3:1 ratio than to the usual Fisherian 1:1.

More impressive evidence comes from species with variable relatedness. When a queen mates multiple times, workers will not favor this 3:1 ratio because the workers are equally related (by 1/4) to half sisters and brothers (because brothers do not have fathers, multiple mating by the queen does not change their relatedness). This means that these workers should rear more males than those in singly mated colonies, and the frequency-dependent nature of sex ratio selection should cause the two kinds of colonies to increasingly specialize (Boomsma and Grafen, 1990, 1991). Workers in colonies with singly mated queens should specialize largely in rearing queens, and workers in colonies with multiply mated queens should specialize in rearing males. This odd prediction of what has come to be called ''split sex ratio theory'' was strikingly confirmed in a study of the ant *Formica exsecta* (Sundström *et al.,* 1996) and has been confirmed in many other species (Queller and Strassmann, 1998; Chapuisat and Keller, 1999; Bourke, 2005).

Besides showing that workers are indeed sensitive to relatedness, the sex ratio studies made an even more important point: There can be conflict within these apparently superorganismal colonies. Queens are equally related to their sons and daughters, so they should prefer the standard 1:1 sex investment ratio (Trivers and Hare, 1976). The resulting conflict can lead to inefficiencies that are decidedly against the interests of the superorganism as a whole. For example, the split sex ratio described above for *Formica exsecta* is achieved only after some waste. Queens in both singly and multiply mated colonies laid the same sex ratio of eggs, but workers in the singly mated colonies achieved their preferred investment in full sisters by killing many of the male larvae (Sundström *et al.,* 1996; Chapuisat *et al.,* 1997).

It seems paradoxical that this elegant evidence for kin selection theory comes from conflict rather than from cooperation, but there is really no contradiction. Kin selection theory shows how individuals can further the reproduction of their own genes, and this is sometimes achieved by cooperation and sometimes by conflict.

AN EVEN MORE INORDINATE FONDNESS FOR SELF

Conflict over sex ratios was an exciting finding, both because of the support that it provided for kin selection theory and because it showed that even sterile workers could find ways to pursue interests that were different from the queen's. It thus poked a hole in the view of a colony as superorganismal, a small hole by itself, but one that opened up a vista of other realms of possible conflict. Hamilton's rule predicts more extensive conflict over the question of who gets to reproduce. Although typically related, individuals are genetically separate, and each individual is usually more related to its own young than to those of a relative. If other things are equal, Hamilton's rule predicts that each individual would prefer to take the reproductive role. Thus, even though advantages like fortress defense or life insurance select for helping instead of going it alone, the issue of helping is not completely resolved because it is often better, still, to be helped.

Conflict over reproduction has long been apparent to researchers working on simple social insect societies that are made up of morphologically identical females, such as *Polistes* wasps. Colonies are initiated by single females or by groups of females, who are often sisters. They do not share reproduction equally. Instead, they form dominance hierarchies (Pardi, 1942; West-Eberhard, 1969; Strassmann, 1981) enforced by time-consuming aggression so intense it can result in death, although some species have conventions that reduce the battling (Hughes and Strassmann, 1988; Seppä et al., 2002). Once the hierarchy is set, the losers function as workers, if they choose to remain at the nest. But they still may not work optimally, instead waiting for a chance to reproduce. In *Liostenogaster flavolineata*, a Malaysian stenogastrine wasp that lacks morphological castes, a queue based on order of arrival determines who succeeds the dominant queen. When females reach the number two spot in the queue, they work less hard (Field *et al.,* 2006). In other words, when the option of reproducing directly appears more likely, they decline to take as many risks on behalf of their relatives.

The success of predictions of sex ratio conflict led researchers to ask how much conflict over reproduction remains in highly eusocial insects, those with morphologically distinct queen and worker castes. Are these colonies subject to the complexities of cross-purpose?

It turns out that even the most advanced societies are not immune to this kind of conflict. When it comes time for honey bee colonies to divide, several half-sister queens are reared with special food in extra-large cells. The old queen leaves with much of the workforce to start a new colony. Then, the first of the new queens to emerge as an adult seeks out all of the other queen cells and uses her sting to kill her sister rivals (Gilley, 2001).

Why honey bees produce these extra queens is not fully clear, perhaps as insurance against one of them dying. But they do limit the conflict to a few individuals by controlling queen production through the special feeding they require. This limitation is extremely common in social insects with queen and worker castes. Queens generally require more food, offering the opportunity to control queen production (Wilson, 1971). It is instructive to see what happens in the unusual cases in which this constraint does not hold.

In one genus of stingless bees, *Melipona*, caste is not determined by differential feeding. Instead, workers and queens develop in cells of the same size, provisioned with the same amounts of food. This presumably leaves the choice of being queen up to each developing female larva. As a consequence, a significant fraction of females (5–20%) develop into queens, with small heads, large abdomens, and lacking the pollen baskets required to be effective workers (Wenseleers and Ratnieks, 2004). Because stingless bees reproduce by colony division, this amount of queen production is far more than the single queen they need, every once in awhile, to head a new colony. The excess queens, useless for work and a potential threat to the old queen, are slaughtered by workers (Wenseleers *et al.*, 2004). The 5–20% reduction in worker production must constitute a significant cost to the colony and clearly shows that cross-purpose can remain important in advanced social insects.

Stingless bees other than *Melipona* determine caste by the usual means of feeding some larvae more, but this does not entirely solve the problem. In some species, in which brood cells are adjacent, a larvae that is supposed to be a worker can tunnel from its own cell into its neighbor's, consume the food stores intended for its neighbor, and develop as a queen (Engels and Imperatriz-Fonseca, 1990). In other species, some individuals with worker-sized food allotments will nevertheless develop into morphological queens (Wenseleers *et al.*, 2005). In *Schwarziana quadripunctata*, these dwarf queens make up only 0.6% of all individuals reared in worker-sized cells but 86% of all queens reared. These queens are less successful than normal-sized queens in attaining reproductive status and are executed more readily by the workers. Still, the strategy appears to be successful often enough to pay, inasmuch as 22% of all reproductive females are dwarf queens (Wenseleers *et al.*, 2005). Some ant genera, such as *Myrmica* and *Solenopsis*, also have some small queens (called microgynes), which may be the result of individual larvae determining their own caste fate in colonies that are initiated by budding (Bourke and Franks, 1995; McInnes and Tschinkel, 1995).

The threat of other queens may lie behind another colony-level design flaw that is usually not obvious but is present in many species: the lack of a backup queen. Consider the fungus-growing ants of the genus *Atta*. A

mature *Atta* colony is a huge and intricate operation. Millions of ants cultivate fungi deep underground. The nest has chambers a person could stand in, gardens tended by a suite of worker castes, including leaf processors who use microbial fungicides and have specialized organs to carry these symbionts (Currie *et al.,* 2006). However, this metropolis of ants has but a single queen, and when she dies the colony dies: the fungus gardens are overrun by rogue fungi, the workers cease to rear brood, and the galleries ultimately collapse. This disaster could be avoided by the relatively simple matter of keeping on hand one or more backup queens. But this would risk unleashing competition among the queens that might harm the interests of both the current queen and her workers.

WORKER REPRODUCTION AND POLICING

The existence of dwarf queens shows that even larvae that are fed less than the normal queen amount can reproduce. This ability can also extend to workers. Workers are females that are morphologically or behaviorally specialized to forage, care for brood, and defend the colony. None of these tasks is enhanced by egg-laying, and yet workers in nearly all species maintain some ability to lay eggs. Workers in many species regularly do so, producing males because they are uninseminated. They have considerable incentive to do this because a worker is more related to her own sons (1/2) than to brothers (1/4) produced by her mother (Trivers and Hare, 1976).

In some species, the queen "polices" these worker-laid male eggs, eating them when she finds them. Indeed the elaborate provisioning and oviposition process of stingless bees described earlier may sometimes be less a matter of cooperative communication about the filling of the cell than a contest over who gets to fill it. All that actually needs to be accomplished is regurgitation of food into the cell by workers, laying of an egg by the queen, and then sealing of the cell by workers. As it is, many more workers than necessary surround the empty cell. When the queen approaches the empty cell she can be very aggressive toward the workers, who ritually either approach her or back away. The queen often aggressively solicits food from the workers, who nearly always refuse to provide it. After the cell is filled, the queen lays an egg in it. Interestingly, the workers close the cell with their abdomen in it, a position in which they might lay an egg, something that might account for the commonness of worker male production in stingless bees (Tóth *et al.,* 2002b). [In some species, workers return later to sealed cells to open them and oviposit (Beig, 1972; Tóth *et al.,* 2002a), which can succeed because the worker-laid male egg hatches quickly, and the larva hunts down and kills the older female larva in the cell.] The fact that these behavior patterns differ considerably

between species is consistent with an "arms race" caused by conflict (Why would a good, efficient communication system need to change rapidly?). In other words, the complexity of the provisioning process may not be purposeful clockwork, but more like a boxing match full of punches and counterpunches. The winner varies from species to species, with queens producing all of the males in some and workers producing most in others (Tóth *et al.,* 2004).

If we do not see these elaborate direct contests in other species, it may be because the stingless bees are rather unusual in mass-provisioning their brood cells with all of the food before oviposition. This focuses the laying process on one cell at a time and brings the contenders together. And of course, workers are favored to produce the males because colonies are headed by single-once mated queens (Peters *et al.,* 1999).

In other social insects, workers have wider opportunities to lay eggs when the queen is absent. Policing by the queen then seems unlikely to succeed in colonies with thousands of workers, so one might expect workers to take advantage of this lack of control and produce many males. In some species they do, but in other species, like the honey bee, the queen still produces all of the males. Here, policing of worker-laid eggs is performed by other workers, in a seeming lapse of class solidarity. Each worker should prefer to lay her own male eggs, but how should she view the contest between other workers and the queen? When the queen is mated multiply, as in honey bees, and workers are usually half-sisters, each worker will prefer the queen's males ($r = 1/4$) to those of other workers ($r = 1/8$) (Ratnieks, 1988). Again we have the inefficiencies of conflict, with some colony members negating what the others have done. Workers may be neuter but, to recall Paley's adjective, they are anything but neutral. They would prefer to reproduce, but they also prefer not to let each other reproduce, and therefore they ensure that the queen wins. Oddly, the stronger the policing, the less actual conflict there may be. In species in which most worker-laid eggs are removed, few workers develop ovaries and lay eggs (Wenseleers and Ratnieks, 2006).

Workers can sometimes win the battle with queens in the most extreme way possible: by killing the queen (Bourke, 1994). Usually this is not a beneficial option, because the workers need the queen to produce both more workers and more reproductive females. But certain social insects have an annual colony cycle with males produced at the end, which makes the queen dispensable at the end of the season. Killing her allows workers to produce all of the males. Again, the choice appears to be affected by relatedness in ways predicted by kin selection theory: queens disappear more in singly mated species because those are the species in which workers favor production of males by other workers (Foster and Ratnieks, 2001).

CONCLUSIONS

Living things are complex, but this complexity is of two broad types. Organisms show complexity of apparent purpose, with all of the parts acting for the whole. Groups, however, are usually dominated by the complexities of cross-purpose; the parts seem goal-directed, but the goals are not shared, and the result is often anything but elegant. The most spectacular exceptions, at the group level, are social insect societies, in which the individuals usually do seem to act toward a common goal.

Any scientific theory purporting to account for biological complexity ought to account for this special nature of social insects. Why do their colonies show a degree of apparent purpose lacking in other aggregations, herds, and flocks? The kin selection extension of natural selection theory does explain this; cooperation results from the opportunity to give sufficiently large benefits to kin.

More importantly, kin selection theory has successfully predicted new findings. Although social insect colonies have clock-like design in many respects, kin selection theory predicts who is throwing sand into the clockworks, as well as which gears might be slipped and which springs sprung. Many of the predicted findings, such as sex ratio conflict and policing, were otherwise completely unexpected. The success of this approach shows that the Darwinian paradigm is capable of explaining not just the adaptations of organisms but also how new kinds of organismal entities come into being.

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9

Symbiosis as an Adaptive Process and Source of Phenotypic Complexity

NANCY A. MORAN

Genomics has revealed that inheritance systems of separate species are often not well segregated: genes and capabilities that evolve in one lineage are often stably acquired by another lineage. Although direct gene transfer between species has occurred at some level in all major groups, it appears to be far more frequent in prokaryotes than in multicellular eukaryotes. An alternative to incorporating novel genes into a recipient genome is acquiring a stable, possibly heritable, symbiotic association and thus enjoying benefits of complementary metabolic capabilities. These kinds of symbioses have arisen frequently in animals; for example, many insect groups have diversified on the basis of symbiotic associations acquired early in their evolutionary histories. The resulting associations are highly complex, often involving specialized cell types and organs, developmental mechanisms that ensure transfer of symbionts between generations, and mechanisms for controlling symbiont proliferation and location. The genomes of long-term obligate symbionts often undergo irreversible gene loss and deterioration even as hosts evolve dependence on them. In some cases, animal genomes may have acquired genes from symbionts, mirroring the gene uptake from mitochondrial and plastid genomes. Multiple symbionts often coexist in the same

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host, resulting in coadaptation among several phylogenetically distant genomes.

Genomic sequence data, coupled with evolutionary analyses, have brought major new insights to our understanding of biological evolution. One of the biggest revelations is the extent to which biological adaptation and phenotypic innovation within a particular genetic lineage have depended on adopting already highly honed functional systems from other lineages, often only distantly related to the recipient. Traditional views of the evolutionary process, forged during the neo-Darwinian synthesis, focused on adaptation occurring as the result of natural selection acting on existing genes within a species. Most such adaptation occurs in small steps, although mutations in existing genes can sometimes cause major phenotypic changes. But the ability to reconstruct evolution at the molecular level, and especially the analysis of full genome sequences, has revealed that integration of genes originating from disparate sources has occurred on a very large scale.

Gene uptake confers novel adaptive capabilities, thereby enabling ecological expansion into new niches. But it also confers phenotypic complexity that is manifested at the genomic, the physiological, and the morphological levels. In many cases, and specifically in multicellular eukaryotes, the route to recruiting foreign genes and novel metabolic capabilities involves symbiotic association, that is, a persistent close interaction with another species. Comparative genomic studies now allow us to reconstruct the history of symbioses and episodes of genome amalgamation and to elucidate their contribution to the complexity evident in the dominant forms of life on earth.

Below, I briefly describe the routes by which organisms stably acquire capabilities evolved in other lineages, with emphasis on insights that have come from recent genome sequencing. I end with examples of the complex phenotypes generated by hereditary symbiosis in insects and with the consequences of this genome integration through symbiosis for animal evolution.

DISPARATE GENE SETS CONFER DISTINCT CAPABILITIES

The evolutionary motivation for assimilating foreign genes stems from the obvious fact that species differ in gene sets and corresponding capabilities. Thus, intimate association between two lineages can readily arise through natural selection acting within each species to fix alleles that promote close association with the other species. Although differences in metabolic capacities among species have long been evident, genomics is

providing a far more detailed view of how these differences have arisen. First, many genes and corresponding capabilities arose after lineage diversification had begun, so that some species descend from ancestors that never possessed the corresponding genes. Examples include fundamental metabolic innovations such as phototrophy (Mulkidjanian *et al.,* 2006) and methanogenesis as well as specialties such as production of particular toxins or pathogenicity mechanisms, including the type III secretion system used by many bacteria for infecting host cells (Saier, 2004).

A second reason that species differ in gene sets and capabilities is that ancestral genes are often lost. Comparisons of genomic content among closely related species are now revealing that gene loss has been an important and ongoing process in evolution in all lineages. For example, tryptophan, required in proteins of all organisms, is produced by a single pathway requiring several enzymatic steps, and reconstructions of the evolution of this pathway point to a single origin before the divergence of the three domains of life (Xie *et al.,* 2003). Many descendant lineages, including all animals and a variety of prokaryotes and parasitic protists, have lost the pathway and are dependent on acquiring tryptophan from ecologically associated species.

The loss of such useful capabilities may seem counterintuitive. But another major insight from comparative genomics is that genes are constantly being eliminated by mutation combined with insufficient purifying selection. The pathways lost most frequently are those with more enzymatic steps and higher energy requirements, suggesting that selection may favor pathway inactivation when the end products can be environmentally acquired. Outstanding examples of gene loss include many host-dependent microbial lineages; obligate pathogens, both bacterial and eukaryotic, lose genes for using substrates not encountered in their restricted environment and lose genes for synthesis of metabolic products that are dependably provided by the host cells (Shigenobu *et al.,* 2000; Gardner *et al.,* 2002; Tamas *et al.,* 2002; Liu *et al.,* 2006; Payne and Loomis, 2006). As a group, animals are unusual in lacking the ability to produce numerous universally required metabolic compounds such as vitamins and amino acids and also in lacking capabilities for producing bioactive secondary compounds that can act as toxins and defenses.

ACQUISITION OF FOREIGN GENES AND CAPABILITIES

For many genes, the distribution among species reflects not only the lineage of origin and subsequent losses in some descendants but also transfer to new lineages. Thus, a species can acquire, more or less instantaneously, traits that originated in unrelated lineages or that were lost ancestrally. Gene transfer is clearly important in prokaryotes, for which

hundreds of genomes have been sequenced. As an illustration of the extent of foreign-gene uptake, a study reconstructing the sources of genes in the genomes of numerous species in the Gammaproteobacteria showed that the overwhelming majority of genes in most genomes were acquired from external sources after these lineages diverged from a common ancestor (Lerat *et al.,* 2005). The impact of gene uptake can be massive even on short time scales. For example, in comparisons of gene sets of distinct *Escherichia coli* strains, for which orthologous DNA sequences are 99% identical, indicating recent shared ancestry, 25% or more of the genes in one genome are absent from other strains, having arrived recently from more distant (often unidentified) sources (Welch *et al.,* 2002). In Bacteria, such incorporation of foreign genes is the major route to the origination of novel capacities (Ochman *et al.,* 2000), as illustrated in *E. coli*, in which recently acquired genes are the basis for strain-specific pathogenicity (e.g., Welch *et al.,* 2002).

Firm estimates are not yet possible for rates of gene acquisition by eukaryotic genomes. For Bacteria, the evidence for rampant gene acquisition is primarily based on comparing related genomes by using complete gene inventories, sequence features, and gene arrangements. To date, the numbers of sequenced genomes for clusters of related eukaryotic species are relatively small for estimating total gene uptake by using comparisons of gene inventories and gene arrangements. Currently, the extent of foreign gene uptake, and specifically genes arriving from Bacteria, does appear to be substantial in certain groups of unicellular eukaryotes, including *Dictyostelium* (Eichinger *et al.,* 2005) and other lineages of amoebae (Andersson *et al.,* 2006). But even in unicellular eukaryotes, duplication and divergence of existing genes appear to be more prominent than gene uptake as a process generating change in genome contents (e.g., Kellis *et al.,* 2004; Aury *et al.,* 2006). Similarly, plant nuclear and plastid genomes have not been found to contain substantial numbers of acquired genes, although acquisition of genes by plant mitochondrial genomes does occur relatively frequently and is currently the major category of gene incorporation by multicellular eukaryotes (Richardson and Palmer, 2007). Despite increasing findings of horizontal transfer even in eukaryotes, the capacity to incorporate new genes underlying enzymatic pathways and processes has severe limits (Kurland *et al.,* 2003). Some groups of organisms rarely incorporate foreign genes, and, even in those that do, such as most free-living Bacteria, many genes underlying important informational and metabolic processes seem to resist horizontal transfer, as illustrated by the case of the tryptophan biosynthetic pathway (Xie *et al.,* 2003).

SYMBIOSIS AS A MECHANISM OF ADAPTATION AND AS A SOURCE OF PHENOTYPIC COMPLEXITY

An alternative to incorporating foreign genes directly into a recipient genome is to develop a close relationship with a species able to provide some beneficial product or process. Symbiotic associations that are mutually beneficial raise immediate issues involving evolutionary stability—issues that Darwin noted and also addressed in *The Origin of Species*: "Natural Selection cannot possibly produce any modification in a species exclusively for the good of another species; although throughout nature one species incessantly takes advantage of, and profits by, the structures of others" (Darwin, 1859a, p. 201). Because different species possess different capabilities, such as different abilities to use substrates or to produce required metabolic compounds, it is now evident that one species can profit through associations with another species and that this benefit can be mutual; that is, providing a benefit to another species need not entail a cost. These differences in capabilities have become more defined through genomic data, which allow us to use genome sequences to map many metabolic capabilities onto the branches of the tree of life. In many circumstances, one symbiotic partner immediately profits from providing some benefit to the other partner; for example, a compound available in excess to one species might act as a limiting substrate to its partner, which in turn can generate from this substrate additional compounds needed by the first species. Because of the different capabilities of different species, mutually beneficial associations can arise *de novo* from organisms that are not coevolved, and these associations can then become stabilized through natural selection acting within each species. Mutual advantage often can be enhanced by natural selection when the two lineages are associated across generations, although heritability of the symbiosis is not a requirement for mutual benefit (Sachs *et al.,* 2004). Genomic data inform us that many symbioses are founded on the differences in metabolic capabilities that are enforced by differences in gene content of genomes.

Symbiosis binds organisms from all domains of life and has produced extreme modifications in genomes and structure (e.g., von Dohlen *et al.,* 2001; Waters *et al.,* 2003; Gilson *et al.,* 2006; Nakabachi *et al.,* 2006). In addition, symbiosis affects genome evolution by facilitating gene transfer from one genome to another and by facilitating the loss from one genome of genes that are present in both symbiotic partners. Both of these events can cause a facultative symbiosis to become an obligate one because one partner becomes dependent on products of genes that are restricted to the genome of the other partner. The result is a complex, fused metaorganism, with different compartments for different portions of its required genes, mechanisms for transporting compounds and gene products between compartments, complex development maintaining the different cell types

in proper proportions and arrangements, and different replication systems and population genetic processes applicable to different parts of the metagenome. In the following, I consider some of the most prominent symbioses in microorganisms and then focus on the role of symbiosis in generating phenotypic complexity in animals.

BACTERIOPHAGE AS GENE VECTORS AND SYMBIONTS OF BACTERIA

Although conventional views of bacteriophage have emphasized their role in killing bacterial hosts, it is now apparent that they often affect host ecology in other, more beneficial ways, including acting as vectors of genes that can enhance bacterial fitness in a particular environment (Comeau and Krisch, 2005; Hendrix, 2002). This realization comes in part from genome sequences for bacteria, which reveal that bacteriophage have made large ongoing contributions to bacterial genome contents and physiological capabilities, often sometimes becoming lasting parts of the bacterial genome even when genes required for the bacteriophage life cycle have been eliminated (Comeau and Krisch, 2005). The richest reservoir of gene diversity lies in the bacteriophage (e.g., Breitbart *et al.,* 2002; Kwan *et al.,* 2005), suggesting that the innovations that they are able to contribute are correspondingly diverse. For example, a very large proportion of pathogenic bacteria studied in humans and other mammals use pathogenicity mechanisms encoded by phage-borne genes (Wagner and Waldor, 2002), some of the competitive mechanisms used among bacterial strains are derived from phage-derived structures (e.g., Nakayama *et al.,* 2000), and the central enzyme components underlying photosynthesis in ubiquitous marine cyanobacteria are transferred among bacterial hosts by bacteriophage (Lindell *et al.,* 2004). Even phage-induced lysis of the bacterial host cell can be a mechanism favoring the growth and fitness of the bacterial host clone, as other cells containing the same phage genes persist and benefit from products released during the death of their sister cells (Wagner and Waldor, 2002).

SYMBIOTIC MICROBIAL CONSORTIA

One consequence of the fact that gene transfer is not without limits even in prokaryotes is the frequent evolution of microbial consortia or microbial syntrophy, that is, close, often obligate, associations of two (or a few) unrelated organisms that depend on one another for metabolic products or maintenance of chemically permissive conditions. Examples of close, coevolved associations include those between methanogenic archaea and bacteria or protists capable of fermentation (e.g., Schink,

2002), phototrophic aquatic consortia consisting of a flagellated bacterium coated with phototrophic bacteria (Overmann and Schubert, 2002; Glaeser and Overmann, 2004), and an archaean–bacterium partnership that links methane oxidation and denitrification (Raghoebarsing *et al.,* 2006). More broadly, metabolic interdependencies among lineages are a major reason that most microbes in soil and other natural habitats cannot be established in pure laboratory culture (Schmidt, 2006). In some systems, detailed knowledge of the interactions of the different bacterial types shows that there has been extensive coadaptation, with recognition mechanisms for promoting the associations and with communication systems for coordinating the behavior of cells from phylogenetically distant groups (Schink, 2002).

SYMBIOSIS AS A ROUTE TO ADAPTATION AND COMPLEXITY IN EUKARYOTES

Molecular phylogenetics, based on sequence data from only a few genes, verified the hypothesis that mitochondria and plastids are derived from bacterial symbionts; these results also identified the bacterial lineages that gave rise to these symbionts and indicated a single primary origin in each case (Woese, 1987; Keeling, 2004; Embley and Martin, 2006; Kurland *et al.,* 2006). Genomic data indicate that both plastids and mitochondria have transferred genes from the bacterial to the host genome, resulting in a genomic mélange (e.g., Martin *et al.,* 2002; Keeling, 2004). Genome sequences have also helped to elucidate further complexities of the mitochondrial and plastid symbioses in some lineages: for example, secondary and tertiary symbioses in which a plastid-containing eukaryote itself becomes a symbiont in a new eukaryotic host, sometimes resulting in bizarre remnant genomes (e.g., Gilson *et al.,* 2006) and complicated histories of gene transfers among several genomes (Keeling, 2004). Beyond cellular organelles, symbioses have arisen innumerable times in eukaryotic hosts. Protists often carry prokaryotic symbionts, and their nuclear genomes may have taken up genes from past symbionts (e.g., Eichinger *et al.,* 2005; Andersson *et al.,* 2005). Much of the complexity of modern eukaryotic cells arises from this divided ancestry involving gene movement between genomes and the evolution of mechanisms for targeting gene products to the correct cellular compartment. Even individual enzymatic pathways or functional systems can be encoded by complex combinations of genes with different histories of direct horizontal transfer, transfer through symbiosis, or vertical inheritance (e.g., Chen *et al.,* 2006; Richards *et al.,* 2006).

Symbioses originating in multicellular eukaryotes are rampant, with highly specialized obligate associations found in fungi, plants, sponges,

and most animal phyla (McFall-Ngai, 2002; Brundrett, 2003; Schardl *et al.,* 2004). Many of these symbioses are vertically transmitted, resulting in continuous association of individual genetic lineages across generations and facilitating the evolution of mutually beneficial features. Others are reestablished each host generation from a dispersing symbiont population.

FORCES FAVORING SYMBIOSIS IN ANIMALS

Animals stand out as a group having lost many ancestral capabilities, making them unusually dependent on other organisms. In the Metazoa generally, gene loss has resulted in the inability to synthesize essential metabolic compounds, yielding a long list of required dietary components, including numerous cofactors (vitamins) as well as the 10 essential amino acids (Payne and Loomis, 2006). The losses of these pathways reflect the evolution of a digestive cavity by animals, which acquire diverse nutrients by eating tissues and cells of other organisms. If nutrients are readily available in the diet, selection to maintain pathways for production of these compounds will be relaxed, resulting in the inactivation of the underlying genes. Comparisons of recently sequenced animal genomes reveal that particular animal lineages have continued to eliminate particular sets of genes. For example, the *Drosophila* genome lacks many genes that are present in both honeybee and mammals, reflecting gene loss in the dipteran lineage (Honeybee Genome Sequencing Consortium, 2006).

Animals also appear to be limited in the ability to incorporate foreign genes directly into their nuclear genomes. To date, the complete genomes of several mammals, nematodes, and insects have not revealed large numbers of foreign genes. [In fact, the initial report that the human genome contains numerous genes acquired from Bacteria (Lander *et al.,* 2001) was later shown to be unwarranted, reflecting artifacts of analysis and limited data from eukaryotic genomes (e.g., Stanhope *et al.,* 2001).]. So, although uptake of nonfunctional DNA does occur (e.g., Sunnucks and Hales, 1996; Kondo *et al.,* 2002) and sometimes may result in adaptive incorporation of functional genes from exogenous sources (e.g., Mallet *et al.*, 2003; Daimon *et al.,* 2003), current evidence indicates that this process is limited in animals. Duplication of existing genes and regulatory changes are far more important. Among potential barriers to gene acquisition in animals are the need for regulating expression in the context of the more complex development and also the separation of germ line. In contrast to organelles (mitochondria and plastids) that arose in single-celled hosts and are present in most or all cells in modern multicellular hosts, symbionts acquired by animals are typically restricted to specialized organs and often live primarily in somatic tissues, where they may be intracellular or

extracellular. This compartmentalization may act as an obstacle to gene transfer from symbiont to host because persistent gene transfer can only occur in germ line cells.

HEREDITARY SYMBIOSIS IN ANIMALS

Before molecular methods were available, Paul Buchner and his students conducted extensive surveys of specialized symbiosis in animals; this work was summarized in a book translated into English in 1965 (Buchner, 1965). Because symbionts are mostly noncultivable under typical laboratory conditions, the approaches of Buchner and his coworkers relied primarily on microscopy to trace the diversity of associations with microbes found in different invertebrate groups, with particular attention to insects. Buchner's central theses included the idea that symbiotic microorganisms shared long evolutionary histories with their host clades and also the premise that the main role of animal symbionts was to provide nutrients to hosts that used deficient diets. The bulk of his work was devoted to describing the complex developmental adaptations that have allowed hosts to maintain stable associations.

Of all of the groups that Buchner studied, he devoted most attention to the sap-feeding insects, some of which possess unusually elaborate symbiotic systems involving multiple microbes. This group of insects serves as an exemplar of the remarkable complexity and variety that can arise in the context of evolving symbioses. One basis of the abundance of symbiotic interactions in this group is the poor diet of most species: plant phloem sap and xylem sap are both particularly unbalanced nutritionally, lacking essential amino acids, and, in the case of xylem sap, vitamins and carbohydrates. Thus, a phloem sap- or xylem sap-feeding animal, while enjoying the advantage of a constant food supply, must collaborate with a microbial symbiont able to synthesize missing nutrients from precursors that are available.

The group of insects that includes cicadas, treehoppers, planthoppers, leafhoppers, and spittlebugs, corresponding to the suborder Auchenorrhyncha in the order Hemiptera, shows a remarkable diversity of symbiotic associations. Buchner referred to this group as "the fairy land of symbiosis," and his student H. J. Müller studied hundreds of auchenorrhynchan species in attempting to reconstruct the evolution of this bewildering diversity of associations (summarized in Buchner, 1965). Individual insects can possess up to six symbiont types, with each symbiont transferred from mother to progeny and packaged during development by means of specialized mechanisms.

Molecular phylogenetic studies have greatly extended our understanding of the origins and evolution of animal symbioses, validating and

extending Buchner's thesis that many of these associations have long evolutionary histories. Such studies have shown repeatedly that nutritional symbionts have evolved in parallel with their hosts, starting with studies of aphids and *Buchnera* and extending to whiteflies, scale insects, psyllids (Baumann, 2005), tsetse flies (Chen *et al.,* 1999), stinkbugs (Hosokawa *et al.,* 2006), carpenter ants (Schröder *et al.,* 1996), and cockroaches (Lo *et al.,* 2003).

The oldest such example of bacterial symbiosis underlying nutrition in an insect is that of *Sulcia muelleri*, a symbiotic clade in the bacterial phylum Bacteroidetes found in most groups of Auchenorrhyncha (Moran *et al.,* 2005b). The *Sulcia* phylogeny matches that of hosts, based on current understanding, although this symbiont has been lost from numerous subclades of Auchenorrhyncha. The most plausible explanation for its occurrence is that an ancestral member of the Bacteroidetes, possibly a gut-dwelling associate, became an obligate symbiont of an insect host that was beginning to feed by sucking liquid from primitive vascular plants. This transition would have occurred by the time of the common ancestor of modern Auchenorrhyncha, implying an origin by the late Permian, at least 270 million years ago, as based on the insect fossil record (Moran *et al.,* 2005b). Thus, a symbiotic event was critical to the emergence of one of the earliest major groups of herbivores on vascular plants and has been retained by many thousands of descendant species (Fig. 9.1).

Sulcia is typical of insect nutritional symbionts in that it inhabits the cytosol of specialized cells, grouped into a specialized host organ called the bacteriome (Fig. 9.2). In this case, this structure is a paired laterally positioned organ in the abdomen of adult insects; this structure appears to be homologous across the Auchenorrhyncha (Moran *et al.,* 2005b). Symbionts are packaged into these specialized cell types during development, requiring specialized mechanisms of part of host and/or symbiont for limiting the location and growth of the bacteria. In aphids, cells destined to become bacteriocytes exhibit distinctive patterns of gene expression very early in development, before colonization by the symbiont population acquired from the maternal bacteriocytes (Braendle *et al.,* 2003).

MULTIPARTITE SYMBIOSES WITHIN INSECT LINEAGES

The elaborate symbiotic systems noted by Buchner for species of Auchenorrhyncha arise from the recruitment of additional symbionts in particular sublineages. In many cases, these later additions become obligate symbionts that coexist with *Sulcia*. The best-studied case to date is that of the sharpshooters, a subfamily of leafhoppers containing several thousand species. Sharpshooters are distinguished from related insects in that they have adopted a xylem sap diet, imposing distinct nutritional

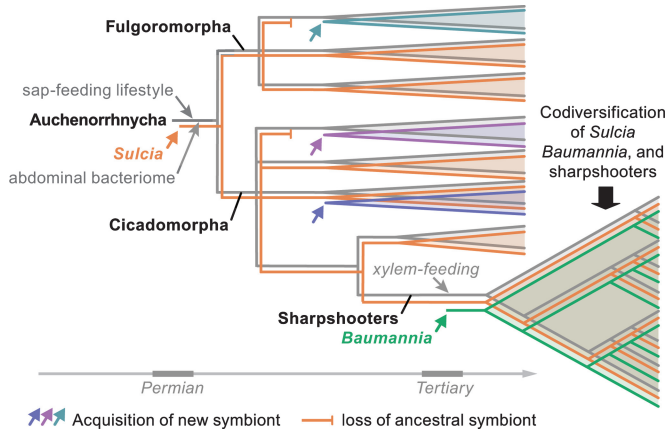

FIGURE 9.1 Schematic diagram of the evolutionary steps in the acquisition of bacterial symbionts and sap-feeding lifestyles in the insect group Auchenorrhyncha (Hemiptera), with emphasis on the sharpshooters (Cicadellinae). The bacterial symbionts *S. muelleri* (Bacteroidetes) and *B. cicadellinicola* (Gammaproteobacteria) colonized at different stages in sharpshooter evolution; they provide nutrients needed to supplement the xylem sap diet (Moran *et al.*, 2005b; Takiya *et al.*, 2006; Wu *et al.*, 2006).

FIGURE 9.2 An individual sharpshooter (*Cuerna sayi*) dissected to reveal the bacteriomes on each side of the abdomen. These structures contain the intracellular symbionts, *Sulcia* and *Baumannia*. Photo by R. Rakitov and D. Takiya, University of Illinois.

needs, especially in the arena of vitamins and energy metabolism. They are also distinguished by the presence of a relatively restricted symbiont, *Baumannia cicadellinicola*, belonging to the Gammaproteobacteria and related to *Buchnera* (Moran *et al.,* 2003; Takiya *et al.,* 2006). Sharpshooters form a much younger group than the Auchenorrhyncha, with fossils not appearing until the Eocene. Phylogenetic analyses based on genes from both symbionts and insect hosts support the following evolutionary reconstruction: *Sulcia* was ancestrally present in a host lineage that acquired *Baumannia* at the same approximate time as the switch to xylem-feeding, consistent with the view that its nutrient-provisioning capabilities were a requirement for this lifestyle. After the acquisition of *Baumannia*, both *Sulcia* and *Baumannia* diversified in parallel with their sharpshooter hosts, through strict maternal transmission, based on the congruence of phylogenetic trees from the three clades (Takiya *et al.,* 2006) (Fig. 9.1).

Other Auchenorrhynchan groups have recruited other bacterial and fungal symbionts, few of which have been studied beyond microscopy studies describing their morphology and transmission. Other cases of successive acquisition of symbionts are numerous, with cases documented in both aphids (Perez-Brocal *et al.*, 2006) and weevils (Lefevre *et al.,* 2004).

GENOME SEQUENCES ELUCIDATE COMPLEMENTARY FUNCTIONAL ROLES OF SYMBIONTS AND HOST

The nutritional role of symbionts associated with specialized organs (bacteriomes) (another of Buchner's central theses) has been elucidated by cloning and sequencing of specific genes, complete genome sequencing, and studies of gene expression (Moran and Degnan, 2006). Thus, of four *Buchnera* genomes now fully sequenced, all are extremely small but nonetheless contain all or most pathways for synthesis of the amino acids that are required by animals (Shigenobu *et al.,* 2000; van Ham *et al.,* 2003; Perez-Brocal *et al.,* 2006).

One genome of *Sulcia* has been partially sequenced, from the host species, *Homalodisca coagulata* (the "glassy-winged sharpshooter") (Wu *et al.,* 2006). As for *Buchnera*, *Sulcia* possesses a very small genome but retains pathways for synthesis of most essential amino acids, nutrients that are in short supply in both phloem and xylem sap, in both of which amino acid profiles are dominated by nonessential amino acids. A complete genome sequence for *Baumannia* of *H. coagulata* confirms that this symbiont plays a critical role in the dependence of sharpshooters on a xylem sap diet. Whereas *Sulcia* retains pathways for amino acid provisioning, *Baumannia* contains a large number of pathways for biosynthesis of vitamins (Wu *et al.,* 2006). The complementarity between capabilities evident from the genomic sequences of the two symbionts is striking. For example,

the single essential amino acid biosynthetic pathway that is retained by *Baumannia*, that for histidine, appears to be the only such pathway missing from the *Sulcia* genome (Wu *et al.*, 2006). The two symbionts live in close proximity within the host bacteriome and sometimes with a single *Sulcia* cell surrounded by closely adjacent *Baumannia* cells (Fig. 9.3).

GENOMIC DECAY IN OBLIGATE SYMBIONTS AND HOST DEPENDENCE

Obligate nutritional symbionts of insects provide prime examples of genome degradation in obligately host-associated bacterial lineages. These symbionts possess the smallest known genomes of cellular life forms with only 182–650 genes for fully sequenced cases (Wernegreen, 2002; Nakabachi *et al.*, 2006; Perez-Brocal *et al.*, 2006). In two symbiont clades, genomes of multiple representatives are available, with two for symbionts of carpenter ants ["*Blochmannia*" species (Degnan *et al.*, 2005)] and four for *Buchnera* symbionts of aphids (Shigenobu *et al.*, 2000; Tamas *et al.*, 2002; van Ham *et al.*, 2003; Perez-Brocal *et al.*, 2006). Divergences in these two cases represent changes 30–200 million years of evolution, yet symbiont genomes show few changes. These cases are the extremes in genome

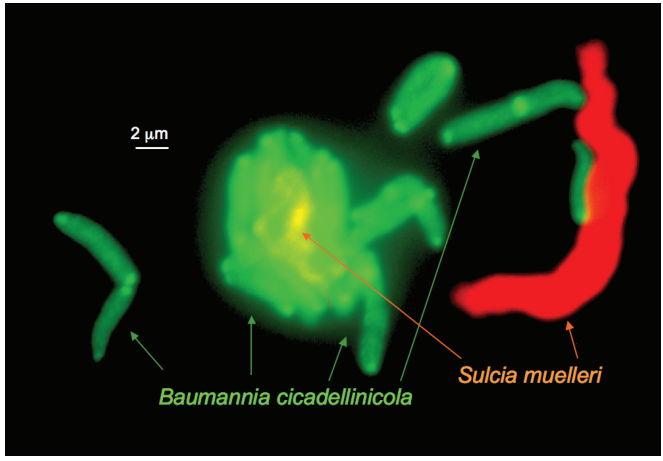

FIGURE 9.3 The two symbionts, *Sulcia* and *Baumannia* from the sharpshooter *Graphocephala atropunctata*. The cells are visualized by using fluorescent *in situ* hybridization with probes for taxon-specific 16S rRNA sequences (Wu *et al.*, 2006). The large *Sulcia* cells are sometimes closely surrounded with *Baumannia* cells. (Photo by P. Tran and N. Moran, University of Arizona.)

stability among known genomes, lacking chromosomal rearrangements and, most significantly from the point of view of host biology, lacking any genes newly acquired from exogenous sources. The only substantial source of divergence is rapid sequence evolution affecting ancestral genes and elimination or inactivation of genes in individual lineages. The latter is of particular interest because such losses are irreversible given the lack of gene uptake; they represent loss of functions that cannot be reinstated. In several cases, eliminated genes are ones that affect host nutrition, such as those underlying pathways for sulfur fixation, arginine biosynthesis, and tryptophan biosynthesis (Tamas *et al.,* 2002; van Ham *et al.,* 2003; Perez-Brocal *et al.,* 2006). The hosts must obtain these compounds from enriched diets available from certain plant species, from manipulation of plant phloem chemistry, or from additional symbionts that have been acquired subsequent to the acquisition of *Buchnera* >100 million years ago, as hypothesized for the smallest *Buchnera* genome sequenced to date (Perez-Brocal *et al.,* 2006).

This genome degradation is not dependent on being intracellular, but rather it reflects long history of obligate host dependence and lack of recombination among strains, enforced by strict maternal transmission. The importance of population genetic structure rather than cellular location is confirmed by the observation that the symbiont, *Ishikawaella capsulata*, of plataspid stinkbugs (Hemiptera) shows reduced genome size and rapid protein evolution despite its location in the gut lumen rather than within cytoplasm of specialized cells (Hosokawa *et al.,* 2006). *Ishikawaella* transmission, which occurs when progeny ingest an inoculum deposited on eggs by the mother, is strictly maternal, resulting in single infections and consequent lack of recombination among lineages. As for intracellular symbionts of other insect groups, *Ishikawaella* shows long-term parallel evolution with hosts, indicating an ancient origin (Hosokawa *et al.,* 2006). These features of transmission enforce asexuality and small population size, as for intracellular symbionts such as *Buchnera* of aphids, and *Ishikawaella* shows similar patterns of gene and genome evolution.

The most extreme known case of degradation of a symbiont genome (other than those of organelles) occurs in *Carsonella ruddii*, the obligate symbiont of psyllids (a sap-feeding insect group related to aphids and whiteflies) (Baumann, 2005). This 160-kb genome contains only 182 protein-coding genes, a number considerably smaller than the proposed minimum gene number for cellular life, based on those required for essential metabolic and informational processes (Nakabachi *et al.,* 2006). One of the most plausible explanations of how this symbiont functions with so few genes is that some genes have been stably transferred to the host genome, with their products reimported to the symbiont cellular compartment. The extent of gene transfer from symbionts to animal hosts will become appar-

ent as more genomes are sequenced for host species with long histories of symbiosis, such as aphids (Brisson and Stern, 2006). The *Carsonella* genome is also extreme in its base composition (16.5% GC content) and in the rate of sequence evolution of proteins; it is remarkable that the insect hosts are dependent on an organism that appears so degenerate.

Long-term coadaptation of hosts with symbionts can enforce dependence beyond the original basis for the symbiosis. For example, aphids require *Buchnera* for normal embryonic development and are unable to reproduce in the absence of *Buchnera* even when diets are supplemented with the nutrients that *Buchnera* normally provides.

ANIMAL SYMBIONTS RETAINING GENOME PLASTICITY

For an animal host, one potential consequence of acquiring a bacterial symbiont might be that it would serve as a portal for ongoing acquisition of novel genes, which is far more common in bacterial than in animal genomes. Although the bacteriome-associated nutritional symbionts provide the most extreme cases of genome stasis known in Bacteria and do not acquire novel genes (Tamas *et al.,* 2002; Degnan *et al.,* 2005), some heritable bacteria continue to undergo recombination, to harbor phage, and to incorporate foreign genes into their chromosomes. In many cases, these symbionts confer benefits such as protection against natural enemies (parasitoids and pathogens) (Oliver *et al.,* 2005; Scarborough *et al.,* 2005) or against variable abiotic conditions such as thermal stress (Russell and Moran, 2006). Although nutritional symbionts usually live in a specialized organ and are strictly required for normal host development, these bacteria are facultative for hosts and more varied in their locations within host bodies. Although they are maternally transmitted with high fidelity, they can also be transferred horizontally, sometimes through paternal transmission (Moran and Dunbar, 2006). As a result, different strains sometimes coinfect the same host individual, resulting in opportunity for recombination and transfer of phage and genes among strains (Moran *et al.,* 2005a). In the case of the symbiont *Hamiltonella defensa*, which provides aphid hosts with protection against parasitoid wasps, phage-borne genes appear to contribute to defensive strategies that are observed to vary among symbiont strains (Oliver *et al.,* 2005; Moran *et al.,* 2005b). These symbionts use some of the same mechanisms for interacting with hosts as do mammalian pathogens, and many of these mechanisms are linked to capacity for gene uptake (Dale and Moran, 2006).

Mutualism is an obvious route for spread of heritable symbionts in a host population and has been the focus of this work. But heritable symbionts can spread among host lineages without conferring a benefit, by manipulating host reproduction to favor their own increase (Werren,

1997). The most well known symbionts in this category belong to the clade referred to as *Wolbachia*, an ancient group that contains members with a variety of kinds of interactions with hosts. In arthropods, *Wolbachia* is primarily exploitative, undergoes transfer among host lineages, and has a plastic genome with ongoing recombination and containing phage-derived elements (Masui *et al.,* 2000; Wu *et al.*, 2004). In contrast, *Wolbachia* in filarial nematodes appear to have been strictly vertically transmitted during host diversification, are required by hosts for normal development, and have a smaller and more static genome, lacking phage (Foster *et al.,* 2005). Population studies indicate that exploitative symbionts can act as a force for reproductive isolation of populations with different infections (e.g., Jaenike *et al.,* 2006). Thus, symbionts likely contribute to the species richness of hyperdiverse taxa such as the insects, not only by enabling expansion of lineages into novel ecological niches through augmentation of metabolic capabilities but also by affecting mating systems and reproductive compatibility of populations. As in the case of symbionts such as *Buchnera* that have evolved as beneficial symbionts, exploitative symbionts can become essential for host reproduction because of coadaptation of host genomes (e.g., Dedeine *et al.,* 2005). Thus, complex development dependence on symbiotic partners is possible even when the original association was not beneficial for the host.

CONCLUSIONS

The literature on symbiosis is vast and growing quickly, largely because of the insights based in genomics. Although symbiosis was once discounted as an important evolutionary phenomenon (e.g., Sapp, 2004), the evidence is now overwhelming that obligate associations among microorganisms and between microorganisms and multicellular hosts have been crucial in many landmark events in evolution, in the generation of phenotypic diversity, and in the origin of complex phenotypes able to colonize new environments. Such evidence is abundant for the symbiotic systems found in insects, which are far better understood than in the recent past, largely because of molecular and genomic studies. Examples from insects show that symbioses can result in specialized organs with unique development, innovations in metabolic capabilities that allow new lifestyles, defenses against natural enemies and other environmental challenges, constraints on evolutionary range, and ongoing acquisition of novel genes and capabilities.

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Part IV

CASE STUDIES: DISSECTING COMPLEX PHENOTYPES

The chapters in Part IV provide examples of how scientists are tackling the empirical challenge of dissecting complex phenotypes. In *The Origin of Species*, Darwin deemed the eye to be an organ of "extreme perfection and complication." He also wrote, "To suppose that the eye with all its inimitable contrivances for adjusting the focus to different distances, for admitting different amounts of light, and for the correction of spherical and chromatic aberration, could have been formed by natural selection, seems, I freely confess, absurd in the highest degree." Nonetheless, "reason tells me, that if numerous gradations from a simple and imperfect eye to one complex and perfect can be shown to exist, each grade being useful to its possessor, . . . then the difficulty of believing that a perfect and complex eye could be formed by natural selection, though insuperable to our imagination, should not be considered subversive of the theory." In Chapter 1, Ayala mentioned how light-sensing organs in mollusks vary from the simple to the highly complex, each type nonetheless of utility to its bearers. In Chapter 10, Francesca Frentiu and others associated with Adriana Briscoe's laboratory delve deeper into the molecular basis of vision by discussing the comparative evolution of genes and proteins underlying color-vision phenotypes in primates and butterflies. The research summarized by these authors demonstrates some remarkable parallels in how particular amino acid sites in photopigments can be involved in color perception in both insects and mammals.

Darwin was interested in the close parallels between natural selection and artificial selection, and in 1868 he published a book on the topic

of phenotypes in domesticated plants and animals. In Chapter 11, Jeffrey Ross-Ibara, Peter Morrell, and Brandon Gaut illustrate modern genetic approaches to dissecting important phenotypes that have evolved under human influence, with special reference to domestic corn. They distinguish top-down genetic approaches (such as QTL mapping) from bottom-up approaches (such as candidate gene assays), and conclude that the latter method, despite some pitfalls, generally holds greater promise for revealing how key phenotypes in crop plants have evolved under domestication from their ancestral wild states.

In Chapter 12, Al Bennett and Richard Lenski address a longstanding question: Is there a necessary cost to adaptation? In other words, does the evolution of a phenotype that is adaptive to a particular environment necessitate deterioration in other traits? If so, what natural selection can achieve via the adaptive process would inevitably be constrained by such fitness *trade-offs*. To examine this issue empirically, the authors monitored multigeneration selection responses of bacteria (*Escherichia coli*) to altered temperature regimes. After 2,000 generations of thermal selection, most colonies that showed improved fitness at low temperatures also showed fitness declines at high temperatures, but this was not invariably the case. The fact that exceptions exist indicates that fitness trade-offs are not an inevitable component of the adaptive evolutionary process.

Bacteria such as *E. coli* are model experimental organisms because they have short generation lengths and are easy to manipulate, but they also have relatively simple phenotypes. Near the other end of the continuum is *Homo sapiens*, which has many complex phenotypes of special interest but is far less tractable to experimental manipulation. In Chapter 13, Cynthia Beall describes the adaptations to high-altitude hypoxia (oxygen shortage) displayed by humans indigenous to the Andean and Tibetan plateaus. Remarkably, the physiological and molecular adaptations to hypoxia differ dramatically between these two populations, suggesting different evolutionary pathways to the same functional outcome. Beall describes how scientists are currently dissecting the evolutionary genetic responses to oxygen deprivation displayed by these two populations, and in so doing reveals some of the special challenges of working with a nonmodel experimental species.

Beetles (Coleoptera) have long been intriguing to biologists. The British geneticist and evolutionist J.B.S. Haldane famously remarked that the Creator must have had an inordinate fondness for beetles because he made so many species of them (at least half a million). A century earlier, Darwin had speculated that the oft-ornate horns that many beetles carry on their heads or thorax were favored by sexual selection as weapons, used in jousts between males over mating access to females. Darwin's fascination with beetles began in childhood and grew in his college years,

as indicated in his autobiography: "no pursuit at Cambridge was followed with nearly so much eagerness or gave me so much pleasure as collecting beetles" (Darwin, 1887b). In Chapter 14, Douglas Emlen, Laura Lavine, and Ben Ewen-Campen describe modern research on the molecular genetics, ontogeny, and phylogenetics of beetle horns. These authors advance fascinating mechanistic scenarios for the evolutionary origins of these peculiar devices, and for subsequent evolutionary alterations in horn shapes, allometries, body locations, and patterns of sexual dimorphism.

This volume then concludes with an essay by Eugenie Scott and Nicholas Matzke on the history, philosophy, and societal impact of a religious movement known as Intelligent Design, and its sharp contrast with scientific explanations for the appearance of biological design that results inevitably from natural selection.

10

Adaptive Evolution of Color Vision as Seen Through the Eyes of Butterflies

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Butterflies and primates are interesting for comparative color vision studies, because both have evolved middle- (M) and longwavelength- (L) sensitive photopigments with overlapping absorbance spectrum maxima (λ_{max} values). Although positive selection is important for the maintenance of spectral variation within the primate pigments, it remains an open question whether it contributes similarly to the diversification of butterfly pigments. To examine this issue, we performed epimicrospectrophotometry on the eyes of five *Limenitis* butterfly species and found a 31-nm range of variation in the λ_{max} values of the L-sensitive photopigments (514–545 nm). We cloned partial *Limenitis* L opsin gene sequences and found a significant excess of replacement substitutions relative to polymorphisms among species. Mapping of these L photopigment λ_{max} values onto a phylogeny revealed two instances within Lepidoptera of convergently evolved L photopigment lineages whose λ_{max} values were blue-shifted. A codon-

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Data deposition: The sequences reported in this chapter have been deposited in the GenBank database (accession nos. AY918903, AY847475, DQ212962, DQ212963, DQ212965, DQ212966, DQ486871, and EF156437–EF156441).

based maximum-likelihood analysis indicated that, associated with the two blue spectral shifts, four amino acid sites (Ile17Met, Ala64Ser, Asn70Ser, and Ser137Ala) have evolved substitutions in parallel and exhibit significant d_N/d_S >1. Homology modeling of the full-length *Limenitis arthemis astyanax* L opsin placed all four substitutions within the chromophore-binding pocket. Strikingly, the Ser137Ala substitution is in the same position as a site that in primates is responsible for a 5- to 7-nm blue spectral shift. Our data show that some of the same amino acid sites are under positive selection in the photopigments of both butterflies and primates, spanning an evolutionary distance >500 million years.

Ever since Darwin, there has been intense interest in the evolution of complex traits, with one of the most notable examples being the evolution of the eyes. Only three phyla have evolved image-resolving eyes: arthropods, mollusks, and chordates (Land and Fernald, 1992). Although there are vast morphological and neurobiological differences among the eyes of these lineages, there are also many similarities that may provide new insight into the molecular mechanisms governing the evolution of this complex trait.

Image-resolving eyes are composed of photoreceptor cells, which contain photopigments, and they also have accessory pigment cells, which shield the photopigments from stray light. Photopigments are made up of a light-sensitive chromophore (e.g., 11-cis-retinal) and an opsin protein. Although the isolated chromophore has an absorbance spectrum maximum (λ_{max} value) at 380 nm (Han *et al.,* 1998), the λ_{max} values of photopigments can vary from 360 to 600 nm through the spectral tuning of the chromophore by specific interactions with amino acids in the chromophore-binding pocket of the opsin protein. Because photopigment sensitivities represent clear adaptations to an animal's light environment (Yokoyama, 1997), the amino acid sites of opsins involved in spectral tuning may be under positive selection, which has been most extensively studied in fish, mammals, and primates (Surridge *et al.,* 2003; Spady *et al.,* 2005; Yokoyama and Takenaka, 2005).

Comparing these photopigment results with those of a more distant lineage such as butterflies could provide a better understanding of the molecular mechanisms of the eye and how these molecules change under selection. The visual systems of butterflies and primates may be under similar selective pressures. Butterflies and primates share a similar light environment and contain species that are nocturnally or diurnally active and species that are fruit-feeders. Like primates that are leaf eaters, butterfly females need to discriminate among foliage types for oviposition, which may have a strong impact on their color vision (see Kelber, 1999,

2001). As well, both use vision in the detection of conspecifics and mates (Mollon, 1989). (Detection of predators is probably visual for butterflies but does not depend on color vision; it probably depends on motion vision instead.) Thus, various aspects of the color vision systems of butterflies and primates may have undergone convergent evolution (see below).

Phylogenetic analyses indicate that the opsin gene family duplicated several times before the radiation of metazoans, giving rise to as many as seven protein subfamilies (Terakita, 2005), including the ciliary and rhabdomeric opsins, each associated with a distinct photoreceptor cell type (Arendt, 2003). All photoreceptor cells expand their membranes to express opsins, but ciliary photoreceptor cells expand their ciliary side, the side closest to the cell body, and express ciliary opsins, whereas rhabdomeric photoreceptor cells expand their apical side and express rhabdomeric opsins (Arendt, 2003). In general, vertebrates have ciliary opsins that are expressed in the photoreceptor cells of the retina, whereas insects have rhabdomeric opsins that are expressed in the photoreceptor cells of the ommatidia of the compound eye.

Color vision adds to the complexity of the eye. With a single spectral class of photoreceptor, only achromatic (brightness-contrast) vision is possible. Both mammalian long-wavelength-sensitive (L) cones and butterfly L photoreceptors provide outputs for brightness processing (Kolb and Scherer, 1982; Jacobs, 1993). Color vision, on the other hand, is the ability to discriminate between different wavelengths of light, regardless of relative intensity and depends on the presence of at least two spectrally distinct classes of photoreceptors, as well as appropriate neuronal connections in the brain. Natural selection has recruited both the vertebrate ciliary opsins and the insect rhabdomeric opsins for use in achromatic and color vision. Moreover, mammals use all their cone photopigments for color vision, and although not yet fully investigated, butterflies likely use all their major spectral receptor types for color vision (Kelber and Henique, 1999; Kelber, 2001; Kelber *et al.,* 2003).

There is variation in both the photopigment sensitivity and the range of color vision in mammals. Color vision among placental mammals is typically dichromatic based on outputs from short-wavelength-sensitive (S) and L cone pigments encoded by distinct opsin genes expressed in the cone photoreceptor cells of the vertebrate retina (Jacobs, 1993; Ahnelt and Kolb, 2000). Some primates have even evolved trichromatic color vision based on an additional middle-wavelength-sensitive (M) cone pigment, which allows them to discriminate colors in the green-to-red part of the visible light spectrum. The most parsimonious explanation for the variation in primate color vision systems is that the common ancestor of primates contained a single S opsin variant, but the ancestor was polymorphic at another locus for alleles encoding M and L cone pigments (Tan *et*

al., 2005). In some lineages, such as in nocturnal prosimians, either the M or the L cone pigment has been lost (Tan and Li, 1999). In other lineages, such as in diurnal New World monkeys, as many as three opsin alleles have been maintained at high frequencies by balancing selection (Mollon *et al.,* 1984). In addition, the functionally distinct M and L allelic variants have been fixed independently at least twice (or evolved convergently) as duplicate genes in the New World monkey genus *Alouatta* (Kainz *et al.,* 1998; Dulai *et al.,* 1999) and in the common ancestor of humans, apes, and Old World monkeys.

Among insects, some butterflies have evolved red–green color vision (Kelber, 1999; Kinoshita *et al.,* 1999), convergently with primates by gene duplication (Kitamoto *et al.,* 1998; Briscoe, 2000, 2001), and also through the use of heterogeneously expressed filtering pigments, in combination with a single L opsin (Zaccardi *et al.,* 2006). In both mammals and butterflies, natural selection has led to the evolution of photopigments with similarly diverse sets of spectral sensitivities in the long wavelength part of the visible light spectrum. For mammals, the L/M pigment λ_{max} = 495–565 nm (Bowmaker and Hunt, 1999), whereas in butterflies, L pigment λ_{max} = 500–600 nm (Briscoe and Chittka, 2001). It is fascinating that the ciliary-type L and M pigments of mammals have evolved similar spectral phenotypes as the rhabdomeric-type L pigments of butterflies, and that some primates and butterflies have evolved red–green color vision in parallel. Because the genetic mechanisms underlying this expanded sensitivity to longer wavelengths of light have been elucidated largely in mammals and primates, we investigated whether similar genetic mechanisms accounted for the parallel phenotypes found in butterflies.

To address this question, we determined the peak sensitivity of the L photopigment from a large number ($n > 20$) of butterflies in the family Nymphalidae. In the course of this survey, we found surprisingly large variation in the λ_{max} values of the L photopigment in eyes of butterflies of the genus *Limenitis*. We therefore decided to concentrate our efforts on evaluating whether positive selection has driven the spectral diversification of these closely related photopigments. Using molecular, population-genetic, and molecular evolutionary approaches, we identified several candidate spectral tuning sites in the chromophore-binding pocket of the butterfly L opsin protein. Our combined approaches suggest that similar amino acid sites are indeed involved in the evolution of color vision in both primates and butterflies.

NYMPHALID BUTTERFLIES COMPRISE A USEFUL SYSTEM FOR STUDYING COLOR VISION

Of the five families of butterflies, we have focused our studies on the diverse family Nymphalidae, because it contains a number of species that are model systems in butterfly ecology and evolutionary biology, such as the monarch butterfly *Danaus plexippus* (mimicry and migration) (van Zandt Brower, 1958; Reppert, 2006), *Heliconius erato* (speciation) (Brower, 1994; McMillan *et al.,* 1997), and *Bicyclus anynana* (evolution and development) (Beldade *et al.,* 2005). Likewise, there are ample anatomical, molecular, and behavioral data for color vision in nymphalids.

Anatomically, the basic unit of the butterfly compound eye is the ommatidium, composed of eight elongate photoreceptor cells (R1–R8) and a small basal ninth cell (R9) (Fig. 10.1 *A* and *B*). The photopigment-containing microvillar membranes produced by all photoreceptor cells of an ommatidium are fused into a single optical-sensing structure known as a rhabdom. Molecular studies in *D. plexippus*, *H. erato*, and *Vanessa cardui* have shown that the photopigments are encoded by two S opsin (UV, ultraviolet; B, blue) and one L opsin gene present in the ancestor of all butterflies and moths. In the main retina of these species, the R1 and R2 photoreceptor cells express either the UV or B opsin mRNAs, and the R3–R9 photoreceptors express the L opsin mRNA (Fig. 10.1*C*) (Briscoe *et al.,* 2003; Sauman *et al.,* 2005; Zaccardi *et al.,* 2006). Some nymphalid butterflies (e.g., *H. erato* and monarchs) also have colored ''lateral'' filtering pigments that coat the rhabdom and modify the wavelengths of light available to photoisomerize the photopigments, whereas others do not (e.g., *Vanessa atalanta).*

Behavioral studies have shown that *H. erato*, although it expresses UV, B, and only one L opsin, discriminates colors in the blue to L range tested (440–640 nm), whereas *V. atalanta*, despite having color vision in the 440- to 590-nm range, is unable to discriminate between longer-wavelength (590–640 nm) colors (Zaccardi *et al.,* 2006). Thus, these two nymphalid butterflies display color vision, with the difference in color vision range between them being due to the presence or absence of heterogeneously expressed filtering pigments.

ABSORBANCE SPECTRA AND OPSIN SEQUENCES OF *LIMENITIS* L PHOTOPIGMENTS

To test for evidence of positive selection of spectral tuning sites in nymphalid L photopigments, we first searched for L photopigments whose λ_{max} values varied among closely related species. The λ_{max} values of L photopigments from a number of nymphalid species were evaluated by using epimicrospectrophotometry, a method (Bernard, 1983) that

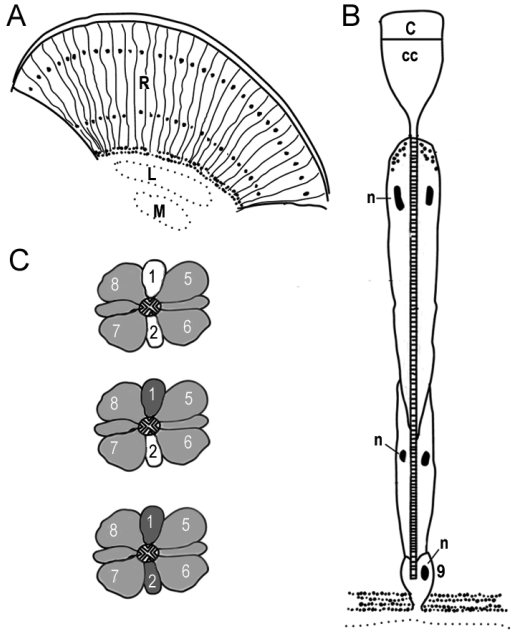

FIGURE 10.1 Butterfly compound eye and opsin expression patterns. (*A*) Diagram of a longitudinal section through the compound eye showing the ommatidial units. Black dots indicate location of photoreceptor nuclei. R, retina; L, lamina; M, medulla. (*B*) Schematic of an ommatidium. C, cornea; CC, crystalline cone; n, nuclei; 9, the ninth photoreceptor cell that sits just above the basement membrane. (*C*) Opsin mRNA expression patterns. The cross-sections of three ommatidia are shown. The cross-hatched area in the middle of each depicts the fused microvillous membranes of the rhabdomeres that contain the visual pigment proteins. Numbers refer to the photoreceptor cells (R1–R8), and the shades of gray refer to the opsin expression patterns: dark gray, UV opsin mRNA; white, blue opsin; light gray, long wavelength opsin. Modified from Sauman *et al.* (2005).

takes advantage of a mirrored tapetum underlying each ommatidium of the nymphalid eye to resolve peak absorbances of the expressed photopigments [see *Materials and Methods* and supporting information (SI) *Methods*]. Using epimicrospectrophotometry, we found that the λ_{max} values of the L photopigments of the five *Limenitis* species ranged from 514 nm in *Limenitis archippus archippus* (Bernard, 1983) and *Limenitis archippus floridensis*, to 530 nm in *Limenitis lorquini* and *Limenitis weidemeyerii*, to 545 nm in *Limenitis arthemis astyanax* (Fig. 10.2). A spectral range of 31 nm within a single butterfly genus is remarkable compared with the other

nymphalid genera we surveyed and is similar to the 33-nm range observed for the human green and red cone pigments. This unusual spectral range among the five L pigments within the genus *Limenitis* provided us with a unique collection of photopigments for further analysis.

To evaluate candidate spectral tuning sites, we next determined the opsin gene sequences that correspond to the encoded L photopigment spectral phenotypes. We thus cloned the full-length L opsin cDNA from *L. arthemis astyanax* (GenBank accession no. AY918903) and found no evidence of gene duplication in this species, as has been found in other butterflies (Briscoe, 1998; Kitamoto *et al.*, 1998). We also cloned partial L opsin gene sequences from each of the other four North American *Limenitis* species. The gene sequences ranged from 1366 to 1391 bp in length and were trimmed of introns. For all five L opsin sequences, only

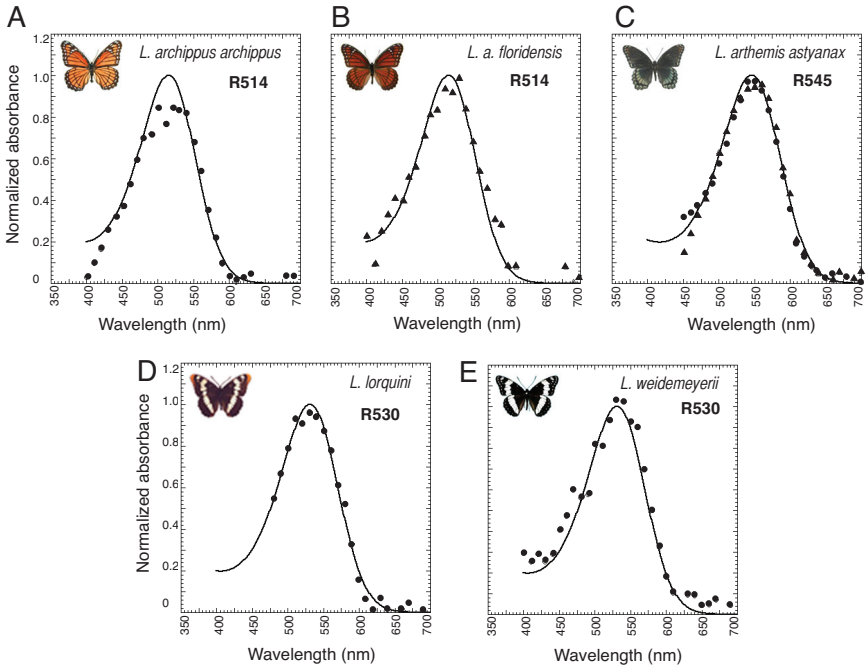

FIGURE 10.2 Normalized absorbance spectra of L photopigments in the *Limenitis* genus measured by epimicrospectrophotometry. Idealized spectra (solid curves) based on the 1987 Bernard template (Palacios *et al.*, 1996). λ_{max} values are shown in upper right corner. R545 from *L. arthemis astyanax*: dots represent dorsal retina, and triangles represent ventral retina.

transmembrane domains I–VI were used in subsequent analyses, because this region includes the chromophore binding pocket (SI Fig. 10.5).

There was a robust correlation between the spectral phenotypes and the number of amino acid substitutions of the L opsin proteins of *Limenitis*. Accordingly, the largest number of amino acid substitutions was observed between the two pigments with the largest spectral difference (31 nm, *L. archippus archippus–L. arthemis astyanax*, 15/263 substitutions), whereas the smallest number of substitutions was between the pigments with the smallest spectral difference (0 nm, *L. archippus archippus–L. archippus floridensis*, 3/263 substitutions; 0 nm, *L. lorquini–L. weidemeyerii*, 0/263 substitutions).

POPULATION GENETIC AND MOLECULAR EVOLUTIONARY ANALYSES SUGGEST L OPSINS ARE UNDER POSITIVE SELECTION

The large range of variation in the λ_{max} values among the *Limenitis* L photopigments (514, 530, and 545 nm) is strikingly similar to that observed in the three-allele system of New World monkeys (≈530, 545, and 560 nm) (Surridge *et al.,* 2003) and suggests that the *Limenitis* L photopigments may also be maintained by positive selection. We therefore undertook four population genetic and molecular evolutionary approaches, the McDonald–Kreitman (MK) test, character mapping, parallel/convergent change analysis, and branch-site test of selection, to define specific spectral tuning sites that may be under positive selection.

MK Test for Selection

Differences in the amino acid sequences of proteins among species may be due to the accumulation of neutral mutations by drift, the fixation of adaptive mutations by positive selection, or a combination of the two. We therefore used the MK test (McDonald and Kreitman, 1991) to examine whether the amino acid differences between the *L. archippus archippus* and *L. arthemis astyanax* pigments had evolved by neutral evolution. Although some demographic scenarios may result in the MK test erroneously indicating adaptive evolution (Eyre-Walker, 2002), generally this test is free of demographic concerns, because neutral and selected sites interspersed throughout a gene share the same phylogeny and have the same effective population size (McDonald and Kreitman, 1991; Nielsen, 2005).

For the MK test, we genotyped 24 *L. arthemis astyanax* individuals for a region (1,056–1,069 bp) on both chromosomes and identified five alleles and 11 polymorphic sites, compared with the *L. archippus archippus* sequence (Table 10.1). We found that the ratio of nonsynonymous to

synonymous fixed differences between species (i.e., the number of substitutions that produce an amino acid change compared with the number of substitutions that do not) deviated strongly from the ratio of nonsynonymous to synonymous polymorphisms within species (Fisher's exact test, two-tailed $P = 0.006$) (Table 10.2). The higher proportion of fixed nonsynonymous substitutions (12) compared with fixed synonymous substitutions (4) rejected the hypothesis of neutral evolution and suggested that most of the observed replacement substitutions are probably due to positive selection-driven fixation of advantageous mutations. The results of this analysis indicate that the *L. archippus* and *L. arthemis astyanax* L opsin genes have evolved under positive selection for divergent functions. This allowed us to focus on specific sites within the encoded protein that are under positive selection and involved in spectral tuning.

Character Mapping of L Opsin λ_{max} Values

Methodological advances have been made in recent years in detecting positive selection at particular amino acids or codon sites. These statistical approaches depend on ancestral state reconstructions of either amino acid or nucleotide sequences at different branching points (nodes) along a gene tree and are greatly strengthened by the availability of functional data mapped onto the tree, which permits the *a priori* selection of branches for investigation. These tests are particularly compelling if convergent phenotypes have evolved along one or more independent lineages of the tree (Zhang, 2006).

To test this possibility, we first reconstructed an opsin gene tree by using L opsin gene data from 12 nymphalid, one papilionid, and one pierid butterfly species for which λ_{max} values are also available (SI Table 10.4), by using maximum-likelihood and Bayesian algorithms. Both methods recovered identical trees with good bootstrap support (>50%) in all except the basal node (Fig. 10.3). Our phylogeny is in general agreement with the most recent phylogeny of butterflies based on molecular and morphological data (Wahlberg *et al.*, 2005).

A character map was constructed by mapping the λ_{max} values onto the gene tree. Visual inspection of the distribution of λ_{max} values on the phylogeny revealed two instances of spectral convergence of L pigment λ_{max} values. Two pigment lineages, one leading from *L. arthemis astyanax* to other *Limenitis* species (Fig. 10.3, Nodes A to B) and the lineage ancestral to the *Siproeta stelenes* and *Junonia coenia* clade (Fig. 10.3, Nodes C to D), have evolved peak spectral sensitivities that are blue-shifted (i.e., shifted to shorter-wavelength light) compared with ancestral nodes.

TABLE 10.1 Allelic Variation in 597 bp of Coding Region of L Opsin Gene in 24 *L. arthemis astyanax* Individuals (48 Chromosomes) and One *L. archippus archippus*

Allele*	N	Polymorphic Sites								
					1	1	1	1	1	2
		1	5	9	2	8	8	9	9	0
		6	1	9	0	4	6	0	2	5
1	28	A	C	C	T	T	C	G	G	G
2	4	—	—	—	—	G	A	T	—	—
3	8	—	—	—	—	—	—	—	—	—
4	5	—	—	T	—	—	—	—	—	—
5	3	—	—	—	—	—	—	—	—	—
L. archippus archippus	2	G	G	—	A	G	A	T	T	A
Amino acid		N	I			V	V	A		V
replacements		↓	↓			↓	↓	↓		↓
		D	M			F	F†	S		I
Position										
			1			6	6	6		6
		6	7			2	2	4		9

A dash (—) indicates identity with the most common allele. Amino acid replacements are given in single-letter notation, and position refers to the numbering of amino acid residues in the alignment.

*GenBank accession nos. EF156437–EF156441.

†A substitution at nucleotide position 186 may lead to a replacement (V→L) but was never observed.

TABLE 10.2 MK Test Results for the L Opsins

	Synonymous	Nonsynonymous	*P* Value
Polymorphic*	9	2	
Fixed divergent†	4	12	0.0063‡

*Number of polymorphic sites among 24 individuals of *L. arthemis astyanax* genotyped for 597 bp of L opsin coding region.

†Divergent with respect to *L. archippus archippus*.

‡Fisher's exact test, two-tailed *P* value.

Parallel/Convergent Change Analysis

Using maximum-likelihood and maximum-parsimony ancestral state reconstructions of amino acid sequences, we next asked whether statistically significant parallel and/or convergent amino acid changes occurred along the two blue-shifted opsin lineages (i.e., along Nodes A to B and C to D) (Kreitman and Akashi, 1995; Zhang and Kumar, 1997). Whether a substitution is a parallel or convergent change depends on the reconstructed amino acid in the ancestral sequences. For example, a parallel change would occur if both ancestral sequences contained the same amino acid

2	2	2	2	2	2	3	3	3	4	4	4	5	5	5	5	5	5
0	1	4	6	8	9	4	6	8	0	1	3	0	2	5	8	8	9
9	6	5	5	5	1	5	6	4	9	5	8	9	9	4	2	8	2
A	T	C	G	T	T	A	C	C	T	T	C	C	T	T	C	C	G
—	—	—	—	C	G	T	A	T	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	T	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	T	—	—	—	T	—	—
G	C	G	A	C	G	T	—	—	G	G	—	T	G	C	—	T	A
N		A	A						S	S		A	F	M			V
↓		↓	↓						↓	↓		↓	↓	↓			↓
S		G	T						A	A		V	V	T			I
									1	1		1	1	1			1
7		8	8						3	3		6	7	8			9
0		2	9						7	9		9	6	4			7

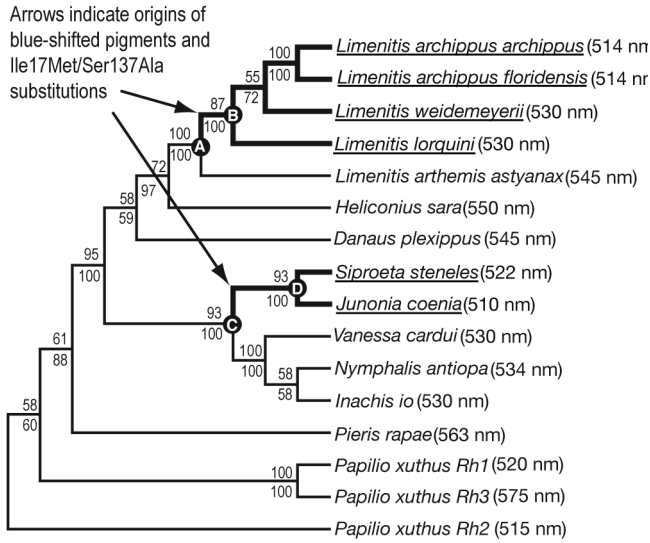

FIGURE 10.3 Character mapping of L photopigment λ_{max} values onto an L opsin gene tree. Numbers shown above branch indicate maximum-likelihood bootstrap support values and numbers below branch show Bayesian clade credibility values. Thick black lines indicate nymphalid branches along which blue spectral shifts occurred that were investigated in the parallel/convergent change and branch-site tests of selection. Extant blue-shifted sequences are underlined. Nodes in which ancestral state reconstructions shown in Table 10.3 were performed are indicated by letters.

TABLE 10.3 Ancestral State Reconstructions Along Blue-Shifted Butterfly L Opsin Lineages Shown in the Opsin Gene Tree (Fig. 10.3)

Amino Acid Site	545 nm Node A	514–530 nm Node B	Amino Acid Site	530 nm Node C	510–522 nm Node D
6	N 0.998	D 1.000	16	V 0.704	F 0.541
17	**I 0.998**	**M 1.000**	**17**	**I 0.997**	**M 1.000**
64	**A 0.996**	**S 1.000**	18	G 0.945	A 0.747
70	**N 0.998**	**S 1.000**	131	L 0.994	F 0.981
137	**S 1.000**	**A 1.000**	**137**	**S 1.000**	**A 1.000**
			258	A 0.640	S 0.717

Parallel amino acid changes are indicated in bold. Numbers above nodes indicate inferred λ_{max} values. Numbers after amino acid residues indicate Bayes Empirical Bayes posterior probabilities.

residue at a site, whereas a convergent change would occur if the ancestral sequences contained different amino acid residues at a site. Convergent changes are statistically less likely and stronger signatures of selection.

We observed two instances of parallel change associated with blue spectral shifts by using both reconstructions. At amino acid residue 17, an isoleucine-to-methionine substitution was observed, and at residue 137, a serine-to-alanine substitution was observed. The number of observed substitutions was significantly greater than expected by chance ($P < 0.01$) (Table 10.3). When the ancestral state reconstruction was expanded to include substitutions that occurred along the terminal branch connecting node D to the even more blue-shifted *J. coenia* opsin, two further parallel amino acid changes were observed associated with parallel blue spectral shifts: an alanine-to-serine substitution at amino acid residue 64 and an asparagine-to-serine substitution at amino acid residue 70.

Branch-Site Models of Selection

Although parallel/convergent change analysis depends on reconstruction of amino acid sequences, a much stronger positive selection inference can be made by using reconstruction of nucleotide sequences. One of the signatures of positive selection on a particular codon site is a much higher rate of substitution, causing amino acid replacements, d_N, than the rate of silent substitutions, d_S, leading to the ratio (ω) of $d_N/d_S > 1$ (Yang and Bielawski, 2000). Using branch-site models of selection, we tested whether specific amino acid sites along the two identified blue-shifted lineages are evolving in a manner consistent with positive selection. For the two

Amino Acid Site	510–522 nm Node D	510 nm *J. coenia*
8	L 0.985	M
20	L 0.998	I
26	T 0.981	A
40	T 0.882	S
61	F 0.541	T
64	**A 0.522**	**S**
70	**N 0.912**	**S**
93	F 0.997	C
180	V 0.758	L

blue-shifted lineages tested, the alternative model (specifically testing for positive selection) was a significantly better fit ($2\Delta\lambda = 3.951$, df = 1, $P < 0.05$) than the null model. The analysis indicated that seven sites were likely to be under positive selection (sites 6, 17, 18, 64, 70, 131, and 137). However, only three (17, 64, and 137; Fig. 10.4 and SI Fig. 10.5) had Bayes Empirical Bayes posterior probabilities >0.85, with two sites, 17 and 137, having posterior probabilities that were >0.95 (data not shown).

This result is provocative, because previous attempts to identify amino acid sites under positive selection in both butterfly and vertebrate photopigments by using similar methods have largely failed despite robust physiological data (i.e., absorbance spectra), suggesting functional divergence and the observation of parallel evolution (Briscoe, 2001; Yokoyama and Takenaka, 2005; but see Spady *et al.*, 2005). Remarkably, four of the identified sites (17, 64, 70, and 137) were the same as those evolving in a manner consistent with positive selection in the parallel/convergent change analysis.

Homology Modeling Places L Opsin Sites Under Selection Close to the Chromophore

A homology model based on the *L. arthemis astyanax* sequence (Fig. 10.4) indicated that four of the sites under selection in the two blue-shifted lineages, sites 17, 64, 70, and 137, directly face the chromophore (Fig. 10.4). In particular, sites 17, 64, and 70 are situated close to the Schiff-base end of the chromophore. The amino acid sites Ile17Met, Ala64Ser, Asn70Ser, and Ser137Ala in the *Limenitis* sequences correspond to Met^{44}, Phe^{91}, Thr^{97}, and Ala^{164} on bovine rhodopsin, respectively. Met^{44} is part of

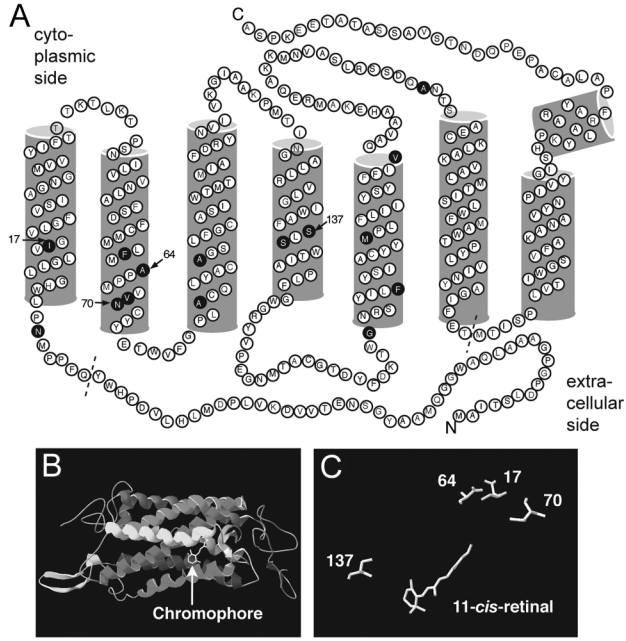

FIGURE 10.4 Topographical and homology models of *L. arthemis astyanax* L opsin. (*A*) Topographical opsin map showing amino acid differences (filled circles) between *L. arthemis astyanax* and *L. archippus archippus* opsins. The diagram is based on a model of bovine rhodopsin (Palczewski *et al.,* 2000). Dashed line indicates boundaries of alignment used in phylogenetic analyses. The amino acid residues are numbered relative to their alignment position. Arrows, amino acid sites under positive selection. (*B*) Homology model of *L. arthemis astyanax* L opsin, with chromophore shown (arrow). (*C*) Candidate spectral tuning sites that directly face the chromophore (11-cis-retinal).

the region surrounding the Schiff base of the 11-cis-retinal chromophore (Palczewski *et al.,* 2000; Okada *et al.,* 2004), and a Met44Thr substitution in bovine rhodopsin causes a 3-nm blue shift (Andres *et al.,* 2003). Phe^{91} produces a small spectral tuning effect when the equivalent site (Asp^{71}) is mutagenized in squid retinochrome, which binds the retinal chromophore and functions as a retinal photoisomerase in squid photoreceptor cells (Terakita *et al.,* 2000). Site 137 corresponds to Ala^{164} in bovine rhodopsin or Ser^{180}/Ala^{180} in human L/M pigments. Intriguingly, in human cone pigments, the substitution Ser164Ala causes a 5- to 7-nm blue shift (Merbs and Nathans, 1993; Asenjo *et al.,* 1994) and is one of the sites

polymorphic among the New World monkey alleles and under balancing selection. Therefore, the correspondence between sites implicated previously in blue spectral shifts in vertebrate opsins and those found in butterflies in this study strongly suggests that some of the same amino acid sites are involved in the evolution of L color vision in both primates and butterflies.

CONCLUSIONS

Expanded, or trichromatic, color vision in some primate lineages evolved through gene duplication and diversification of an ancestral polymorphic M/L pigment gene. Expanded color vision is advantageous over a more restricted color vision range in individuals foraging for ripe fruits and young leaves, especially under dim light (Caine and Mundy, 2000; Smith *et al.*, 2003; Osorio *et al.*, 2004). We have shown that, like the polymorphism observed in New World monkeys, the photopigment genes of *Limenitis* have evolved under positive selection. Remarkably, we have further shown that two closely related butterfly species, *L. archippus* and *L. arthemis astyanax* (which can hybridize in nature), show a commonly observed primate polymorphism. Like the primate cone pigments that contain this substitution, the butterfly photopigments are blue-shifted in sensitivity. This suggests that genetic mechanisms of spectral tuning may have evolved in parallel across 500 million years of evolution.

MATERIALS AND METHODS

Epimicrospectrophotometry

The technique was used as described (Bernard, 1983; Briscoe and Bernard, 2005). Briefly, the visual pigments of a butterfly ommatidium are contained within a rhabdom waveguide. Light propagates down the waveguide, being absorbed by rhodopsins as it goes. Light reaching the end of the rhabdom is reflected by the tapetum, a specialized tracheolar layer, back out of the eye as eyeshine. The reflectance spectrum of eyeshine was measured in dark-adapted live insects, with a series of dim monochromatic flashes set as a reference spectrum. Reflectance spectra were then measured after partial bleaching of the rhabdom of its L visual pigment after a series of photoisomerizing flashes. The rhodopsin photoproduct-free difference spectrum yields a two-way absorbance spectrum. An estimate of λ_{max} is then obtained by least-squares fitting to template absorbance spectra (Palacios *et al.*, 1996).

Sample Collecting

All *Limenitis* specimens were collected as adults in the field (*L. archippus archippus*, Goose Lake, Dane County, Wisconsin; *L. archippus floridensis*, Yankeetown, Florida, and Archbold Biological Station, Lake Placid, Florida; *L. lorquini*, Ash Canyon, Carson City County, Nevada; *L. weidemeyerii*, Willow Creek, Catron County, New Mexico, and Kelber Pass, Colorado; and *L. arthemis axtyanax*, Due West, South Carolina, $n = 2$; Madison, WI, $n = 1$; Amherst, MA, $n = 1$; and Lowndes County, Georgia, $n = 1$). The remaining 19 *L. arthemis astyanax* specimens used for the population sample were gifts from Austin Platt and were collected in Green Ridge State Forest, Maryland.

PCR, Cloning, and Sequencing

L. arthemis astyanax cDNA was synthesized from total RNA from one frozen head by using the Marathon cDNA Amplification Kit (BD Biosciences Clontech, Mountain View, CA). The full-length L opsin cDNA, including 3′ and 5′ UTRs, was obtained by amplification of 3′ RACE products by using a degenerate primer (5′-GAA CAR GCW AAR AAR ATGA-3′) followed by amplification of 5′ RACE products by using a gene-specific reverse primer (5′-CAG AGC CCC AAA TGG TCA CTA A-3′). The resulting products were cloned into the pGEM T-Easy Vector System II (Promega, Madison, WI) and sequenced at the University of California, Irvine, DNA core sequencing facilities.

Genomic DNA from the other *Limenitis* species was extracted from individual adult butterflies (one per species) by using phenol-chloroform. First, a highly conserved 300-bp part of the gene was amplified by the PCR by using degenerate primers 5′-GAA CAR GCW AAR AAR ATG A-3′ and 5′-CCR TAN ACR ATN GGR TTR TA-3′, which was cloned and sequenced as above. These sequences allowed the design of species-specific reverse primers that were used in combination with a degenerate forward primer (5′-CAY YTN ATH GAY CCN CAY TGG-3′) to amplify a 1,366- to 1,391-bp fragment. For the *L. arthemis astyanax* population sample, PCR products were then directly sequenced. Heterozygous individuals were identified by visual inspection. Haplotypes were confirmed by cloning into the pGEM T-easy vector as described above.

For the MK test (McDonald and Kreitman, 1991), introns were manually removed from the 1,056- to 1,069-bp region of the 24 *L. arthemis astyanax* and one *L. archippus archippus* individuals genotyped, leaving 597 bp of coding region that was aligned. The number of sites within the *L. arthemis astyanax* population sample that was polymorphic for synonymous and nonsynonymous substitutions was counted and com-

pared with the number of sites that was fixed between species for synonymous and nonsynonymous substitutions by using a Fisher's exact test.

Phylogenetic Reconstruction

Sequences for *Limenitis* species were added to other L opsin sequences obtained from GenBank for which physiological data were also available. The resulting alignment was edited to retain only coding sequence, 263 aa in length, spanning transmembrane domains I–VI (Fig. 10.4). Phylogenetic relationships were reconstructed from nucleotide data by using maximum-likelihood and Bayesian methods, with the moth *Manduca sexta* L opsin sequence as an outgroup by using all three nucleotide positions. The optimal DNA substitution model for the maximum-likelihood phylogenetic analysis was determined by using a hierarchical likelihood ratio test in Modeltest (Posada and Crandall, 1998). A maximum-likelihood analysis was conducted in PHYML with TrN93 + I + G (invariant sites and gamma-distributed rates for sites) substitution model, and the reliability of the tree obtained was tested by bootstrapping with 500 replicates. Bayesian phylogenetic analyses were performed by using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). Because MrBayes 3.1 does not implement the TrN93 DNA substitution model, we used the next-less-complex (HKY85+I+G) and the next-more-complex (GTR+I+G) models in two separate analyses. Both models were run for 10^6 generations, with a sampling frequency of 102, using three heated and one cold chain and with a burnin of 2.5×10^3 trees.

Parallel Change Analysis and Branch-Site Tests of Selection

The Bayesian and maximum-likelihood tree topology obtained was used to perform all selection tests. For the parallel change analysis, amino acid substitutions along each lineage in the opsin gene tree were reconstructed by using maximum parsimony in MacClade (Maddison and Maddison, 2005) and maximum likelihood in PAML (Yang, 1997). Parallel amino acid changes were detected along butterfly lineages that also displayed parallel phenotypic evolution in the L visual pigment λ_{max} value. The statistical significance of these amino acid changes was tested by using the method (Zhang and Kumar, 1997) implemented in the program Converge.

For the branch-site test of selection, we used branch-site models (Yang and Nielsen, 2002; Zhang *et al.*, 2005) that allow the d_N/d_S ratio (ω) to vary both among sites and among lineages because these models may be more likely to detect positive selection affecting only a few sites. Specifically, we used test 2 of Zhang *et al.* (2005) to construct a likelihood ratio test

with Model A and Model A1 (null model). Both models require *a priori* specification of the lineages likely to have experienced positive selection. Visual inspection of Fig. 10.3 allowed us to test two blue-shifted lineages that may have experienced positive selection. Sites that may be under positive selection in the spectrally shifted lineages were identified with a Bayes Empirical Bayes approach (Yang *et al.*, 2005).

Homology Modeling

We used homology modeling to study the relationship between the *L. arthemis astyanax* opsin structure and function using the methods described (Briscoe, 2002). The full-length *L. arthemis astyanax* L opsin protein sequence was manually aligned with the bovine template 1U19.pdb (Okada *et al.*, 2004), and the alignment was submitted to the Swiss-Model server (www.expasy.ch/swissmod) (Schwede *et al.*, 2003). The atomic coordinates were then viewed with SwissPdb Viewer (www.expasy.ch/spdbv) (Guex and Peitsch, 1997), and candidate spectral tuning sites were mapped onto the 3D homology model.

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11

Plant Domestication, a Unique Opportunity to Identify the Genetic Basis of Adaptation

JEFFREY ROSS-IBARRA, PETER L. MORRELL, and BRANDON S. GAUT

Despite the fundamental role of plant domestication in human history and the critical importance of a relatively small number of crop plants to modern societies, we still know little about adaptation under domestication. Here we focus on efforts to identify the genes responsible for adaptation to domestication. We start from a historical perspective, arguing that Darwin's conceptualization of domestication and unconscious selection provides valuable insight into the evolutionary history of crops and also provides a framework to evaluate modern methods used to decipher the genetic mechanisms underlying phenotypic change. We then review these methods, framing the discussion in terms of the phenotype–genotype hierarchy. Top-down approaches, such as quantitative trait locus and linkage disequilibrium mapping, start with a phenotype of interest and use genetic analysis to identify candidate genes. Bottom-up approaches, alternatively, use population genetic analyses to identify potentially adaptive genes and then rely on standard bioinformatics and reverse genetic tools to connect selected genes to a phenotype. We discuss the successes, advantages, and challenges of each, but we conclude that bottom-up approaches to understanding domestication as an adaptive process hold greater promise both for the study of

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adaptation and as a means to identify genes that contribute to agronomically important traits.

Plant domestication fundamentally altered the course of human history. The adaptation of plants to cultivation was vital to the shift from hunter–gatherer to agricultural societies, and it stimulated the rise of cities and modern civilization. Humans still rely on crops that were domesticated >10,000 years ago in such diverse places as Central America, New Guinea, and the Fertile Crescent. Nonetheless, modern humans are reliant on a surprisingly small number of crops: Nearly 70% of the calories consumed by humans are supplied by only 15 crops (Table 11.1). The cereals are particularly important, with five crops (rice, wheat, maize, sugarcane, and barley) contributing more than half of the calories consumed.

Despite the critical importance of these crops, in most cases little is known about their domestication. Some obvious questions pertain to the domesticators: Who were they? How did they identify the incipient crop? What were their cultivation methods? Other questions concern crop history: What was the wild progenitor of the modern crop? Did domesti-

TABLE 11.1 Major World Crops Ranked by Metric Tonnage

Rank by Tonnage*	Common Name	Rank by Calories Consumed*	Ploidy	Propagation	Life History
1	Sugarcane	4	8×	O,V	P
2	Maize	3	2×	O	A
3	Wheat	2	6×	O	A
4	Rice	1	2×	S	A
5	Potatoes	6	4×	O,V	AP
6	Sugar beet	8	2×	O	A
7	Soybeans	5	2×	S	A
8	Cassava	9	4×	O,V	AP
9	Palm kernel	7	2×	O	P
10	Barley	11	2×	S	A
11	Sweet potatoes	15	4–6×	O,V	AP
12	Tomatoes	30	2×	S	A
13	Watermelons	38	2×	O	A
14	Bananas	19	3×	V	P
15	Brassicas	37	2×	O	A

O, outcrossing; S, selfing; V, vegetative; P, perennial; A, annual; AP, perennial species generally cultivated as annuals.

*Data are from the Food and Agriculture Organization of the United Nations (www.fao.org, 2004).

cation occur more than once? If so, where? The application of phylogeographic methods is beginning to inform the answers to this latter set of questions (Heun *et al.*, 1997; Harter *et al.*, 2004), but the picture for any one crop remains far from complete.

In this chapter we focus on a third set of questions that revolve around the phenotypic changes associated with domestication. The first question is whether phenotypic changes driven by artificial selection are an apt analogy for adaptation in nature. We take a historical perspective on this issue, arguing that Darwin's conceptualization of domestication provides valuable insight into our view of crop history and provides a framework for evaluating methods used to decipher the genetic mechanisms underlying phenotypic change. We then review these methods, framing the discussion in terms of the phenotype–genotype hierarchy, evaluating both "top-down" and "bottom-up" approaches. We conclude by arguing that an appreciation of domestication as an adaptive process has the potential to reveal far more about the genes contributing to agronomic traits than has been learned to date.

IS ARTIFICIAL SELECTION ANALOGOUS TO NATURAL SELECTION? A HISTORICAL PERSPECTIVE

In the opening chapter of the *Origin of Species*, Charles Darwin introduced the idea of natural selection with an analogy to domestication (Darwin, 1859b). The importance of domestication to Darwin's thinking is evident even in early sketches of his work (Darwin, 1909), and Darwin himself claimed that the example of domestication was fundamental to the formulation of his theory (C. Darwin, 1859b, 1868, 1958; for a different perspective see Rheinberger and McLaughlin, 1984). But for Darwin domestication was more than a useful analogy: he saw it as a model of adaptation from which inferences about the nature of variation and selection in natural systems could be drawn (Cornell, 1984; Rheinberger and McLaughlin, 1984).

Darwin's assertion of the importance of domestication in understanding the evolutionary process was not universally accepted, however. One of the most vocal critics of Darwin's views on domestication was Alfred Russell Wallace. Even in their joint publication announcing the theory of natural selection, Wallace denies the relevance of domestication: "We see, then, that no inferences as to varieties in a state of nature can be deduced from the observation of those occurring among domestic animals" (Darwin and Wallace, 1858). "It has always been considered a weakness in Darwin's work," he later writes, "that he based his theory, primarily, on the evidence of variation in domesticated animals and cultivated plants" (Wallace, 1889).

Wallace found fault with two aspects of domestication as a heuristic for understanding adaptation in nature. He argued first that the analogy was flawed: artificial selection requires an intelligent selector, whereas no such force acts in natural systems. Additionally, he insisted that the selection itself was fundamentally different, leading to intrinsically different kinds of variation. Domesticated species, he wrote, ''are abnormal, irregular, artificial; they are subject to varieties which never occur and never can occur in a state of nature: their very existence depends altogether on human care; so far are many of them removed from that just proportion of faculties, that true balance of organization, by means of which alone an animal left to its own resources can preserve its existence and continue its race'' (Darwin and Wallace, 1858).

Both of Wallace's lines of argument find modern audiences, from those who see a fundamental difference between the conscious selection of humans and natural processes (Stebbins, 1950) to those who argue that variation in domesticated species differs from that in nature (Coyne and Lande, 1985). Yet Darwin directly addressed these ideas with his explicit recognition of unconscious selection (Darwin, 1909). Darwin (1868) divided human-mediated selection into two components: methodical selection, ''systematically endeavor[ing] to modify a breed according to some predetermined standard,'' and unconscious selection, ''that which follows from men naturally preserving the most valued and destroying the less valued individuals, without any thought of altering the breed.'' Unconscious selection, he posited, was no different from natural selection. Humans change the conditions in which cultivated species live and reproduce, and this change exerts selection on the population even in the absence of a choice or predetermined goal by the cultivator.

Although the term ''unconscious selection'' fell out of use for many years (Darlington, 1963), students of crop evolution nonetheless recognized its fundamental role in domestication. Both Vavilov (1926) and Engelbrecht (Zeven, 1973) viewed the initial stages of domestication as determined entirely by unconscious selection, and modern workers widely cite the central role of unconscious selection, sometimes referred to as automatic (Harlan, 1992) or unintentional (Rindos, 1984), in effecting observed variation in domesticated species. These authors argue that many of the phenotypic changes associated with domestication are likely to have arisen via unconscious selection and, like Darwin, view unconscious selection, and much of the process of domestication, as illustrative of the process and effects of natural selection.

The phenotypic changes associated with adaptation under domestication are substantial. Many of these changes are shared across a broad array of domesticated plants; this suite of changes is commonly referred to as the ''domestication syndrome'' (Hammer, 1984). Common features

of the domestication syndrome are larger fruit or grain, reduced branching, gigantism, the loss or reduction of seed dispersal, the loss of seed dormancy, changes in photoperiod sensitivity, and the loss or reduction of toxic compounds (Hammer, 1984; Gepts, 2004). For example, the major cereal crops in Table 11.1 (rice, wheat, maize, and barley) all experienced a series of parallel phenotypic shifts brought about by domestication, including reduced seed dispersal, reduced branching or tillering, decreased seed dormancy, synchronized seed maturation, an increase in grain size, and larger inflorescences.

We emphasize three salient points about the phenotypic changes associated with domestication. First, with the possible exception of characteristics such as color or fruit size that were clearly desirable by humans, most features of the domestication syndrome are likely the result of unconscious selection (Hammer, 1984; Heiser, 1988; Harlan, 1992; for a more inclusive view see Darwin, 1868). Second, the traits most clearly resulting from unconscious selection are those that would have been difficult for early cultivators to notice or that would have changed without any direct effort. Seed dormancy, for example, would be selected against by almost any method of cultivation, even without a conscious decision to plant only nondormant individuals. Finally, like its natural counterpart, unconscious selection is not limited to visible phenotypes; much of the adaptation under domestication may have involved physiological or developmental changes corresponding to the new edaphic, photosynthetic, hydrological, and competitive regimes associated with cultivation.

TWO APPROACHES TO FINDING ADAPTIVE GENES

Dramatic shifts in phenotype associated with domestication are not only important as evolutionary examples; they have broad economic and societal consequences. There is substantial interest in discovering the genes and genetic mechanisms that contribute to phenotypic changes associated with domestication, because their identification may facilitate trait manipulation through modified breeding strategies (McCouch, 2004). We discuss two approaches to this goal, starting at opposite ends of the phenotype–genotype hierarchy. To date, most research has followed what we call a top-down approach, which begins with a phenotype of interest and then identifies causative genomic regions via genetic analyses such as quantitative trait locus (QTL) and linkage disequilibrium (LD) mapping (Fig. 11.1 *Left*). An alternative approach is to build on Darwin's view of domestication, starting with the concept of adaptation and moving from the bottom up. In this approach, population genetic methods are used to search for the signal of adaptation in a set of genes, and traditional molecular methods are used to move from gene to phenotype (Fig. 11.1 *Right*). Here we introduce

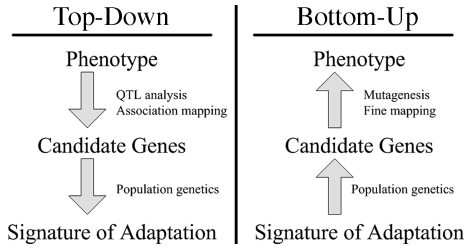

FIGURE 11.1 Schematic of the phenotype–genotype hierarchy as represented by top-down and bottom-up approaches.

each of these approaches, discuss the methodologies available for their implementation, and assess their strengths and weaknesses.

FROM THE TOP DOWN: QTL AND LD MAPPING

To date, all of the successes at identifying genes underlying the adaptive changes during domestication have originated from top-down approaches, beginning with the phenotype and using genetic analyses to uncover genomic regions and eventually candidate genes responsible for the phenotype of interest. The most successful method for finding these genes has been QTL mapping, but association or LD methods are rapidly gaining favor in the plant genomics community. While it is beyond the scope of this article to provide a comprehensive review of QTL and LD mapping, we review some empirical findings and highlight some of the challenges of spanning the gap between phenotype and genotype.

QTL Mapping

Given a trait of interest, QTL mapping was the first (and is still the most widely used) method available for localizing the genetic basis of a trait (e.g., Sax, 1923). QTL mapping has led to all of the major successes in the identification and cloning of genes underlying domestication traits (Doebley *et al.*, 2006). The best-known examples come from tomato and maize. In the mid-1980s Tanksley and coworkers (Paterson *et al.*, 1988) initiated QTL analysis of fruit mass in a cross between wild and domesticated tomato, localizing six QTLs. With extensive mapping efforts, they were able to isolate a region encompassing the major QTL *fruitweight2.2* (*fw2.2*). They also demonstrated the phenotypic effect of *fw2.2* with transgenic analysis (Frary *et al.*, 2000). At about the same time Doebley and coworkers

(Doebley *et al.,* 1990; Doebley and Stec, 1991) mapped differences in plant architecture and plant yield between maize and its wild ancestor, teosinte. Subsequent mapping and mutation analyses led to the isolation of major genes that govern phenotypic differences between maize and teosinte, including *teosinte branched1* (*tb1*), a gene controlling lateral branching (Doebley *et al.,* 1995), and *teosinte glume architecture* (*tga*), which contributes to differences in inflorescence architecture (Wang *et al.,* 2005).

These successes highlight the value of the QTL approach, but the method is not without its limitations. It can, for example, be difficult to develop mapping populations for perennial, inbreeding, and vegetatively propagated crops. Thus, some of the 15 crops in Table 11.1, such as bananas and palm trees, are intractable for study by QTL approaches. It is also important to remember that the results of QTL analysis often depend on the environment (Paterson *et al.,* 1988) as well as the parental lines used in the cross (Doebley and Stec, 1991; Li *et al.,* 2006a). Caution is therefore warranted in interpreting the generality of QTLs, especially in cases of multiple domestication or local adaptation. There are also numerous statistical issues, the most important of which is the limited power to accurately estimate the number and size of QTLs, an observation that has become known as the Beavis effect (Beavis, 1994, 1998). Although this limitation has not proven problematic for cloning genes of large phenotypic effect, statistical power poses a major concern for more classically quantitative traits like size, weight, or yield that are likely to be determined by a larger number of QTLs of smaller phenotypic effect, and statistical concerns become even more problematic for the estimation of complex phenomena such as epistasis (Carlborg and Haley, 2004).

QTL studies have provided and will continue to provide considerable utility for identifying genes and genomic regions that contribute to phenotypes of interest. Moreover, the rate at which such genes are identified will continue to increase as genomic data become available for more species; this increase is already evident in the 2006 publication year, which witnessed an explosion of the isolation of genes contributing to major phenotypic differences between domesticates and their wild ancestors. Although not solely attributable to QTL approaches, genes isolated in 2006 included two rice shattering genes (Konishi *et al.,* 2006; Li *et al.,* 2006b), a rice kernel color gene (Sweeney *et al.,* 2006), a wheat shattering gene (Simons *et al.,* 2006), and a wheat senescence gene affecting nutritional content (Uauy *et al.,* 2006). Even so, only a handful of genes have been isolated by these approaches (Doebley *et al.,* 2006), and the total output has been surprisingly small given both the large amount of money and human capital invested in QTL studies and the economic and societal importance of a relatively small number of plants (Table 11.1). Furthermore, the genes isolated to date are genes of very large effect, i.e., the ''low hanging fruit''

(Doebley *et al.,* 2006). Substantially more effort will likely be required to identify and clone genes of smaller effect.

LD Mapping

In the hope of overcoming some of the limitations of QTL analysis, plant researchers have moved toward LD mapping as an additional means to identify genomic regions that contribute to phenotypes. In practice, LD mapping can be separated into two types, each focusing on a different level of genetic analysis. The first, like most QTL approaches, aims to identify genome-wide variation that associates with phenotypic variation. This requires measures of genetic variability in markers representing most of the genome and tests of phenotype–genotype association for each marker. The second type of association analysis attempts to pinpoint the causative genetic mutation(s) that effect phenotype; these latter studies typically focus on variation in one or few candidate genes rather than whole genomes.

The primary advantage of LD mapping is that it can rely on population samples; there is no need for crosses and the production of large numbers of progeny. This is an obvious benefit for the study of bananas, palms, or other long-lived perennial species (Table 11.1) and in general allows studies to proceed more rapidly. In addition, the population sample may contain many more informative meioses (i.e., all those that have occurred in the evolutionary history of the sample) than a traditional QTL mapping population. As a result, the phenotype of interest may be associated with a much smaller chromosomal segment than in a QTL population, in theory providing greater mapping resolution.

Like QTL methods, however, there are several features of experimental design that need to be carefully considered when undertaking LD mapping. First, distinguishing true associations from statistical noise requires large sample sizes, both for statistical power and to correct for multiple tests (Long and Langley, 1999; Macdonald and Long, 2004). Even with large sample sizes, researchers may have to assume that the effects of individual mutations are additive; testing for epistatic interactions between hundreds of markers further exacerbates the problem of multiple tests (Macdonald *et al.,* 2005). One way to reduce this problem is to test for associations between phenotypes and haplotypes (or ''haplotype blocks'') rather than individual markers (Clark, 2004). But unless haplotypes can be inferred experimentally (Morrell *et al.,* 2006), as in selfing taxa such as barley and rice (Table 11.1), the necessary computational inference of haplotypes can prove an impediment to this approach.

Another design challenge is sample origin. Geographic structure or other departures from panmixis can result in spurious associations in

which a genotype is associated with a geographic region rather than a phenotype. This will become especially problematic for phenotypes that vary by geographic region, such as flowering time or photoperiod sensitivity. Many important crops (e.g., barley, rice, and soybean) are derived from wild populations with extensive geographic structure (Lin *et al.,* 2001; Morrell *et al.,* 2003; Kuroda *et al.,* 2006). This structure is often reflected in the domesticate as well, especially in cases involving multiple independent domestications (Londo *et al.,* 2006; Morrell and Clegg, 2007). Unfortunately, for many of the crops in Table 11.1 we have little information about the location of domestication or population structure in wild populations. The conspicuous exceptions are rice, maize, barley, and wheat, whose domestication histories are becoming more clear (Matsuoka *et al.,* 2002; Salamini *et al.,* 2002; Londo *et al.,* 2006; Morrell and Clegg, 2007). Studies of human diseases (Weiss and Clark, 2002) suggest that basic research on demographic history and population structure will be crucial to the success of LD mapping in plants.

The final design challenge that we will consider here is marker (usually SNP) density. LD mapping studies are very powerful when the causative mutation is genotyped (Risch and Merikangas, 1996; Long and Langley, 1999). If the causative mutation is not genotyped, it is still possible to identify association via markers that are in LD with the causative mutation. However, the extent of LD can vary dramatically among plant species (Flint-Garcia *et al.,* 2003; Morrell *et al.,* 2005), among genomic regions within plants (Gaut *et al.,* 2007), and among population samples (Tenaillon *et al.,* 2001; Ching *et al.,* 2002; Caldwell *et al.,* 2006). The distribution of LD is also affected by homologous gene conversion, which predominantly disrupts short-range LD patterns (Jeffreys and May, 2004; Padhukasahasram *et al.,* 2004; Morrell *et al.,* 2006). Study design, statistical analysis, and controlling for biological challenges such as population structure are very active areas of research (Rosenberg and Nordborg, 2006; Yu and Buckler, 2006), but several large-scale plant LD mapping studies are currently underway despite having little background information about the extent of LD and geographic structure in the populations being studied.

The difficulties inherent to LD mapping are reflected in the literature. In a genome-wide association study, Aranzana *et al.* (2005) confirmed several *Arabidopsis* QTLs for flowering time and pathogen resistance but also noted a high rate of false positive associations. Workers using large wild-caught populations of *Drosophila* have been unable to verify associations identified in lab populations, suggesting that some results may not be replicable regardless of sample size, the number of SNPs genotyped, or the care taken in study design (Macdonald and Long, 2004; Macdonald *et al.,* 2005). Furthermore, failure to identify an association between a candidate gene and a phenotype of interest is likely underreported.

Despite these drawbacks, LD mapping has had some successes. One early example successfully linked phenotypic variation in malting quality in barley to haplotype variation at the β-amylase2 gene, a locus involved in starch hydrolysis. Differences in the coding region of barley β-amylase2 affect thermostability of the enzyme (Ma *et al.,* 2001; Clark *et al.,* 2003), and SNP genotyping confirmed that cultivars with high malting quality and the high-thermostability enzyme share a common haplotype (Polakova *et al.,* 2003; Malysheva-Otto and Roder, 2006). Resequencing of candidate genes has also been used in foxtail millet and rice to determine the genetic basis of waxy or sticky grains. Mutations at the *waxy* (granule-bound starch synthase) locus result in changes in amylose content in the endosperm, resulting in the sticky grains popular in eastern and southern Asia (Domon *et al.,* 2002; Kawase *et al.,* 2005; Olsen *et al.,* 2006). LD mapping has also been used to verify associations inferred from QTL or other approaches (Thornsberry *et al.,* 2001; Szalma *et al.,* 2005; Balasubramanian *et al.,* 2006; Breseghello and Sorrells, 2006b).

A promising future direction for LD mapping is the use of synthetic populations derived from a relatively small number of founders (Breseghello and Sorrells, 2006a), facilitating QTL and LD mapping in a single population while minimizing complications due to population structure (Flint-Garcia *et al.,* 2005; Breseghello and Sorrells, 2006a; Yu and Buckler, 2006).

History, Adaptation, and Population Genetics

Extensive work is required to isolate a candidate gene for a particular trait, but a phenotype–genotype association is no guarantee that the trait or its candidate gene has been historically important or is an adaptation. It is tempting to conclude that observable phenotypic differences are adaptive, particularly in domesticated organisms where selection is strong and the direction of selection can be surmised. However, many of the differences between domesticates and their progenitors may not be adaptive, at least from a human perspective; for example, QTLs decreasing protein content in wheat (Uauy *et al.,* 2006) and seed size in sunflower (Burke *et al.,* 2002) are unlikely to have been directly selected during domestication. A number of alternative processes can explain observed phenotype–genotype associations, including genetic drift, selection on a correlated trait, pleiotropy, or even natural selection working in opposition to anthropogenic selection. It therefore behooves us to endeavor to test adaptive hypotheses rather than assume them to be true (Gould and Lewontin, 1979).

To understand the process of adaptation during domestication, one must first consider the genetic history associated with domestication.

Domestication of all plants and animals led to a reduction in genetic diversity (Ladizinsky, 1985; Doebley, 1989; Gepts, 2004), and thus all genes in any domesticated plant necessarily have a history that includes a recent demographic event, the bottleneck associated with domestication (Fig. 11.2). Population subdivision in the wild ancestor, ongoing introgression between the crop and wild relatives, and multiple domestication events can also have demographic impacts. Genes important for domestication were also subjected to conscious or unconscious directional selection, experiencing a reduction in variation over and above that associated with any demographic events (Fig. 11.2). The level of diversity remaining at a given locus in a domesticate is thus expected to be inversely proportional to the locus's adaptive importance during domestication. Thus, the major genes contributing to agronomically important traits may lack variation entirely (Whitt *et al.*, 2002).

With a candidate gene in hand, molecular population genetic methods can be used to test adaptive hypotheses. Conceptually, the approach is simple: under the selection scenario described in Fig. 11.2, one expects that genes contributing to adaptive traits will have low genetic variation relative to nonselected genes. In addition, a strongly selected gene may have other discriminating features, such as an excess of low frequency polymorphisms or high intralocus LD (Przeworski, 2002). It is thus essen-

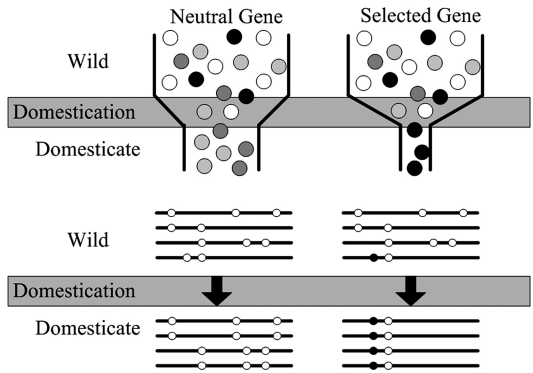

FIGURE 11.2 Schematic representation of a population bottleneck and its effect on a neutral gene and a selected gene. In *Upper*, shaded circles represent genetic diversity. The bottleneck reduces diversity in neutral genes, but selection decreases diversity beyond that caused by the bottleneck alone. *Lower* illustrates sequence haplotypes of these two hypothetical genes. The neutral gene lost several haplotypes through the domestication bottleneck, but the selected gene is left with only one haplotype containing the selected site.

tial to assay genetic polymorphism in a number of randomly selected reference genes and compare them to the candidate gene. However, this is often difficult to do well, requiring many randomly sampled genes (see below) and computationally intensive simulation methods to estimate the underlying demographic model.

FROM THE BOTTOM UP: MOLECULAR POPULATION GENETICS

In marked contrast to the top-down or phenotype-first approaches already discussed, bottom-up approaches start by identifying genes with the signature of adaptation using population genetics and then make use of a broad array of genetic tools to identify the phenotypes to which these genes contribute. Bottom-up approaches are relatively new, and many of the methodologies are still being developed, but we believe that they have the potential to revolutionize crop genetics. Here we briefly introduce some of the methods and outline the challenges involved in identifying candidate genes using population genetics.

Fitting a Demographic Model

Ideally, bottom-up approaches begin by assaying genetic diversity in hundreds of loci, preferably from a sample of ≈100 individuals representing both the domesticate and its wild ancestor. Given sequence polymorphism data, several factors will affect the ability to detect the signal of adaptation, including the strength and history of selection, rates of mutation and recombination, and the demographic history of the population (Wright and Gaut, 2005). As mentioned above, demographic considerations are particularly important for crop plants, likely invalidating standard population genetic tests designed to detect the signal of selection. The standard tests typically assume that populations evolve according to the idealized Wright–Fisher model, with panmictic populations of constant population size. When these assumptions are inaccurate, as they certainly are for most domesticated species (Fig. 11.2), tests to detect selection can be wildly inaccurate. For example, computer simulations show that Tajima's *D*, a commonly used test statistic for selection, identifies up to 25% of loci as selected after a change in population size due to a bottleneck, even when there has been no selection (Wright and Gaut, 2005). Another recent method incorrectly infers selection up to 90% of the time when Wright–Fisher assumptions do not hold (Jensen *et al.*, 2005). Thus, departures from standard assumptions dramatically decrease the reliability of tests for selection and can distort the signature of selection beyond recognition (Slatkin and Wiehe, 1998; Przeworski *et al.*, 2005). Clearly, one should view with skepticism studies of domesticated crops

that employ standard population genetics tests to infer selection and thus the historical importance of a gene.

How, then, does one address the problem of demography? One way is to develop a demographic model that provides a reasonable fit to available data and then apply statistical tests of selection under that demographic model. The estimation of demographic history from DNA sequence data was first applied to maize (Eyre-Walker *et al.,* 1998; Hilton and Gaut, 1998). In these early studies, sequence variation was assessed at a handful of loci from maize and its wild ancestor, teosinte. It was explicitly assumed that there had been no artificial selection on these genes. Observed polymorphism data were compared with data simulated under a historical coalescent model that included a population bottleneck. The size and duration of the bottleneck were varied via simulation, and bottleneck parameters that best fit the observed data were determined. For example, over a time frame of 2,800 years (an estimate of the duration of domestication based on archaeological data), the effective size of the population maintained through the bottleneck was estimated to be ≈2,900 individuals (Hilton and Gaut, 1998). This indicates that high genetic diversity in maize is not necessarily due to a large founding population. Taken further, such inferences can be applied to better understand the agricultural practices of early domesticators (Hillman and Davies, 1990).

Since these initial studies, it has become possible to gather sequence polymorphism data from hundreds of loci. With many loci, it is no longer necessary or appropriate to assume that none of the genes has been targeted by selection, and it becomes possible both to infer the proportion of genes under selection and to identify those genes. At the same time, coalescent models have improved and can now include demographic factors such as recombination, population growth, and introgression (Hudson, 2002).

The bottom-up approach should be especially powerful when applied to domesticated species, for three reasons. First, archaeological remains provide independent information about the timing of the domestication bottleneck, and its effects are relatively well understood. Second, artificial selection is strong and domestication is recent on an evolutionary time scale, so that the signature of selection should be highly detectable in patterns of genetic diversity (Przeworski, 2002; Olsen *et al.,* 2006; Teshima *et al.,* 2006). Third, polymorphism can be compared between a crop and its wild ancestor, greatly increasing inferential power (Voight *et al.,* 2005) and helping to discriminate among evolutionary events before, during, or after domestication (Wright and Gaut, 2005). Examples of this demographic approach have appeared in the literature with increasing frequency (95) and have been incorporated into testing for selection in humans and *Drosophila* as well (Tenaillon *et al.,* 2004). At present, however, the process

of estimating a demographic model is time-consuming and computationally intensive and requires substantial population genetic expertise.

Thus far, the bottom-up approach to studying domestication has been applied only to maize. Wright *et al.* (2005) formulated a demographic model of maize domestication using sequence polymorphism data from 793 genes in 14 maize inbred lines and 16 haploid plants from its wild progenitor, teosinte. With these data, Wright *et al.* (2005) first sought to estimate a plausible demographic model and then asked whether the data were more likely if directional selection on a subset of loci was included in the model. Applying a novel likelihood ratio approach to this problem, they estimated that 2–4% of their loci were linked to a target of artificial selection during domestication. Their approach also allowed them to rank loci in terms of evidence for selection. The list of selected genes is enriched for functions related to transcription factors, genes implicated in plant growth, and genes involved in amino acid biosynthesis. Moreover, genes identified as targets of selection clustered nonrandomly around previously identified QTLs for domestication traits (Wright *et al.,* 2005) and are more highly expressed than random genes only in the maize ear (K. M. Hufford and B.S.G., unpublished results), an organ expected *a priori* to be the target of selection.

Empirical Ranking

The demographic approach for finding candidate "adaptive" genes is model-intensive. As an alternative to estimating the demographic model, several studies have simply ranked genes empirically (Toomajian *et al.,* 2006; Voight *et al.,* 2006). This is an acceptable, but not optimal, solution based on a straightforward idea. Under the selection scenario described in Fig. 11.2, one expects that genes contributing to adaptive traits should have low genetic variation or skewed allele frequencies compared with nonselected genes (Tajima, 1989). Without knowing the exact demographic model, it thus makes sense to assay genetic polymorphism in a number of genes, compare them, and rank them by summary statistics. The candidate gene, if selected, should fall into the extreme tail of the distribution of summary statistics like S, the number of SNPs in the gene, or Tajima's D, a measure of the allele frequency spectrum. If the gene is extreme, then the polymorphism data are consistent with an adaptive hypothesis. This idea can be applied to a genome-wide sample of genes to identify candidate genes *de novo* via bottom-up methods, or, alternatively, to compare a candidate gene identified by top-down approaches to a sample of reference loci.

Although empirical ranking is a suitable approach, its efficacy depends greatly on the particulars of individual evolutionary histories

and the number of sampled loci used. Simulations show that the false discovery rate of this method may be high for recessive genes, for genes selected from standing variation, and for populations that have undergone demographic change (Teshima *et al.,* 2006), all factors likely to have played a role in adaptive events under domestication. Similarly, although statistical methods are available to explicitly test for selection using this approach, recent results suggest that the false positive rate of these statistics is also high (but can be controlled, e.g., Thornton and Jensen, 2006). To illustrate these potential problems empirically, we describe results from resampling the data of Wright *et al.* (2005). We simulated scenarios in which a researcher tests for selection in a candidate gene and ranks the candidate relative to a sample of reference loci. To do this, we chose both a ''candidate'' locus and a ''reference'' sample of loci, sampling with replacement from the complete data set. For each candidate locus we asked whether it was extreme in terms of low genetic diversity (measured by *S*) or in its frequency distribution (measured by Tajima's *D*). We made two comparisons for each locus, first using the observed measure in maize, then using the difference between maize and teosinte. We treated each of the ≈800 loci as a candidate locus and compared it to a random set of reference loci. We initially set the size of the reference sample to five loci, then repeated the experiment with samples of 10, 20, and 50. The entire process was repeated 1,000 times, giving an estimate of the distribution of numbers of candidate genes rejected for each reference sample size.

Results of this resampling are shown in Fig. 11.3, presented for both summary statistics and a scenario in which the neutrality of a candidate locus is rejected if either of the statistics is extreme. Two valuable insights emerge from this exercise. The first is that using small samples of reference loci gives very poor results. With a sample of five loci for comparison, our simulations rejected >12% of the candidate loci for each of the statistics and rejected ≈35% of the loci if the most extreme of the two statistics was used. Samples of size 10 improve the situation, but our simulations still rejected more than twice as many loci as the model-based approach (Wright *et al.,* 2005). The second insight is that including ancestral individuals improves the test greatly. Because loci in domesticated plants have undergone a demographic bottleneck, a substantial number of loci have lost much or all of their variation, even in the absence of selection: of the 793 loci in the data set, 90 have no diversity. Using only observed diversity in maize for comparison, these 90 loci are always rejected as extreme, regardless of the sample size. When comparative data from teosinte are included it becomes clear that some of the loci with zero diversity in maize have low levels of diversity in teosinte as well, suggesting that these are low-diversity genes rather than genes disproportionately affected by domestication.

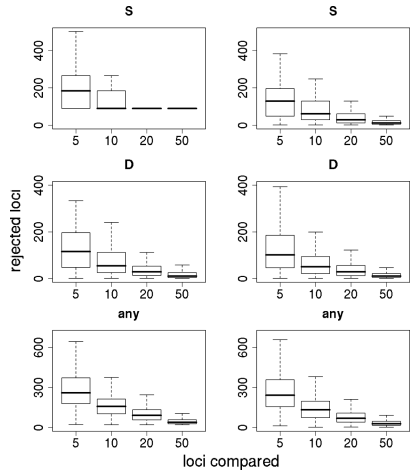

FIGURE 11.3 Resampling tests to examine empirical ranking methods for finding candidate genes. *Left* represents sequence data from maize alone; *Right* demonstrates the difference in statistics between maize and teosinte. The statistics are the number of SNPs (*S*) (*Top*), Tajima's *D* (*Middle*), and a combination of both (*Bottom*) (see text). For each graph, the heavy line represents the median number of genes, of ≈800, that are inferred to be under selection. Boxes represent the central 50% of the data, and lines extend out to 3/2 of the interquantile range.

From Gene to Phenotype via Molecular Genetics

The biggest drawback to bottom-up approaches is that the candidate genes are not associated with a phenotype. In theory this can be rectified by using the array of genetic tools available for many model species. For the first five species listed in Table 11.1, for example, a number of genomics tools exist to aid in connecting a candidate gene to a phenotype: databases for ESTs, microarray, and gene expression data; targeted mutagenesis lines; genetic maps; and partial or complete genome sequences. For model crops such as maize and rice, reverse genetics methodologies have been transformed into high-throughput pipelines suitable for the analysis of large numbers of genes (McCallum *et al.,* 2000). The link from gene to phenotype for nonmodel species, however, may be daunting.

Although high-throughput analysis of phenotype is a distant possibility for many species in Table 11.1, bioinformatics and standard reverse genetics techniques can still provide a bounty of information regarding possible phenotypes. For many crops without extensive genetic resources, valuable information can nonetheless be gleaned from comparative

genomic analysis of gene function or expression in related species. At worst, comparative bioinformatics can provide insight into the general class of gene, potentially offering information about the role the gene has played during domestication. Similarly, many standard reverse genetics approaches such as RNA interference and transgenic methods can lead to significant clues as to gene function.

PERSPECTIVE: TOP-DOWN VS. BOTTOM-UP

Both top-down and bottom-up approaches will continue to prove useful for the study of adaptation to domestication. With the current rate of increase of genomic information for many crop species, we expect that the dramatic increase in top-down success stories seen in 2006 will continue for some time, identifying some of the genes of large effect that contribute to the phenotypes associated with domestication. Anytime the goal is to identify genes underlying a specific phenotype of interest, these top-down approaches will continue to be the best choice. We argue, however, that top-down approaches have a severe and insuperable limitation for the study of adaptation: the requirement of identifying a phenotype *a priori*.

It is plausible (and even likely) that alleles influencing fruit size in tomato (Nesbitt and Tanksley, 2002) or inflorescence structure in maize (Bomblies and Doebley, 2005) have evolved as adaptations to domestication; the available genetic evidence does not speak to a history of selection, and without comparative genetic or experimental evidence of selection, adaptive hypotheses for these genes must remain, at best, hypotheses. We must be careful not to assume adaptation simply because a gene correlates with a trait of agronomic importance, and the converse is equally true: there are likely many genes that, although not responsible for obvious morphological change, will nonetheless show evidence of selection and adaptation under domestication.

Furthermore, the genetic history of crops creates a dilemma for QTL and LD mapping approaches: mapping requires segregating genetic diversity at the gene of interest, but genes governing historically important phenotypes are expected to have low genetic diversity in the domesticate (Fig. 11.2). QTL and LD approaches that do not include wild populations are likely to miss many of the genes contributing to agronomic traits that were important during early domestication. In contrast to QTL studies that can use wild × domesticate crosses, LD mapping is faced with a Catch-22: including both wild and domesticated individuals will lead to spurious associations due to sample origin, but purely wild populations will be depauperate for the domesticated phenotype (and genotype) of interest.

In addition to their freedom from the constraints of *a priori* phenotypic choices, bottom-up approaches have several advantages for finding genes

that contribute to adaptive traits and that will be useful in an agronomic context. These advantages include the following: (*i*) segregating variation is not required to identify genes of interest; (*ii*) far fewer plant samples are needed than for LD mapping, with only tens (<100) of samples (Teshima *et al.*, 2006) often sufficing as opposed to hundreds or thousands (Long and Langley, 1999); (*iii*) like LD mapping, bottom-up approaches can be applied to species that reproduce slowly and lack genetic tools; and (*iv*) they allow inferences about demographic history, providing historical insights into the process of domestication. We should note, of course, that bottom-up and top-down approaches are not mutually exclusive; for example, bottom-up approaches in maize are also being used to identify candidate genes for LD mapping (Yu and Buckler, 2006).

Although they have advantages, bottom-up approaches are also not a panacea, for at least four reasons. First, their success will vary among species, depending on levels and distribution of genetic diversity. For example, initial surveys of genetic diversity in sorghum have failed to identify selected genes (Hamblin *et al.*, 2006). This failure is in part a limitation of the study system, because sorghum has low genetic diversity, but it may also reflect inefficient sample design. Simulation studies suggest that these methods should be quite powerful with moderate (<100) sample sizes, even with diversity levels as low as those found in sorghum (Teshima *et al.*, 2006, and M. Przeworski, personal communication), but empirical studies relied on a sample of 17 domesticated individuals and only one wild plant (Hamblin *et al.*, 2006). Second, genes identified as selected may have been targets of selection or may be linked to a target of selection (through ''hitchhiking''). For example, selection on the rice gene *waxy* appears to have affected patterns of sequence diversity in 29 additional genes. This lack of resolution is, however, a shortcoming shared with QTL and LD methods, because in all cases it is difficult to differentiate between a target (or ''causal'') marker and linkage effects (Weigel and Nordborg, 2005). In fact, when the genomic locations of genes are available, the expected chromosomal resolution of bottom-up approaches is at worst similar to QTL and LD mapping. Third, like top-down approaches, bottom-up approaches may not be feasible for all crops. The limitation here is not generation time (as in QTL studies), but rather levels of genetic diversity, polyploidy, and population structure. Polyploidy makes population genetic analysis difficult, requiring careful separation of homeologs and their independent evolutionary histories. As with LD mapping, unrecognized population structure can be problematic for population genetic analyses, producing patterns that can be mistaken for selection. Finally, bottom-up approaches share a major limitation with both association and QTL mapping. All three methods identify candidate genes or regions, but verification requires additional functional characterization (Weigel and Nordborg, 2005). It is worth noting that in many cases

this last step, connecting a candidate gene to a phenotype via functional studies, is often not much easier for top-down approaches than for candidate genes identified by using population genetics.

Over the past 25 years, top-down approaches have yielded a list of ≈30 genes with well characterized phenotypic effects in plants (Doebley *et al.,* 2006). It is known that these 30 are genes of major effect, i.e., either Mendelian factors or major QTLs, but for most it has not been determined whether they have played an important adaptive role historically. In contrast, limited application of bottom-up approaches in maize have identified ≈50 genes with a signature of adaptation. It is a statistical certainty that some of these will prove to be false positives, but it is also likely that some of these genes contribute to phenotypes that would not or could not be studied via QTL or LD mapping.

In the last year, the number of published, large-scale studies seeking to identify selected genes has exploded. Screens for selection have been applied to polymorphism data from humans (Bustamante *et al.,* 2005; Voight *et al.,* 2006) and *Arabidopsis* (Toomajian *et al.,* 2006) as well as maize (Wright *et al.,* 2005; Yamasaki *et al.,* 2005). To a much more limited extent bottom-up approaches are being applied to other domesticated species, i.e., rice (Olsen *et al.,* 2006; Zhu *et al.,* 2007), flax (Allaby *et al.,* 2005), sorghum (Hamblin *et al.,* 2006), and dogs (Pollinger *et al.,* 2005). We argue that there is an opportunity, in fact, a pressing need, for a broad-based initiative to implement bottom-up approaches in 15–20 important crops, not unlike a multispecies HapMap project. Such an initiative would be relatively inexpensive given new sequencing technologies and would have far-reaching consequences beyond identifying candidate genes. Important side benefits would include broader-based information on LD, SNP discovery on a panel of sufficient size to limit ascertainment biases, and evolutionary analyses of polymorphism in a genomic context. Data compared across species may also provide insights into the process of adaptation. For example, such data could inform the age-old question as to whether parallel phenotypic changes, such as the domestication syndrome, evolve via parallel genetic mechanisms (Paterson *et al.,* 1995). Wide-scale implementation of bottom-up approaches across species would be of potential agronomic benefit, but would also provide a unique opportunity to identify the genetic basis of adaptation.

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12

An Experimental Test of Evolutionary Trade-Offs During Temperature Adaptation

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We used experimental evolution to test directly the important and commonplace evolutionary hypothesis that adaptation, increased fitness within the selective environment, is accompanied by trade-off, a loss of fitness in other nonselective environments. Specifically, we determined whether trade-offs at high temperature generally and necessarily accompany genetic adaptation to low temperature. We measured the relative fitness increment of 24 lineages of the bacterium *Escherichia coli* evolved for 2,000 generations at 20°C and the relative fitness decrement of these lines at 40°C. Trade-offs at the higher temperature were examined for their generality, universality, quantitative relationship, and historical contingency. Considering all 24 lines as a group, a significant decline in fitness was found at 40°C (mean decline = 9.4%), indicating the generality of the trade-off effect. However, in a lineage-by-lineage analysis, only 15 of 24 showed a significant trade-off, and one lineage increased fitness at high temperature. Thus, although general, trade-offs were not universal. Furthermore, there was no quantitative association between the magnitude of adaptive fitness increment at 20°C and fitness decline at

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40°C, and no effect of lineages' historical thermal environment on either their improvement at 20°C or the extent of their trade-off at high temperature. We do not yet know the underlying mechanisms responsible for the trade-off, but they are sufficiently prevalent to drive a general effect. However, approximately one-third of the experimental lineages achieved low-temperature adaptation without detectable high-temperature trade-offs; therefore, it cannot be necessary that every change conferring benefit in cold environments has a negative effect on function in warmer environments.

Evolutionary adaptation to a new environment necessarily involves the enhancement of certain traits, leading to improved function and an increase in fitness. However, adaptation may be accompanied by deterioration in other traits, which are presumably of less or no importance in the new environment. This decline in some characters during adaptation is termed a trade-off and is often viewed as a cost or constraint associated with adaptation (e.g., Futuyma and Moreno, 1988; Stearns, 1992; Futuyma, 1998; Sibly, 2002; Novak *et al.,* 2006).

The assumption of cost associated with gain has been a fundamental premise of biological and evolutionary thought for centuries. For example, Darwin (1859b, pp. 147–148) states that ". . . natural selection is continually trying to economise in every part of the organisation. If under changed conditions of life a structure before useful becomes less useful, any diminution, however slight, in its development, will be seized on by natural selection, for it will profit the individual not to have its nutriment wasted in building up an useless structure." The assumption of trade-offs continues to be an important component of thinking about adaptive evolution: ". . . improvements cannot occur indefinitely, because eventually organisms come up against limitations. . . . At that point, improvements in one trait may be achievable only at the expense of others—there is a trade-off between the traits" (Sibly, 2002). This way of thinking has embedded itself into the models and mindsets we use to study life history and morphological and physiological evolution. For instance, in regard to environmental adaptation, Levins' (1968) principle of allocation explicitly incorporates fitness trade-offs and consequent niche shifts. Adaptation to cold environments, for instance, is predicted to entail the loss of performance in warm environments. Subsequent models in evolutionary physiology about thermal niche structure and biological responses to climate change have usually assumed trade-offs (e.g., Lynch and Gabriel, 1987; Pease *et al.,* 1989; but see Gilchrist, 1995).

Although evolutionary trade-offs are widely assumed, demonstrating their existence can be difficult. Several approaches have been used,

including comparative studies on different taxa, phenotypic manipulation, analysis of genetic correlations, and selection experiments, but most of these have interpretive limitations (Reznick, 1985; Futuyma and Moreno, 1988; Futuyma, 1998). Comparative studies, for instance, are essentially correlational, without access to knowledge about the ancestral condition or the evolutionary sequence of gains and losses of functions. Selection experiments, in which selection is imposed on one trait and correlated change is measured in another, are generally considered by evolutionary biologists to be the most powerful approach for demonstrating the existence of trade-offs (Futuyma, 1998; Sibly, 2002).

In this study, we use 24 experimentally evolved lineages of the bacterium *Escherichia coli* to analyze whether adaptation to low temperature (20°C) is accompanied by a loss of fitness at high temperature (40°C). We analyze these trade-offs from four perspectives:

(*i*) Generality; is there a significant loss of fitness at high temperature across all lineages considered together?

(*ii*) Universality; do all of the lineages individually demonstrate a loss of fitness at high temperature?

(*iii*) Quantitative relationship; does the magnitude of adaptation influence the magnitude of trade-off? That is, do the cold-adapted lineages with the highest fitness at low temperature also have the lowest fitness at high temperature?

(*iv*) Historical contingency; does the prior thermal selective history of a lineage influence either the extent of its adaptation to low temperature or the magnitude of trade-off in fitness at high temperature?

RESULTS

Adaptation to 20°C

Table 12.1 provides the measured fitness values at 20°C of each of the 20°C evolved lineages and its immediate historical progenitor, both obtained relative to their common ancestor. The resulting changes in fitness (ΔW) at 20°C are shown in Table 12.2. The temperature that each derived line had experienced before its evolution at 20°C had no significant effect on the extent of adaptation to 20°C ($F_{3,20} \pm 0.497$, $P = 0.69$). Given this absence of a historical effect and the absence of any other phylogenetic relationships among these lineages [star phylogeny from the common ancestral clone (Anc; Fig. 12.1)], we analyzed the significance of adaptation in all 24 lineages both individually and collectively. Mean ΔW for all 24 lines was 0.118 (±0.025 95% confidence limit, $P < 0.0001$), and ΔW was significantly positive ($P \leq 0.05$ in Table 12.2) in 22 of the 24 lines.

TABLE 12.1 Fitness at 20°C of the 20°C-Selected Lineages and Their Historical Progenitors, Each Measured Relative to the Common Ancestor

	−1	−2	−3
32	1.026 ± 0.050	1.081 ± 0.026	1.022 ± 0.019
32/20	1.124 ± 0.065	1.266 ± 0.076	1.121 ± 0.024
37	0.979 ± 0.045	0.997 ± 0.034	0.897 ± 0.042
37/20	1.072 ± 0.053	1.124 ± 0.080	1.050 ± 0.045
42	0.947 ± 0.061	1.005 ± 0.039	0.992 ± 0.059
42/20	1.019 ± 0.041	1.089 ± 0.089	1.128 ± 0.057
32–42	1.018 ± 0.035	1.085 ± 0.014	1.001 ± 0.035
32–42/20	1.143 ± 0.052	1.113 ± 0.039	1.191 ± 0.042

Rows are historical selective temperature regimes; columns are lineage designations (see Fig. 12.1). Entries are mean values ± 95% confidence limits, with $n = 6$ replicate fitness assays for each entry.

TABLE 12.2 Change in Fitness (ΔW) at 20°C of 20°C-Selected Lines Compared with Their Historical Progenitors

	−1	−2	−3
32/20 vs. 32	0.098	0.185	0.099
	$P = 0.006$	$P < 0.001$	$P < 0.001$
37/20 vs. 37	0.093	0.128	0.154
	$P = 0.003$	$P = 0.002$	$P < 0.001$
42/20 vs. 42	0.073	0.084	0.136
	$P = 0.015$	$P = 0.025$	$P = 0.001$
32-42/20 vs. 32-42	0.125	0.028	0.190
	$P < 0.001$	$P = 0.056$	$P < 0.001$

Cell entries are changes in mean fitness from Table 12.1 and P values of one-tailed t tests.

Therefore, there was a general adaptation to selective temperature in this experiment, and this adaptation was also significantly manifest in >90% of the individual lineages. The mean fitness increment is not significantly different ($P = 0.14$) from that of six replicate lines selected at 20°C directly from Anc (Mongold *et al.*, 1996).

Trade-Off at 40°C

Table 12.3 gives the relative fitness, measured at 40°C, of each lineage that evolved at 20°C and its immediate progenitor. The resulting change in fitness at 40°C (ΔW) is reported in Table 12.4. Changes in fitness (ΔW) were not significantly affected by historical environment ($F_{3,20} = 0.282$, $P = 0.84$).

+1	+2	+3	Mean
1.066 ± 0.022	0.998 ± 0.058	1.030 ± 0.129	1.037 ± 0.032
1.173 ± 0.062	1.109 ± 0.054	1.234 ± 0.078	1.153 ± 0.057
1.004 ± 0.037	0.987 ± 0.044	0.994 ± 0.022	0.976 ± 0.042
1.126 ± 0.071	1.111 ± 0.041	1.105 ± 0.061	1.098 ± 0.032
0.986 ± 0.059	0.977 ± 0.045	1.033 ± 0.038	0.990 ± 0.030
1.053 ± 0.047	1.268 ± 0.092	1.119 ± 0.051	1.113 ± 0.051
0.967 ± 0.063	1.015 ± 0.027	0.998 ± 0.036	1.014 ± 0.041
1.068 ± 0.047	1.131 ± 0.046	0.994 ± 0.135	1.116 ± 0.054

+1	+2	+3
0.107	0.110	0.204
$P < 0.001$	$P = 0.003$	$P = 0.003$
0.122	0.124	0.111
$P = 0.001$	$P < 0.001$	$P < 0.001$
0.067	0.291	0.086
$P = 0.022$	$P < 0.001$	$P = 0.003$
0.100	0.117	−0.005
$P = 0.004$	$P < 0.001$	$P = 0.466$

The mean ΔW for all 24 lines was −0.094 (±0.047 95% confidence limit, $P < 0.0001$). In general, therefore, there was a significant loss of fitness at high temperature, demonstrating a trade-off associated with adaptation to lower temperature. This mean fitness decrement at 40°C is not significantly different ($P = 0.16$) from that of six replicate lines selected at 20°C directly from Anc (Mongold *et al.,* 1996). However, there is considerable heterogeneity in the response among the individual lineages in their performance at this high temperature. Using $P \leq 0.05$ as the criterion for testing the trade-off, 15 lines had a significant decrement in fitness at 40°. Eight lines did not significantly decrease in fitness, and one (42/20 + 3) actually experienced a significant increase in fitness at 40°C ($\Delta W = 0.122$) while it evolved at and adapted to 20°C ($\Delta W = 0.086$). To verify the fitness

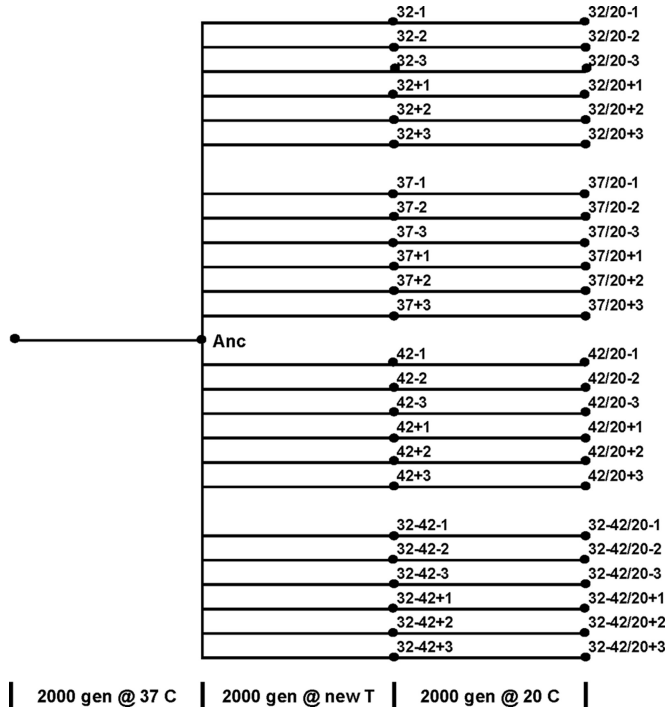

FIGURE 12.1 Phylogeny and nomenclature of the experimental lineages of *E. coli* used in this study. The ancestral organism (Anc) was obtained from a lineage that evolved under defined laboratory conditions at 37°C for 2,000 generations. A clone was sampled and cultured in six replicate populations that evolved in each of four thermal environments: 32°C, 37°C, or 42°C, or a daily alteration between 32°C and 42°C. After 2,000 generations, a clone was isolated from each lineage and propagated for another 2,000 generations at 20°C. Note that each of the 20°C selected lineages are equally related to Anc (separated by 4,000 generations) and are equally distant from each other (separated by 8,000 generations).

increment of 42/20 + 3 at 40°C, which went against our *a priori* expectation, the 40°C measurements were repeated with 12-fold replication for three lines, one that demonstrated a trade-off (42/20 − 1), one that did not significantly change fitness (42/20 + 2), and the single line that incremented fitness at high temperature (42/20 + 3). The previous results were repeated for each line: 42/20 − 1 had a negative ΔW ($P < 0.001$), 42/20 + 2 had no significant change in fitness ($P + 0.06$), and 42/20 + 3 had a significantly positive ΔW ($P = 0.04$). Of the six lines selected at 20°C directly from Anc

(Mongold *et al.*, 1996), four had significant decrements in fitness at 40°C, whereas two showed no significant trade-off (unpublished data).

Previous studies have noted that correlated responses are often more variable than direct responses to selection (Travisano and Lenski, 1996), and the heterogeneity in directional responses measured for fitness changes at 40°C is consistent with that pattern. To examine this issue further, we calculated the among-lineage variance components for fitness increments measured at 20°C and at 40°C and their associated confidence limits (Sokal and Rohlf, 1981). The among-lineage variance component is 0.0025 at 20°C and 0.0108 at 40°C, with 95% confidence intervals of 0.0010 to 0.0059 and 0.0057 to 0.0230, respectively. Neither of the corresponding 95% confidence intervals overlaps the point estimate at the other temperature. Hence, this difference in genetic variability among lines is significant and in the direction that supports the hypothesis of greater heterogeneity in the correlated responses than in the direct responses to selection.

The quantitative relationship between fitness gain at 20°C and fitness loss at 40°C is shown in Fig. 12.2. Although 20 of the 24 lineages fall within the trade-off quadrant (i.e., lower right section) of Fig. 12.2, there is no significant quantitative relationship between the magnitude of the selected gain and correlated loss ($r = 0.006$, $P > 0.50$).

DISCUSSION

Generality and Universality

The results of our study illustrate some of the complexities of analyzing and interpreting evolutionary patterns, even those that come from a carefully designed and replicated experiment. The general hypothesis that trade-offs occur as a result of evolutionary adaptation is certainly supported by these experimental results. Associated with the pervasive adaptation of the evolved bacteria to low temperature was a significant loss of fitness at high temperature. In the analysis of all 24 lineages together, mean ΔW at 40°C significantly declined ($P < 0.0001$). This trade-off is the general pattern observed and was also the general prediction that motivated this study.

However, the trade-offs were far from universal among the 24 experimental lineages. In a line-by-line analysis, only 15 followed the general pattern, and about this same proportion was also observed in six additional lineages derived from Anc adapting directly to 20°C (Mongold *et al.*, 1996). Nine of the 24 lineages analyzed here did not significantly decline in fitness at 40°C, even with the relatively low stringency criterion of $P \leq 0.05$; applying a Bonferroni correction for multiple comparisons would result in an additional five nonsignificant decrements. Although

TABLE 12.3 Fitness at 40°C of 20°C-Selected Lineages and Their Historical Progenitors, Each Measured Relative to the Common Ancestor

	−1	−2	−3
32	0.963 ± 0.021	1.028 ± 0.064	0.980 ± 0.029
32/20	0.849 ± 0.063	0.549 ± 0.178	0.900 ± 0.047
37	1.102 ± 0.029	0.616 ± 0.071	0.993 ± 0.031
37/20	0.881 ± 0.067	0.608 ± 0.145	0.889 ± 0.021
42	1.153 ± 0.050	1.089 ± 0.059	1.117 ± 0.041
42/20	0.984 ± 0.054	0.934 ± 0.061	0.974 ± 0.033
32–42	1.056 ± 0.040	1.079 ± 0.058	1.064 ± 0.067
32–42/20	0.916 ± 0.100	0.963 ± 0.044	1.010 ± 0.057

Contents and notations are as in Table 12.1.

TABLE 12.4 Change in Fitness (ΔW) at 40°C of 20°C-Selected Lines Compared with Their Historical Progenitors

	−1	−2	−3
32/20 vs. 32	−0.114	−0.478	−0.080
	$P < 0.001$	$P < 0.001$	$P = 0.003$
37/20 vs. 37	−0.220	−0.007	−0.104
	$P < 0.001$	$P = 0.454$	$P < 0.001$
42/20 vs. 42	−0.169	−0.156	−0.144
	$P < 0.001$	$P < 0.001$	$P < 0.001$
32-42/20 vs. 32-42	−0.140	−0.116	−0.053
	$P = 0.004$	$P = 0.001$	$P = 0.088$

Contents and designations are as in Table 12.2.

one might be tempted to ''explain away'' these nonsignificant results as false negatives that reflect limited statistical power, that explanation was rejected, as follows. For 3 of the 24 lines, their correlated fitness changes at 40°C were positive rather than negative and, in one case, the correlated improvement was significant. To avoid the possibility of a false positive in the unexpected direction, additional fitness assays were performed (with twice the original level of replication) for that exceptional line, as well as for two others that showed significant and nonsignificant trade-offs. The very same patterns emerged in these new and independent assays for all three strains, including confirmation of the unexpected gain in fitness at 40°C in the line of interest. Hence, contrary to the general pattern and prediction, one line significantly increased in fitness at high temperature while adapting to low temperature.

+1	+2	+3	Mean
0.488 ± 0.066	1.064 ± 0.037	0.982 ± 0.049	0.917 ± 0.224
0.527 ± 0.145	1.046 ± 0.043	0.919 ± 0.051	0.798 ± 0.222
1.008 ± 0.056	0.919 ± 0.065	1.047 ± 0.041	0.947 ± 0.182
0.941 ± 0.039	0.947 ± 0.037	0.952 ± 0.026	0.870 ± 0.138
1.113 ± 0.040	1.136 ± 0.044	1.082 ± 0.073	1.115 ± 0.028
1.077 ± 0.067	1.114 ± 0.052	1.204 ± 0.109	1.048 ± 0.107
1.104 ± 0.039	1.079 ± 0.059	1.114 ± 0.067	1.083 ± 0.024
1.003 ± 0.237	0.984 ± 0.077	0.942 ± 0.083	0.970 ± 0.038

+1	+2	+3
0.039	−0.018	−0.063
$P = 0.271$	$P = 0.214$	$P = 0.023$
−0.068	0.028	−0.094
$P = 0.014$	$P = 0.182$	$P < 0.001$
−0.036	−0.021	0.122
$P = 0.133$	$P = 0.221$	$P = 0.019$
−0.101	−0.095	−0.172
$P = 0.152$	$P = 0.016$	$P = 0.001$

Therefore, although the trade-off pattern is general, it cannot be universal. Which is more important in considering and discussing evolutionary trade-offs? Is the glass two-thirds full, one-third empty, or partly inverted? The prediction must be that trade-offs will generally occur, but they may fail to happen in some or even many individual instances, and correlated responses may sometimes even be opposite in sign to those expected under the trade-off hypothesis.

Quantitative Relationship Between Direct and Correlated Responses

The previous section dealt with the qualitative aspects of the trade-off hypothesis, as indicated by the sign of the correlated response. Levins' (1968) principle of allocation also predicts a quantitative association between the magnitude of adaptation to one environment and its trade-off

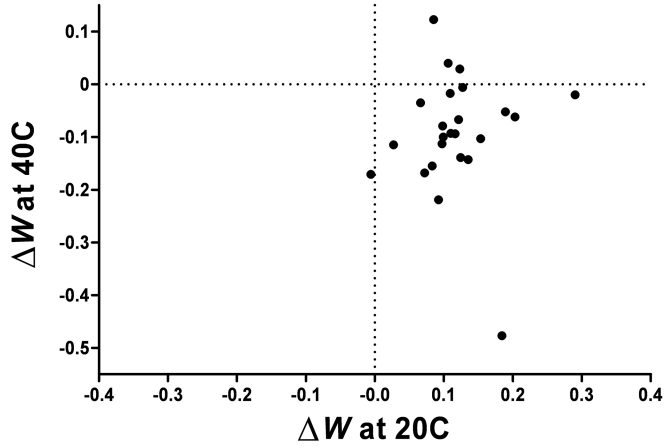

FIGURE 12.2 Correlation between change in fitness measured at 20°C and at 40°C after experimental evolution at 20°C. Although 20 of 24 points lie in the quadrant associated with an evolutionary trade-off (fitness gain at 20°C and loss at 40°C), the correlation between them is not significant ($r = 0.006$, $n = 24$, $P > 0.50$).

in another. Commonsensical notions of trade-off would also seem to make a similar prediction: the greater the magnitude of fitness gain at lower temperature, the greater the anticipated loss of fitness at high temperature. However, no such correlation was observed; trade-offs, when they occur, appear random with respect to the degree of adaptation to 20°C (Fig. 12.2). For example, line 42/20 + 2 had the highest fitness gain at 20°C, but it did not have even a statistically significant trade-off at high temperature. One factor contributing to the absence of any compelling quantitative relationship is the divergence in the direction of the correlated fitness response measured at 40°C, as discussed in the previous section. Another factor is that the genetic variation among the lines is much lower for the direct fitness response at 20°C than for the correlated response measured at 40°C, making it more difficult to detect the underlying quantitative relationship, if any.

Historical Contingency

The 24 lineages that evolved at 20°C for 2,000 generations had previously evolved in the same medium under one of four different thermal regimes: constant 32°C, 37°C, or 42°C or a daily alteration between 32°C and 42°C (Bennett *et al.*, 1992). Hence, we could test whether this prior selective history influenced their subsequent evolution at 20°C. However,

there is no evidence of an effect of historical thermal environment on the extent of their adaptation to low temperature or on the fitness trade-off observed at high temperature. Lineages previously adapted to 32°C, for example, adapted no more or less to 20°C than did lineages previously adapted to 42°C. This negative result was previously reported for these lineages on the basis of fewer replicate assays of fitness measures (Mongold *et al.,* 1996). The genetic adjustments made during adaptation to their historical thermal environments evidently neither helped nor hindered subsequent adaptation to 20°C. Likewise in regard to trade-offs, prior adaptation to diverse temperatures did not differentially predispose or prevent trade-offs.

Investigating the Nature of Trade-Offs

The basis of some trade-offs is readily apparent as a result of differential allocation of time, space, or energy, as described earlier in the quote from Darwin (1859b). The cause of other trade-offs may be less obvious and can involve antagonistic pleiotropy, mutation accumulation, or both, in genes encoding functions under relaxed selection (Cooper and Lenski, 2000; Cooper *et al.,* 2001a). Within this experimental series, there are several lineages that have achieved low-temperature adaptation without high-temperature trade-offs. Therefore, it cannot be the case that every mutational change conferring benefit in cold necessarily has a negative effect on performance in heat. For the majority of the lineages that do demonstrate trade-offs, two different population-genetic mechanisms might be responsible for the decline of fitness at high temperature, antagonistic pleiotropy, or mutation accumulation (Rose and Charlesworth, 1980; Rose, 1991; Holt, 1996; Cooper and Lenski, 2000). Investigation of the genetic bases underlying low-temperature adaptation in these lineages is now in progress, and the implications of the genetic mechanisms for performance at high temperature are therefore presently unknown. In the future, however, it may be feasible to identify and revert modified alleles to their ancestral states and then directly measure their quantitative impact on both adaptation and trade-off. Under antagonistic pleiotropy, such reversion should simultaneously decrease fitness at low temperature and increase it at high temperature. Under mutation accumulation, by contrast, reversion should repair function at high temperature without a simultaneous decrement in low-temperature function. The diversity of quantitative associations between adaptation and trade-off (including the complete absence of the latter) could result from either mechanism or some combination of both. Previous studies on trade-offs in thermal and catabolic performance using the progenitor experiment to this evolution experiment have favored antagonistic pleiotropy over mutation accumu-

lation (Cooper and Lenski, 2000; Cooper *et al.*, 2001a,b, 2003). Whatever mechanisms are operating in our system, they result in a general, but not universal, trade-off effect.

The Utility of an Experimental Approach to Evolution

We believe that our study again demonstrates the power of an experimental approach to test and inform evolutionary theory (Bennett and Lenski, 1999). It forces the restatement of a qualitative assumption into a quantitative hypothesis and then allows rigorous testing of that hypothesis. Experiments can provide a sufficient number of replicated lineages, measurements, and controls so the hypothesis in question can be statistically evaluated. The evolving lineages can also generate enough biological novelty so the diversity of adaptations to a common environment and their underlying physiological and genetic bases can be studied further (Bennett, 2003).

METHODS

Study Organisms

The 24 lineages of *E. coli* used in this study were originally derived by Mongold *et al.* (1996). All of these lines evolved from a single ancestral strain (here designated Anc), which itself was obtained from a lineage that had evolved on minimal glucose medium in serial dilution culture for 2,000 generations at 37°C (Lenski *et al.*, 1991). The 24 lines founded by Anc were maintained in the same medium and serial transfer regime for another 2,000 generations with six replicate populations propagated in each of the following thermal conditions: constant 32°C, 37°C, or 42°C or a daily alteration between 32°C and 42°C (Bennett *et al.*, 1992). Clonal isolates of these 24 lines, each derived from a single colony, were then cultured for another 2,000 generations at 20°C (Mongold *et al.*, 1996). That temperature corresponds to the lower boundary of the thermal niche of Anc, where the boundary was defined operationally by its ability to sustain itself at a stable population density in the face of a 100-fold daily dilution (Bennett and Lenski, 1993). The phylogeny and nomenclature of these lineages are shown in Fig. 12.1. Each of the 20°C adapted lines is independent of each other; they have not shared a common ancestor for 4,000 generations and are therefore phylogenetically separated from each other by 8,000 generations. Clonal isolates of all lines, including Anc and the intermediate lineages adapted to diverse temperatures, are stored frozen at −80°C and were used in these analyses. The 20°C adapted lines have diverse evolutionary thermal histories, and therefore the effect of

historical adaptive environment on rate and extent of adaptation to low temperature can be undertaken as well (Travisano *et al.,* 1995; Mongold *et al.,* 1996).

Experimental Measurements

The performance metrics used here are the fitnesses of a derived line relative to the common ancestor, measured at both the low selected temperature of 20°C and a high temperature (40°C); 40°C was conservatively used as the high temperature instead of the upper thermal niche boundary of Anc of 42°C (Bennett and Lenski, 1993), because four of six 20°C-adapted lines were incapable of growth at 42°C, but all six were able to grow and maintain themselves in serial dilution culture at 40°C (figure 3 in Mongold *et al.,* 1996). The rationale and methodology for measuring relative fitness (*W*) are given in Lenski (1991). Here it is calculated and expressed as the ratio of the number of doublings of a derived line relative to that of its ancestor when the two are grown together and compete for the same pool of nutrients.

Details for the measurement of *W* in this system have been reported (Lenski *et al.,* 1991; Bennett *et al.,* 1992). Briefly, both an evolutionary derived line and the ancestor are taken from storage at −80°C, separately inoculated into flasks containing rich LB medium, and incubated at 37°C for 1 day. These cultures are then diluted into minimal glucose medium [Dulbecco's medium (DM) supplemented with 25 μg of glucose per milliliter] and incubated at 37°C for another day. They are then diluted again into fresh DM and incubated at the experimental test temperature (20°C or 40°C in this study). Flasks were incubated in New Brunswick incubator shakers (New Brunswick Scientific, Edison, NJ) at 20°C and 37°C and at 40°C in a New Brunswick water bath shaker. These preliminary incubations ensure that the bacteria are growing and are phenotypically acclimated to the medium and the experimental temperature. On the next day, both the derived line and the ancestor are diluted together into a common flask; a small sample is immediately plated onto agar for enumeration, and the flask is incubated at the experimental test temperature for 1 day, during which time the bacteria experience a lag phase, exponential growth, depletion of the nutrients, and stationary phase. After 24 h, a sample from the mixed culture is then plated again, colonies of each competitor are counted, and the ratio of the number of doublings of the derived and ancestral types is calculated from the change in densities of the two types relative to the initial sample taken immediately after mixing. The ancestor used in this experiment exists in two genetically marked forms, one capable of using the sugar arabinose and the other not. These two forms differ in their colony color on tetrazolium–arabinose indicator agar, but the marker itself is neutral in

the minimal glucose medium used for the competition assays (Lenski *et al.*, 1991). Competitions are always performed between reciprocally marked ancestral and derived lines, such that the colony color serves only as a marker to distinguish evolutionary derivation. In these experiments, each determination of W was done with six replicate measurements, and the mean and 95% confidence limits are reported.

Analyses

Change in fitness (ΔW) is measured by comparing W of a 20°C line relative to Anc with that of its immediate historical progenitor, also assessed relative to Anc. (Direct competitions are not possible because both competitors share the same genetic marker state.) For example, the extent of adaptation of the 32/20 – 1 line to 20°C is determined by W of 32/20 – 1 at 20°C minus W of 32 – 1 at 20°C. Values of ΔW significantly >0 in the selective environment (20°C) indicate evolutionary adaptation, whereas values significantly <0 in the nonselective environment (40°C) indicate a trade-off.

Adaptation by each derived line to 20°C was analyzed by a one-tailed t test on the six replicate measurements of ΔW at 20°C. Mean ΔW of each of the 24 derived lines at 20°C was used to determine the generality of the adaptive response (one-tailed t test on 24 lines). Trade-off by each derived line at 40°C was analyzed with a one-tailed t test on the six experimental replicate measurements of ΔW at 40°C, except as noted, when we explored, independently tested, and confirmed the finding that one lineage in fact showed a correlated improvement at this temperature. Mean ΔW of each of the 24 derived lines at 40°C was used to determine the generality of the trade-off response (one-tailed t test on 24 lines). The quantitative nature of the trade-off was analyzed by determining the correlation coefficient between ΔW at 20°C and ΔW at 40°C for each of the 24 lineages. The effects of historical thermal environment on adaptation to 20°C and trade-off at 40°C were analyzed with one-way ANOVAs on historical temperature (32°C, 37°C, 42°C, or 32–42°C).

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13

Two Routes to Functional Adaptation: Tibetan and Andean High-Altitude Natives

CYNTHIA M. BEALL

Populations native to the Tibetan and Andean Plateaus are descended from colonizers who arrived perhaps 25,000 and 11,000 years ago, respectively. Both have been exposed to the opportunity for natural selection for traits that offset the unavoidable environmental stress of severe lifelong high-altitude hypoxia. This paper presents evidence that Tibetan and Andean high-altitude natives have adapted differently, as indicated by large quantitative differences in numerous physiological traits comprising the oxygen delivery process. These findings suggest the hypothesis that evolutionary processes have tinkered differently on the two founding populations and their descendents, with the result that the two followed different routes to the same functional outcome of successful oxygen delivery, long-term persistence and high function. Assessed on the basis of basal and maximal oxygen consumption, both populations avail themselves of essentially the full range of oxygen-using metabolism as populations at sea level, in contrast with the curtailed range available to visitors at high altitudes. Efforts to identify the genetic bases of these traits have included quantitative genetics, genetic admixture, and candidate gene approaches. These reveal generally more genetic variance in the Tibetan population and more potential for natural selection. There is evidence that natural selection is ongoing in the Tibetan

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population, where women estimated to have genotypes for high oxygen saturation of hemoglobin (and less physiological stress) have higher offspring survival. Identifying the genetic bases of these traits is crucial to discovering the steps along the Tibetan and Andean routes to functional adaptation.

People have occupied many different habitats since leaving Africa, probably during the past 100,000 years (Trinkaus, 2005). Behavioral buffering and biological adaptability have enabled human occupation of environments spanning large ranges in features such as temperature, UV radiation, and diet. However, only biological adaptability has contributed to our success in occupying high-altitude lands (to ≈5,400 m), because traditional technology could not buffer us from the unavoidable environmental stress of high-altitude hypoxia (less than the normal amount of oxygen in the air because of reduced atmospheric pressure).

Indigenous human populations on the Tibetan and Andean Plateaus are descendents of colonizers who arrived at most ≈25,000 and 11,000 years ago, respectively (Aldenderfer, 2003). Abundant evidence documents the reduced physical function of low-altitude natives visiting high altitudes who engage many homeostatic responses yet do not restore preexposure function (Ward *et al.,* 2000). The two high-altitude populations can be viewed as the current outcome of separate replications of a natural experiment in which an ancestral founding population moved from low to high altitude, and its descendents have been exposed for millennia to the opportunity for natural selection to improve function under high-altitude hypoxia. Both experiments have been successful, as indicated by the rise of great civilizations, long-term persistence, and population growth. However, the experiments have proceeded differently, as indicated by large quantitative differences in physiological traits related to offsetting the stress of high-altitude hypoxia. Evolutionary theory suggests that features of physiology or metabolism that are distinctive as compared with ancestral conditions or other populations represent functional adaptations. The purpose of this paper is to present evidence for Tibetan–Andean contrasts in functional adaptations that offset the stress of hypoxia and to consider the evidence for a genetic basis for these differences between Tibetan and Andean high-altitude natives.

The environmental stress of high altitude is hypoxia that, in turn, creates the conditions for physiological hypoxia (less than the normal amount of oxygen in the organism). The severity of high-altitude hypobaric hypoxia is illustrated in Fig. 13.1 by the regular decrease in the partial pressure of oxygen in the atmosphere with increasing altitude. Studies of adaptation to high-altitude hypoxia usually focus on populations living at

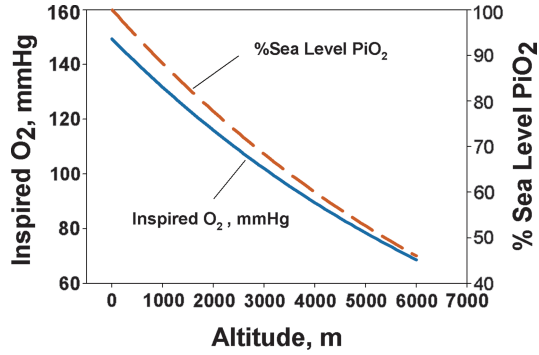

FIGURE 13.1 Ambient oxygen levels, measured by the partial pressure of oxygen (solid line) or as a percent of sea-level values (dashed line), decrease with increasing altitude, a situation called high-altitude or hypobaric hypoxia. The atmosphere contains ≈21% oxygen at all altitudes.

≥2,500 m, where physiological effects become more easily detectable with more severe stress. Many studies report about populations living in the range of 3,500–4,500 m, because many people live in that altitude range on both plateaus, and because those residents must deal with severe stress and may be most likely to exhibit adaptive responses. At 4,000-m elevation, every breath of air contains only ≈60% of the oxygen molecules in the same breath at sea level. This is a constant feature of the ambient environment to which every person at a given altitude is inexorably exposed. Less oxygen in inspired air results in less oxygen to diffuse into the bloodstream to be carried to the cells for oxygen-requiring energy-producing metabolism in the mitochondria. Humans do not store oxygen, because it reacts so rapidly and destructively with other molecules. Therefore, oxygen must be supplied, without interruption, to the mitochondria and to the ≥1,000 oxygen-requiring enzymatic reactions in various cells and tissues (Raymond and Segre, 2006).

The oxygen level is near zero in human mitochondria at all altitudes (Hochachka and Rupert, 2003). This condition is described as "primitive," because it has changed little for the past 2.5 billion years despite wide swings in the amount of atmospheric oxygen (at times it has been 10,000-fold lower; Bekker *et al.*, 2004; Huey and Ward, 2005) and "protective" in the sense that it circumvents potentially damaging reactions of oxygen with other molecules (Massabuau, 2003). Fig. 13.2 describes the transport of oxygen in humans along a "cascade" of falls in oxygen level from inspired air to the capillaries from which it will diffuse into the mito-

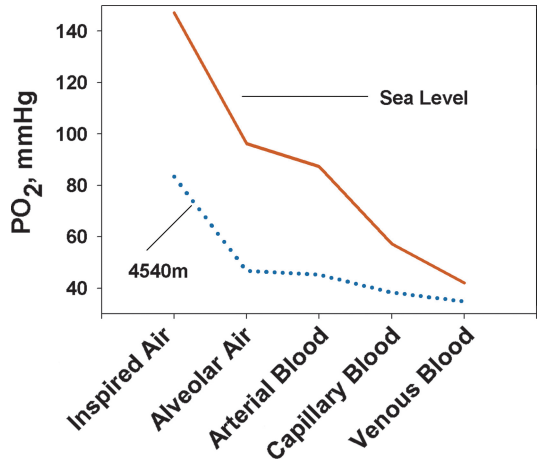

FIGURE 13.2 The oxygen transport cascade at sea level (solid line) and at the high altitude of 4,540 m (dotted line) illustrates the oxygen levels at the major stages of oxygen delivery and suggests potential points of functional adaptation (data from Hurtado, 1964).

chondria. The inspired oxygen pressure at the higher altitude of 4,540 m is much lower, so the pressure differences among different stages of oxygen transport are smaller, and the diffusion rate is lower. Fig. 13.2 identifies several points of potential adaptation with respect to sustaining the process of mitochondrial generation of energy at very low oxygen levels.

POTENTIAL AND ACTUAL POINTS OF ADAPTATION TO HYPOXIA

Energy Production

Lowlanders traveling to high altitude display homeostatic responses to the acute severe hypoxia. The responses are energetically costly, as indicated by an increase in basal metabolic rate (BMR; the minimum amount of energy needed to maintain life with processes such as regulating body temperature, heart rate, and breathing). BMR is increased by ≈17–27% for the first few weeks upon exposure to high altitude and gradually returns toward sea-level baseline (Butterfield *et al.*, 1992). In other words, for acutely exposed lowlanders, the fundamental physiological processes required to sustain life at high altitude require more oxygen despite lower oxygen availability. At the other extreme of energy expenditure, a mea-

sure of the upper limit to oxygen delivery is the highest oxygen uptake an individual can attain during work. This upper limit is decreased by ≈20–30% during the first weeks and gradually returns toward normal over the course of 1 year (although it does not return to preexposure sea-level baseline) (Buskirk, 1976; Baker, 1976; Ward *et al.,* 2000; Marconi *et al.,* 2006; Wu and Kayser, 2006). The result is a relatively narrow scope for increasing oxygen consumption above the basal requirement for supporting other functions, including growth, reproduction, and physical activity.

In contrast to acutely exposed lowlanders and despite the equally low level of oxygen pressure in the air and lungs, both Andean and Tibetan highlanders display the standard low-altitude range of oxygen delivery from minimal to maximal. Both populations have the normal basal metabolic rate expected for their age, sex, and body weight (Picon-Reategui, 1961; Mazess *et al.,* 1969; Beall *et al.,* 1996), implying that their functional adaptations do not entail increased basal oxygen requirements. Furthermore, Andean and Tibetan highlanders have maximal oxygen uptake expected for their level of physical training (Beall, 2002; Marconi *et al.,* 2006; Wu and Kayser, 2006). For example, a comparative analysis of 17 samples of Tibetan and Andean men living at an average altitude of ≈3,900 m finds estimated maximum oxygen consumptions of 46 and 47 ml/O_2 per kilogram, respectively, that are similar to values for untrained men at sea level and ≈10–20% higher than those reported for six low-altitude native samples residing at the same altitudes (Beall, 2002). Thus, they can use at high altitude the same full range of aerobic potential for activities requiring oxygen delivery that others use at low altitude. This represents a functional change from the ancestral acute response to altitude, and it suggests that the high-altitude native populations have adaptations that do not elicit elevated oxygen consumption. Unexpectedly, as described below, the similar functional endpoints are reached differently among Tibetan as compared with Andean high-altitude natives, who differ quantitatively in measures of oxygen delivery along the transport cascade. In turn, this raises questions about the possible mechanisms and evolutionary steps along the two adaptive routes.

Ventilation

One potential point of adaptation in oxygen delivery is ventilation, which, if raised, could move a larger overall volume of air and achieve a higher level of oxygen in the alveolar air (Fig. 13.2) and diffusion of more oxygen. An immediate increase in ventilation is perhaps the most important response of lowlanders acutely exposed to high altitude, although it is not sustained indefinitely and is not found among members of low-altitude populations born and raised at high altitude, such as Europeans

or Chinese (Moore, 2000; Ward *et al.*, 2000). Tibetan, but not Andean, highlanders have retained this temporary ancestral response, as indicated by elevated resting ventilation, as compared with Andean highlanders and low-altitude populations at low altitude. For example, a comparative analysis summarizing the results of 28 samples of Tibetan and Andean high-altitude natives at an average altitude of ≈3,900 m reported an estimated resting ventilation of 15.0 liters/min among the Tibetan samples as compared with 10.5 liters/min among the Andean samples (Beall, 2001). Fig. 13.3 illustrates the higher resting ventilation of Tibetans as compared with Andean highlanders evaluated using the same protocol at ≈4,000 m. The mean resting ventilation for Tibetans was >1 SD higher than the mean of the Andean highlanders (Beall *et al.*, 1997a).

The control of ventilation has been evaluated in the two populations by quantifying the reflexive increase in ventilation induced by exposure to a standardized experimental hypoxic stress, a measure called the hypoxic ventilatory response (HVR). The HVR of low-altitude populations is abruptly and markedly elevated upon acute exposure to high altitude, returns to normal levels after a few days, and falls below normal levels after months or years (Weil *et al.*, 1971; Zhuang *et al.*, 1993; Sato *et al.*, 1994). Tibetans express a normal HVR as compared with sea-level populations in their native altitude, whereas Andean highlanders have HVRs generally lower than sea-level values. A comparative analysis summarizing reports on 25 samples of Tibetan and Andean high-altitude natives at an average altitude of ≈3,900 m found that the average HVR of Tibetans was approximately double that of the Andean high-altitude natives (Beall, 2001). The higher HVR of Tibetans is illustrated in Fig. 13.3 by a comparison of paired samples evaluated at ≈4,000 m (Beall *et al.*, 1997a). The implication is that the Tibetan respiratory physiology has changed from the ancestral functional response of a temporary increase in ventilation and HVR to a pattern of sustaining those responses indefinitely.

Oxygen in the Bloodstream

The higher ventilation levels among Tibetans that move more oxygen through the lungs, along with the higher HVRs that respond more vigorously to fluctuations in oxygen levels, might be expected to result in more oxygen in the bloodstream. However, the level of oxygen in the arterial blood (Fig. 13.2) of a sample of Tibetans at ≈3,700 m was lower than that of a sample of Andean high-altitude natives at the same altitude (54 as compared with 57 mmHg; 1 mmHg = 133 Pa) (Winslow *et al.*, 1989; Zhuang *et al.*, 1996). In addition, hemoglobin, the oxygen-carrying molecule in blood, is less saturated with oxygen among Tibetans than among their Andean counterparts (Beall *et al.*, 1997b, 1999). Fig. 13.3 illustrates the

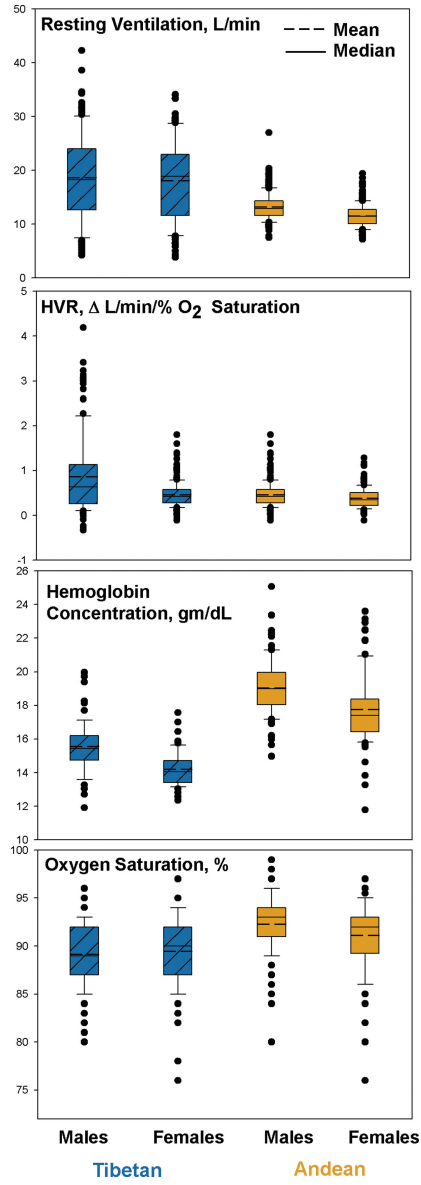

FIGURE 13.3 Boxplots comparing pairs of Tibetan and Andean samples, measured at ≈4,000-m altitude by using the same recruiting and measurement protocols, illustrate the marked quantitative differences in resting ventilation, HVR, hemoglobin concentration, and percent of oxygen saturation (recalculated from data reported in Beall *et al.*, 1997a, 1997b, 1998, 1999).

lower percent of oxygen saturation of hemoglobin in a sample of Tibetans at ≈4,000 m. The increased breathing of Tibetans does not deliver more oxygen to the hemoglobin in the arteries.

Another potential adaptation in the bloodstream is a higher concentration of hemoglobin itself. However, Tibetans have lower hemoglobin concentrations than their Andean counterparts at the same altitude (e.g., Winslow *et al.,* 1989; Beall *et al.,* 1998). An analysis summarizing the results of 53 samples of Tibetan and Andean high-altitude native men at an average altitude of ≈3,900 m reported an estimated mean hemoglobin concentration of 16.9 g/dl among Tibetan men as compared with 18.1 g/dl among Andean men (Beall, 2001). Fig. 13.3 illustrates the markedly lower hemoglobin concentrations in a sample of Tibetan men and women as compared with their Andean counterparts at ≈4,000 m. [The average hemoglobin concentrations were 15.6 and 19.2 g/dl for Tibetan and Andean men, respectively, and 14.2 and 17.8 g/dl for women (Beall *et al.,* 1998).] Hemoglobin concentration is influenced by many factors, including erythropoietin, a protein that causes differentiation of the precursors that will become hemoglobin-containing red blood cells. Tibetans have slightly lower erythropoietin concentrations than Andean highlanders at the same altitude (Winslow *et al.,* 1989). When matched for volume of red blood cells, a procedure that would effectively compare the highest Tibetan and the lowest Andean values, Andean highlanders have much higher erythropoietin levels, which implies that some sensor is responding as if the stress were more severe, even though the samples were collected at the same altitude of ≈3,700 m.

Together, oxygen saturation and hemoglobin concentration determine arterial oxygen content. Fig. 13.4 illustrates that the calculated arterial oxygen content in a sample of Tibetans is substantially lower than among Andean highlanders, who actually have higher arterial oxygen content than sea-level natives at sea level. On average, neither Andean nor Tibetan highlanders restore the usual sea-level arterial oxygen content. Instead, Andean highlanders have overcompensated for ambient hypoxia according to this measure, whereas Tibetan highlanders have undercompensated. Indeed, Tibetans are profoundly hypoxic and must be engaging other mechanisms or adapting at different points in the oxygen transport cascade to sustain normal aerobic metabolism.

Blood Flow and Oxygen Diffusion

Other potential points of functional adaptation include the rate of flow of oxygen-carrying blood to tissues and the rate of oxygen diffusion from the bloodstream into cells.

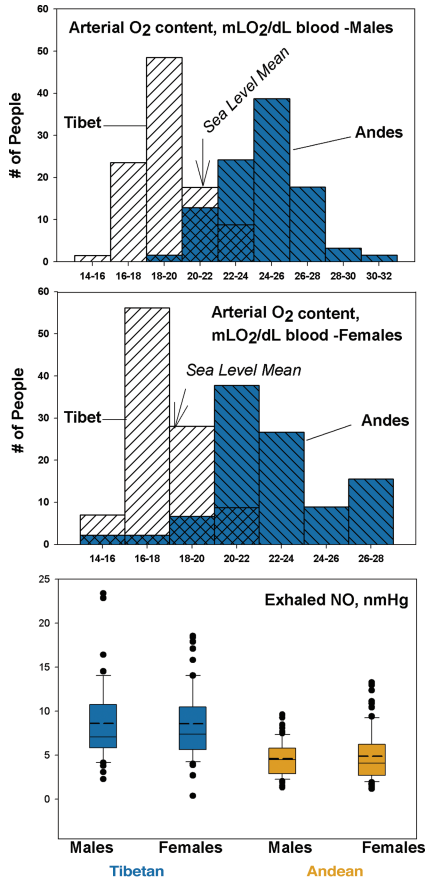

FIGURE 13.4 The calculated arterial oxygen content of Tibetan men and women is profoundly lower than their Andean counterparts measured at ≈4,000 m (data from Beall, 2006), whereas the exhaled NO concentration is markedly higher (recalculated from data reported in Beall *et al.*, 2001).

Because blood flow is a function of the diameter of blood vessels, dilating factors could, in principle, improve the rate of oxygen delivery. Sea-level populations respond to high-altitude hypoxia by narrowing the blood vessels in their lungs, the first point of contact with the circulation. Known as hypoxic pulmonary vasoconstriction, that reflex evolved at sea level to direct blood away from temporarily poorly oxygenated toward better oxygenated parts of the lung. High-altitude hypoxia causes poor

oxygenation of the entire lung and general constriction of blood vessels to the degree that it raises pulmonary blood pressure, often to hypertensive levels (Groves *et al.,* 1993; Ward *et al.,* 2000). In contrast, most Tibetans do not have hypoxic pulmonary vasoconstriction or pulmonary hypertension. This is indicated by essentially normal pulmonary blood flow, as measured by normal or only minimally elevated pulmonary artery pressure (Groves *et al.,* 1993; Hoit *et al.,* 2006). Although there are no studies of paired Tibetan–Andean samples evaluated by the same investigators, a comparison of a Tibetan sample from 4,200 m and an Andean sample from 3,700 m using the same technology reveals a mean pulmonary artery pressure of 31 mmHg for the Tibetan 28%, lower than the mean of 43 mmHg for the Andean (35 mmHg is often considered the upper end of the normal sea-level range) (Antezana *et al.,* 1998; Hoit *et al.,* 2006). Andean highlanders are consistently reported to have pulmonary hypertension (Groves *et al.,* 1993). Thus, pulmonary blood flow is another element of oxygen delivery for which Tibetans differ from Andean highlanders in the direction of greater departure from the ancestral response to acute hypoxia.

A probable reason for the normal pulmonary artery pressure among Tibetans is high levels of the vasodilator nitric oxide (NO) gas synthesized in the lining of the blood vessels. Low-altitude populations acutely exposed to high-altitude down-regulate NO synthesis, a response thought to contribute to hypoxic pulmonary vasoconstriction (Duplain *et al.,* 2000; Busch *et al.,* 2001). In contrast, NO is substantially elevated in the lungs of Tibetan as compared with Andean highlanders and lowlanders at sea level (Fig. 13.4) (Beall *et al.,* 2001). Among Tibetans, higher exhaled NO is associated with higher blood flow through the lungs (Hoit *et al.,* 2006).

Several other lines of evidence highlight the importance of high blood flow for Tibetans. These include greater increase in blood flow after temporary occlusion (Schneider *et al.,* 2001) and higher blood flow to the brain during exercise (Huang *et al.,* 1992) as compared with lowlanders. Pregnant Tibetans increase blood flow to the uterine arteries, increase oxygen delivery to the uterus and placenta more than acutely exposed lowlanders, and give birth to heavier babies (Moore *et al.,* 2001). In contrast, pregnant Andean high-altitude natives increase oxygen delivery to the uterus and placenta by increasing ventilation and oxygen saturation, a response that correlated with giving birth to heavier babies (Moore *et al.,* 1986, 2004). These two means for increasing uteroplacental oxygen delivery during pregnancy occur at a point in the life course where natural selection for improving function could be particularly effective. This is because infant birth weight is associated with infant survival and maternal reproductive success. Generally, Tibetans appear to have relatively high blood flow that may contribute significantly to offsetting their low arterial oxygen content.

A denser capillary network could potentially improve perfusion and oxygen delivery, because each capillary would supply a smaller area of tissue, and oxygen would diffuse a shorter distance. Tibetans (the study sample were Sherpas, an ethnic group that emigrated from Tibet to Nepal ≈500 years ago) who are born and raised at high altitude have higher capillary density in muscles as compared with Andean high-altitude natives, Tibetans born and raised at low altitude, or lowlanders (Fig. 13.5) (Hoppeler *et al.*, 2003). Those findings suggest that another route used particularly by Tibetans to overcome profoundly low arterial oxygen content is a high rate of diffusion. Diffusion could be further enhanced by easier dissociation of oxygen from hemoglobin. However, oxygen dissociation is normal in both Tibetan and Andean populations (Winslow *et al.*, 1981, 1989; Moore *et al.*, 1992).

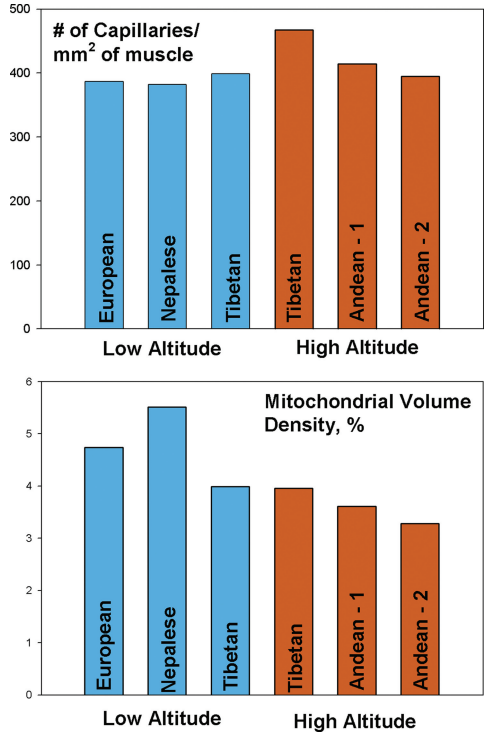

FIGURE 13.5 High-altitude native Tibetans have higher capillary density than their Andean counterparts or populations at low altitude; Tibetan and Andean highlanders both have lower mitochondrial volume than low-altitude populations (data from Hoppeler *et al.*, 1990, 2003; Desplanches *et al.*, 1996; Kayser *et al.*, 1996).

The last potential point of adaptation is at the level of the mitochondrion itself. Acutely exposed lowlanders lose mitochondria in leg muscles during the first 3 weeks at altitude. Similarly, both Tibetan (Sherpas) and Andean high-altitude natives have a lower mitochondrial volume in leg muscle tissue than sea-level natives at sea level (Fig. 13.5) (Hoppeler *et al.*, 2003). However, Tibetans born and raised closer to sea level (at 1,200 m) also have few mitochondria, indicating that, for them, expression of this trait does not require exposure to high altitude. The functional implications of fewer mitochondria are unclear, because overall oxygen-requiring metabolism is not lower. Among Tibetans, a smaller mitochondrial volume somehow supports a relatively larger oxygen consumption, perhaps by higher metabolic efficiency (Kayser *et al.*, 1996; Gelfi *et al.*, 2004; Marconi *et al.*, 2006).

To emphasize the magnitude of these population differences in mean values of healthy Tibetan and Andean high-altitude natives living under the same hypoxic stress, the marked differences at these points in oxygen transport can be quantified further by using a measure of ''effect size,'' calculated by subtracting the Tibetan mean from the Andean mean and dividing by the pooled variance of the samples (Cohen, 1988). An effect size of ≥0.8 is conventionally considered large; it means there is no overlap of ≈48% or more of the observations in the two samples being compared. By this criterion, each of the following contrasts (described above) is large; the higher Tibetan mean for ventilatory traits, the lower Tibetan mean for hematological traits, particularly for hemoglobin for which the mean differences are >2 SD, the higher Tibetan mean for exhaled NO and muscle capillary density, and the lower Tibetan mean values for arterial oxygen level, oxygen saturation, and pulmonary artery pressure (Table 13.1). Together, these large effects at many points in the oxygen delivery cascade are evidence of two different sets of adaptive responses to millennia of residence at high altitude.

ARE THESE FUNCTIONAL ADAPTATIONS HERITABLE?

To evaluate the hypothesis that natural selection accounts for the functional physiological characteristics of Tibetan highlanders relative to Andean highlanders or of highlanders relative to lowlanders, a primary consideration is the presence of heritable variation in the traits under consideration. However, the genetic underpinnings of these quantitative traits are mostly unknown (with the exception of nitric oxide). These traits are also influenced by individual characteristics, including age and sex.

Quantitative genetic techniques can be used to estimate the heritability (h^2), the proportion of total variance in a trait attributable to the genetic relationships among individuals in the population. Theoretical values

for h^2 can range from 0 (no genetic variance) to 1 (all of the variance is genetic), with higher h^2 values implying a greater potential for natural selection. It is calculated by using samples containing biological relatives and comparing the observed similarities in trait values of relatives with expectations based on the proportions of their shared genes. An absence of significant h^2 does not mean there is no genetic influence on these traits; it simply means that no genetic variance is expressed, and therefore there is no potential for natural selection at the time of measurement. Genetic homogeneity could reflect past natural selection. Table 13.1 shows that Tibetan samples generally have higher h^2 and thus greater potential for natural selection on many of the oxygen delivery traits described above. The presence among Tibetan, but not Andean, high-altitude natives of significant h^2 for resting ventilation and oxygen saturation is indirect evidence of population genetic differences, because it reflects the presence of at least two alleles in the Tibetan sample (but not the Andean sample). Interestingly, a sample of people with one high-altitude native Tibetan parent and one low-altitude native Chinese parent was found to have high resting ventilation similar to Tibetans but low HVR, similar to Chinese residents at high altitude (Curran *et al.,* 1997).

Another approach to detecting the genetic bases of these traits, so far applied only in the Andean population, is the use of admixture analysis (Brutsaert *et al.,* 1999, 2003, 2004). This approach takes advantage of the history of Spanish and African migration and intermarriage with the indigenous population in the Andean region and uses a panel of ancestry-informative genetic markers to quantify the proportion of Native American, European, and West African ancestry of each study participant. The contribution of the ''proportion of Native American ancestry,'' called genetic admixture, to variation in HVR has been estimated for samples of low-altitude natives from the Andean region who were acutely exposed to high altitude. A higher proportion of Native American ancestry was associated with lower ventilation during exercise and a lower HVR. However, there was no association of admixture with oxygen saturation upon acute exposure. These results support the hypothesis of distinctive genetic bases to the relatively low ventilation and HVR exhibited by Andean highlanders and support the finding of no heritable variance in oxygen saturation. Like the quantitative genetic approach, genetic admixture studies do not identify any specific genetic locus.

There is evidence for a major gene (an inferred locus whose alleles have a large quantitative effect) on oxygen saturation among Tibetans. Individuals with the inferred autosomal dominant allele average 6–10% higher oxygen saturation than their homozygous counterparts at the same altitude (Beall *et al.,* 2004). Although the specific locus and alleles remain unknown, an individual's genotypic probability can be estimated. In a

TABLE 13.1 Comparisons of Oxygen Transport Traits for Tibetan and Andean High-Altitude Natives Living at Comparable Altitudes Between 3,500 and 4,500 m (Expressed as Effect Size *d*, Percent of Nonoverlapping Observations in the Cited Samples, and as Heritability h^2)

Trait	*d*
Resting ventilation, liters/min	Male, 1.0 Female, 1.1
Tidal volume, ml	Male, 1.1 Female, 0.8
Respiration rate, breaths per minute	Male, −0.2 Female, −0.2
HVR, liters/min per saturation, %	Male, 0.8 Female, 0.8
Oxygen saturation of hemoglobin, %	Male, −0.9 Female, −0.5
Hemoglobin concentration, g/dl	Male, −2.2 and −0.7 Female, −2.4
2,3-Bisphosphoglycerate mutase concentration	Male, −0.7
Erythropoietin concentration, milliunits/ml	Male, −0.2
Exhaled NO, nmHg	Male, 1.2 Female, 1.1
Partial pressure of O_2 in arterial blood, mmHg	Male, −0.8
Mean pulmonary artery pressure, mmHg	Male, −1.3
Pulmonary artery systolic pressure, mmHg	Male and female, −1.3
Calf muscle capillary density, no/mm^2	Male, 1.3
Mitochondrial volume density, %	Male, −0.3

Negative effect sizes reflect lower Tibetan mean values.

Percent Nonoverlap of Observations	h^2	Refs.
≈55% (*n* = 320 Tibetan, 542 Andean)	Tibetan, 0.32 Andean, not significant	Beall et al. (1997a)
≈55% (*n* = 320 Tibetan, 542 Andean)	No data	Beall *et al.* (1997a)
≈15% (*n* = 320 Tibetan, 542 Andean)	No data	Beall *et al.* (1997a)
≈47% (*n* = 320 Tibetan, 542 Andean)	Tibetan, 0.35 Andean, 0.22	Beall *et al.* (1997a)
≈47%, ≈33% (*n* = 354 Tibetan, 381 Andean)	Tibetan, 0.35 Andean, NS	Beall *et al.* (1997b, 1999)
>82%; ≈43% (*n* = 136 Tibetan, 174 Andean)	Tibetan, 0.64 Andean, 0.89	Winslow *et al.* (1989), Beall *et al.* (1998)
≈43% (*n* = 30 Tibetan, 30 Andean)	No data	Winslow *et al.* (1981, 1989)
≈15% (*n* = 30 Tibetan, 29 Andean)	No data	Winslow *et al.* (1989)
≈55% (*n* = 105 Tibetan, 144 Andean)	No data	Beall *et al.* (2001)
≈47% (*n* = 10 Tibetan, 20 Andean)	No data	Winslow *et al.* (1989), Zhuang *et al.* (1996)
≈65% (*n* = 5 Tibetan, 11 Andean)	No data	Groves et al. (1993)
≈65% (*n* = 57 Tibetan, 14 Andean)	No data	Antezana et al. (1998), Hoit et al. (2005)
≈65% (*n* = 5 Tibetan, 10 Andean)	No data	Kayser *et al.* (1991), Desplanches *et al.* (1993, 1996)
≈21% (*n* = 5 Tibetan, 10 Andean)	No data	Kayser *et al.* (1991), Desplanches *et al.* (1993, 1996)

sample of nearly 700 women residing at ≈4,000 m, those estimated with high probability to be homozygous or heterozygous for the high oxygen saturation allele had more surviving children (3.7 as compared with 1.6 for women estimated to be homozygous for the low saturation allele), primarily because of lower infant mortality. Using these observations and assigning a Darwinian fitness coefficient of 1.0 to the women with the high-saturation genotype, the relative Darwinian fitness of women with the low-saturation genotype was only 0.44. For comparison, in the classic case of an environment with endemic falciparum malaria, a Darwinian fitness coefficient of 0.66 applies to homozygotes for normal hemoglobin A (Firschein, 1961). High-altitude hypoxia may be an even stronger agent of natural selection than falciparum malaria. These findings suggest that the frequency of the high saturation allele may be increasing rapidly in the Tibetan population.

With respect to identifying specific genetic loci contributing to high-altitude functional adaptation, efforts so far have not been successful. They have mainly used the strategy of identifying plausible candidate genes and examining them for distinctive alleles or allele frequencies. Unusual genetic variants or allele frequencies have not been detected in the mitochondrial genome of Tibetans (Torroni *et al.,* 1994). A plausible candidate is the gene for myoglobin, a protein that contributes to oxygen storage and diffusion in skeletal and cardiac muscle. A screen of the myoglobin gene exon 2 for novel variants or deviations from Hardy–Weinberg equilibrium in a sample of Tibetans found little that was distinctive, apart perhaps from a higher frequency of one variant as compared with a U.S. sea-level population (Moore *et al.,* 2002). Myoglobin gene expression was reported to be very high among Tibetans, regardless of altitude of residence (Gelfi *et al.,* 2004).

Candidate genes for pulmonary vasodilators have been examined, based on the reasoning that alleles for high levels could improve blood flow and oxygen diffusion in the lung (Wilkins *et al.,* 2002). One study reported a high frequency of a ''wild-type'' endothelial NO synthase (one of three enzymes catalyzing the synthesis of NO) haplotype in a Tibetan (Sherpa) sample as compared with a low-altitude sample (Droma *et al.,* 2006). That study reported a lower level of circulating NO metabolites in the serum of the high-altitude sample. That finding is contrary to expectation based on the finding of high exhaled NO among Tibetans.

Another candidate gene is the transcription factor hypoxia-inducible factor 1 (HIF1) often called the ''master regulator'' of oxygen homeostasis, because it induces ≥70 genes that respond to hypoxia (Semenza, 2000, 2002, 2004). An investigation of polymorphisms in the HIF1A gene of Tibetans (Sherpas) found a dinucleotide repeat in 20 Tibetans that was not found in 30 Japanese lowlander controls (Suzuki *et al.,* 2003). However, no

phenotypic data were reported. On the one hand, it seems unlikely that a transcription factor regulating the induction of dozens of genes accounts for the Tibetan–Andean differences, because a change of this sort would have many downstream effects. Perhaps more likely is genetic variation in one or more of the ≥70 genes induced by HIF1 or in the biochemical pathways in which they participate. On the other hand, considering that the Tibetan–Andean differences involve many traits and apparently have accumulated in a relatively short time of ≤25,000 years, perhaps a change in a regulatory mechanism is the underlying mechanism.

In summary, measures of oxygen transport reveal that Andean and Tibetan populations have large quantitative differences in numerous physiological and molecular traits involved in oxygen delivery. The hypothesis is that evolutionary processes have tinkered differently in the two founding populations and their descendents, with the result that the two populations followed different routes to the same functional outcome of successful oxygen delivery. That conclusion will remain tentative, however, until the responsible genes are identified.

ACKNOWLEDGMENTS

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14

On the Origin and Evolutionary Diversification of Beetle Horns

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Many scarab beetles produce rigid projections from the body called horns. The exaggerated sizes of these structures and the staggering diversity of their forms have impressed biologists for centuries. Recent comparative studies using DNA sequence-based phylogenies have begun to reconstruct the historical patterns of beetle horn evolution. At the same time, developmental genetic experiments have begun to elucidate how beetle horns grow and how horn growth is modulated in response to environmental variables, such as nutrition. We bring together these two perspectives to show that they converge on very similar conclusions regarding beetle evolution. Horns do not appear to be difficult structures to gain or lose, and they can diverge both dramatically and rapidly in form. Although much of this work is still preliminary, we use available information to propose a conceptual developmental model for the major trajectories of beetle horn evolution. We illustrate putative mechanisms underlying the evolutionary origin of horns and the evolution of horn location, shape, allometry, and dimorphism.

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The origin and subsequent evolutionary diversification of complex morphological structures have long puzzled biologists (Darwin, 1859b; Goldschmidt, 1940; Mayr, 1960). These traits may arise suddenly, and their size and complexity, as well as their lack of visible homology with existing structures (novelty), were thought for many years to be incompatible with traditional gradualistic views of genetic variation, selection, and evolution (Müller and Wagner, 1991). Modern analytical techniques, including phylogenetic analyses and molecular genetics, have greatly improved our understanding of how complex structures arise, revealing in several instances how subtle perturbations to existing developmental mechanisms can generate substantial and unprecedented changes in animal form (Nitecki, 1990; Müller and Wagner, 1991). These studies demonstrate unequivocally that both novelty and complexity can arise from simple changes to development, and they illuminate how an understanding of development can inform studies of character evolution. We illustrate this approach using the example of beetle horns, skeletal outgrowths that function as weapons in intraspecific combat.

Beetles with horns include some of the most magnificent and bizarre organisms alive today. The sizes of these horns relative to the sizes of the beetles that bear them can dwarf even the most extreme antlers of ungulates, and the diversity of horn forms is breathtaking. Darwin used beetle horns when he first described sexual selection (Darwin, 1871) and Teissier and Huxley used beetle horns when they first described the concept of relative growth and allometry (Huxley, 1932; Teissier, 1935).

How did the first beetle horns arise? And once present, how were these structures modified so dramatically in form? In this chapter, we approach these questions from two vantages, comparative phylogenetic studies of horn evolution and developmental studies of the regulation of horn growth, and show that these disparate biological perspectives converge on the same basic conclusions regarding horn evolution: beetle horns do not appear to be difficult structures to gain or lose, and they appear capable of rapid and radical changes in form. We end this chapter with a conceptual model for how beetle horns evolve. Specifically, we identify three developmental mechanisms that are now thought to underlie the principle trajectories of beetle horn evolution. This integration of perspectives comprises an important step in our attempts to elucidate the myriad ways in which these exaggerated structures have radiated in form. It also illustrates the more general theme of this colloquium: that "Darwinian" processes of selection, combined with subtle genetic variations in basic developmental processes, can account for the origin, and the subsequent diversification of even the most extreme animal structures.

A NATURAL HISTORY OF BEETLES WITH HORNS

Beetle horns are weapons: they are used in combat between rival males over access to females (Eberhard, 1978; Iguchi, 2001; Hongo, 2003). These contests tend to occur in physically restricted substrates, such as on branches or bamboo shoots or more commonly, inside the confines of tunnels. Tunnels can be the hollowed-out stems of plants, such as sugar cane, or burrows excavated into the soil. Regardless, long horns aid males in these battles over reproductive access to females [males with the longest horns win (Rasmussen, 1994; Pomfret and Knell, 2006)], and this can translate into higher fertilization success for these long-horned individuals (Hunt and Simmons, 2001). Thus, beetle horns are conspicuous morphological structures of known functional significance, and the more than a century of interest and observation of these animals, combined with the recent behavioral studies listed above, provide a rich ecological context for the historical and developmental studies we are about to describe.

Beetles with horns are primarily confined to the scarab superfamily (Coleoptera: Scarabaeoidea). Extant scarabs are diverse and successful, and include the bess beetles (Passalidae), stag beetles (Lucanidae), dung beetles (Scarabaeinae), flower beetles (Cetoniinae), May and June beetles (Melolonthinae), the chafers (Rutelinae), and the rhinoceros beetles (Dynastinae) (Ratcliffe and Jameson, 2006). The earliest scarabs are thought to have been robust animals with bodies adapted to a lifestyle of burrowing (Iablokoff-Khnzorian, 1977; Crowson, 1981; Scholtz and Chown, 1995; Krell, 2006), and they may have excavated tunnels into the soil beneath dinosaurs (Scholtz and Chown, 1995) or the stems of plants (Lameere, 1904).

It is currently not known whether these ancestral scarabs had horns. Despite considerable effort in reconstructing the early history of the scarabs (Iablokoff-Khnzorian, 1977; Browne and Scholtz, 1995, 1999; Kohlmann, 2006; Smith *et al.,* 2006), little attention has been given to the question of whether or not these animals had horns. The long-standing view has been that these ancestors probably did not have horns. Despite the literally thousands of scarab species with exaggerated horn morphologies, the majority of extant scarabs are hornless. In addition, the family and subfamilies most predominated by species with large horns (Geotrupidae, Scarabaeinae, Dynastinae) are widely separated within the scarabs as a whole (Fig. 14.1), leading to the view that scarab horns must have evolved independently many different times (Arrow, 1951).

However, almost all of the extant subfamilies of scarabs possess rudimentary horns, and many of the predominantly ''hornless'' groups (e.g., Cetoniinae, Rutelinae) contain at least a few species with dramatic horns [e.g., *Theodosia viridaurata* (Cetoniinae), *Ceroplophana modiglianii* (Rutelinae)]. Where present, these horns generally occur in the same basic

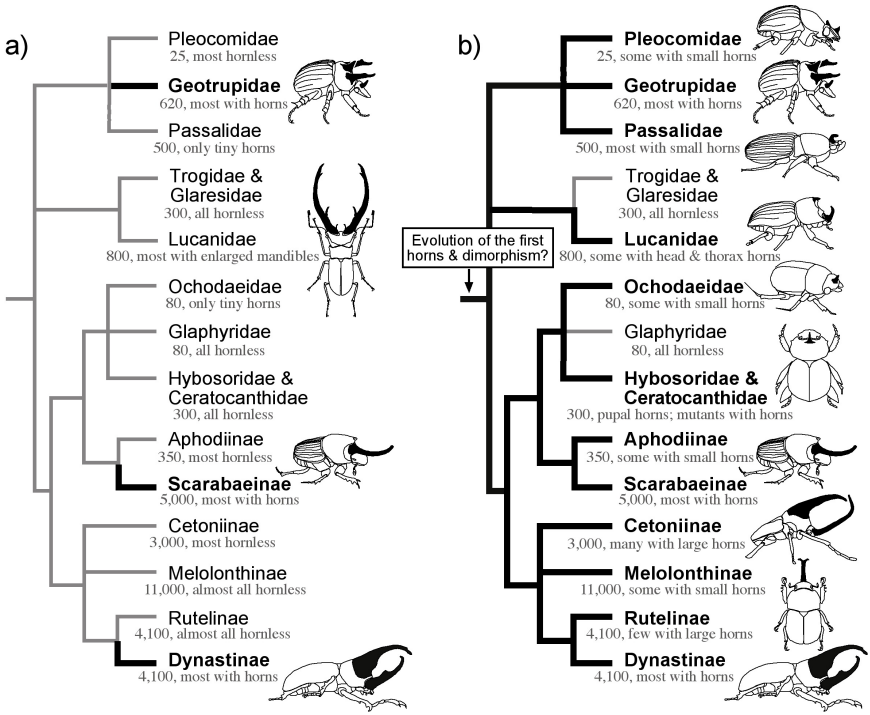

FIGURE 14.1 Partial phylogeny for the families and subfamilies of scarab beetles (Coleoptera: Scarabaoidea) illustrating two alternative reconstructions of the evolution of horns. The majority of species with exaggerated horns are concentrated within three distantly related clades (Geotrupidae, Scarabaeinae, Dynastinae) that collectively represent only 20% of extant scarab species. For this reason, horns are thought to have arisen multiple times independently within the superfamily (thick black branches in **a**), leading Darwin and others to speculate on the "special tendency" of the scarabs towards evolution of enlarged horns. However, all but three of the included clades contain either rudimentary horns, at least a few genera or species with enlarged horns, pupal horns, or mutant individuals with horns. When these horns occur, they generally resemble the horns of "traditional" horned species and they exhibit similar patterns of sexual dimorphism and male dimorphism in their expression. Based on these observations we recently proposed that the ancestor to the scarabs may have had both horns, and mechanisms for suppressing horn growth (horn dimorphism) (**b**). Tree topology from Smith *et al.* (2006). Species numbers and taxon descriptions from Ratcliffe and Jameson (2006) http://www-museum.unl.edu/research/entomology/Guide/guide-introduction/guideintro.html.

body regions as the horns of more "typical" horned scarabs (the anterior surface of the thoracic pronotum, which is a body region that was already universally enlarged in scarabs and thought to be an adaptation to a burrowing lifestyle, and the dorsal surface of the head). They also tend to have the same basic forms, and they nearly always exhibit the same patterns of horn "dimorphism" (females, small males lack the horns) characteristic of more typical horned scarabs. These observations led us recently to propose that the earliest scarabs may have had horns, as well as the developmental capacity to suppress horn growth facultatively (i.e., horn dimorphism; Fig. 14.1b) (Emlen *et al.*, 2006).

This alternative view of early horn evolution raises the exciting possibility that all descendant scarabs inherited the developmental capacity both to produce and to suppress horns. Extant species lacking horns, in this case, would be secondarily hornless. If true, it might take only relatively subtle genetic modifications to their development to reverse the suppression of horn growth, perhaps accounting for the "irregular" appearances of horned taxa nested within clades of otherwise hornless scarabs. It also could account for the surprising occurrence of mutant horned individuals, which sporadically appear within hornless taxa (Ziani, 1995; Ballerio, 1999).

Resolving the horn morphologies of the earliest scarabs may take some time. New scarab fossils are discovered each year (Krell, 2006), and these may reveal the shapes of these Jurassic beetles. Certainly, a great deal of information will come from the ongoing studies of horn development (described below). Comparing these mechanisms in different scarab subfamilies will provide important clues as to whether these weapons shared a common origin in their distant past. However, rooting the scarab tree is not necessary for drawing important conclusions about historical patterns of horn evolution. All phylogenies for the scarabs agree that their history was replete with a multitude of gains of horns. Regardless of whether these events represent a series of independent gains of new horns, or recurrent re-gains of ancestral horns, or both, the patterns of horn evolution that emerge from comparative studies of scarab morphology are indisputable. Two conclusions are especially clear.

Conclusion 1: Horns Are Easy to Gain and Lose

Modern phylogenetic reconstructions of horn evolution reveal a history rich with gains and losses of these structures. One study of 48 species from the dung beetle genus *Onthophagus* (a mere 2% of this genus and <0.1% of the scarabs) concluded that there had been nine losses of one horn type and at least 15 gains of additional horn types, together contributing to over 25 changes in the physical location of horns (e.g., head

versus thorax; see Emlen *et al.,* 2005b). Another study of 45 genera sampled across the subfamily Scarabaeinae suggested that there had been at least three losses and eight gains of horns (Emlen and Philips, 2006). Inferring ancestor states becomes problematic in these cases (Emlen *et al.,* 2005a; Moczek *et al.,* 2006a), but all studies agree that beetle horns have arisen and been lost many, many times.

Conclusion 2: Beetle Horns Change Rapidly and Dramatically in Form

One recent attempt to characterize the vast diversity of scarab horn morphologies revealed the following four principal trajectories of horn evolution (Emlen *et al.,* 2006).

Horns vary in their physical location **(Fig. 14.2).** There are five major regions of the body from which the horns can extend: three dorsal segments of the head (vertex, frons, clypeus) and the center or sides of the thoracic pronotum. Horns appear to be gained or lost independently at each of these body regions, and species can have all possible combinations of these horn types (Emlen *et al.,* 2005b). Within any of these regions, horn location also may change; for example, when a single central head horn splits into a lateral pair of horns (Fig. 14.2 *Lower*).

Horns vary in shape **(Fig. 14.3).** Even closely related species often differ extensively in horn shape, and phylogenetic studies suggest that there have been multiple and repeated transformations in horn shape (Emlen *et al.,* 2005b). Common changes in shape appear to include the splitting of a single horn into two or even three horns accompanied by changes in horn location (see above), the addition of forks or branches to horns, and the transition from straight to curved horns.

Horns vary in their allometry (the scaling of horn lengths with among individual variation in body size). Allometric relationships reflect both the relative size of the horn and the developmental coupling of horn growth with among-individual variation in body size (see below). Horn allometries can diverge rapidly in the wild (Kawano, 2002; Moczek *et al.,* 2002; Rowland, 2003) and under selection in the laboratory (Emlen, 1996), and the slopes, intercepts, and even the shapes of these allometries differ markedly among extant taxa (Fig. 14.4).

Horns vary in their dimorphism. Scarab species differ in the presence/absence of horn dimorphism and even in the nature of their dimorphism (Eberhard and Gutierrez, 1991; Emlen *et al.,* 2005a; Moczek, 2006b) (Fig. 14.5). Two forms of dimorphism are widespread in these beetles,

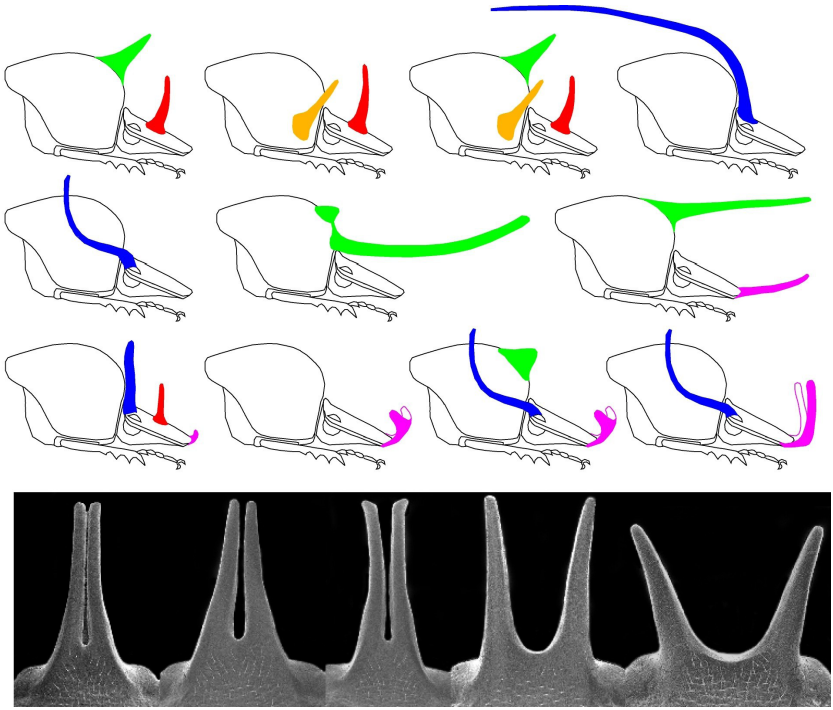

FIGURE 14.2 Evolution of horn location. Head and thorax shown for 11 species of *Onthophagus* (Coleoptera: Scarabaeinae). Horns can extend from at least five body regions—the dorsal surface of the front, middle, or base of the head, corresponding to the clypeus, frons, and vertex head segments, respectively, and the center or sides of the thoracic pronotum. Top row: *O. sagittarius* (female); *O. lanista*; *O. pentacanthus*; *O. raffrayi*. Middle row: *O. taurus*; *O. nigriventris*; *O. brooksi*; Bottom row: *O. demarzi*; *O. sharpi*; *O. praecellens*; *O. andersoni*. Bottom: within-species variation in horn shape. Head horns of five males sampled from a single population of an unidentified *Onthophagus* species from Africa, illustrating a transition between a single horn at the center of the head and a pair of horns at the sides of the head.

male dimorphism and sexual dimorphism. In most species with male dimorphism, males smaller than a critical, or threshold, body size dispense with horn production, resulting in horn lengths that scale according to a very different relationship than in large males. Females also often dispense with horn production, sometimes entirely, as in species where females never produce horns. In other cases females do produce the horn but the relative sizes of female horns differs from that of the males. Horn dimorphism also appears to have been gained and lost repeatedly in the

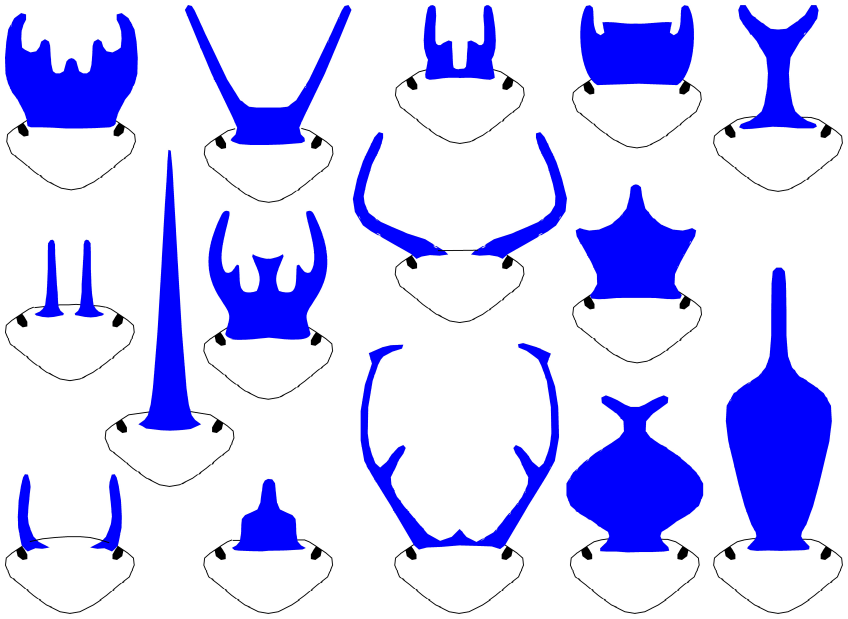

FIGURE 14.3 Evolution of horn shape. Head horns shown for 15 species of *Onthophagus*. Common changes in horn shape include the splitting of a single horn into two or more horns, the addition of forks or branches, and transitions from straight to curved. Top row: *O. victoriensis*; *O. rupicapra*; *O. sugillatus*; *O. capella*; *O. ouratta*. Middle row: *O. aeruginosus*; *O. pentacanthus*; *O. fuliginosus*; *O. taurus*; *O. leai*. Bottom row: *O. gazella*; *O. nuchicorn*is; *O. rangifer*, and two *Paleonthophagus* species. Specied morphologies for *O. victoriensis, O. rupicapra, O. sugillatus, O. capella, O. ourata, O. pentacanthus, O. fuliginosus* and *O. leai* were derived from Matthews (1972); *Paleonthophagus* species were derived from Fig. 11 of Kabakov (1990).

history of the scarabs. One study of 31 species of the genus *Onthophagus* revealed at least 20 reversals in the presence/absence of horn dimorphism (Emlen *et al.*, 2005a).

Combined, these four trajectories account for most of the extant diversity in horn forms. But identifying these trajectories does a great deal more than describe taxonomic patterns; it also provides an essential first step toward elucidating the underlying mechanisms responsible for generating this diversity of animal forms. Only by identifying biologically meaningful trajectories of morphological change is it possible to begin to consider how these changes are generated and which developmental and physiological mechanisms are involved. In the following sections, we briefly describe three mechanisms now thought to be involved in the development of a

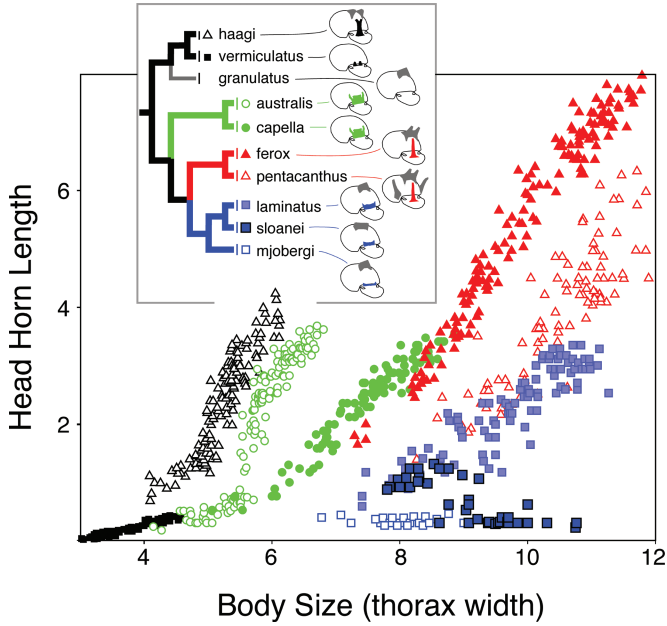

FIGURE 14.4 Evolution of horn allometry. Horn length–body size scaling relationships shown for the head horns of nine Australian species of *Onthophagus*, representing a well-supported monophyletic clade within the phylogeny of Emlen *et al.* (2005b).

beetle horn, and we illustrate how each of these mechanisms could contribute to the above trajectories of horn evolution.

THREE STEPS TO BUILDING A BEETLE HORN

Step 1: Making an Axis of Outgrowth

Beetle horns form as long tubes, localized regions of epidermal tissue that undergo a burst of proliferation at the end of the larval period, just before pupation (Fig. 14.6) (Emlen and Nijhout, 1999). As these horn cells proliferate, they fold in on themselves to produce a compact disc of epidermal tissue that unfurls to its full length during the pupal molt. In these respects, beetle horns develop in a way very similar to the traditional appendages in beetles and other insects (e.g., wings or legs), and it now appears that similar mechanisms may be involved.

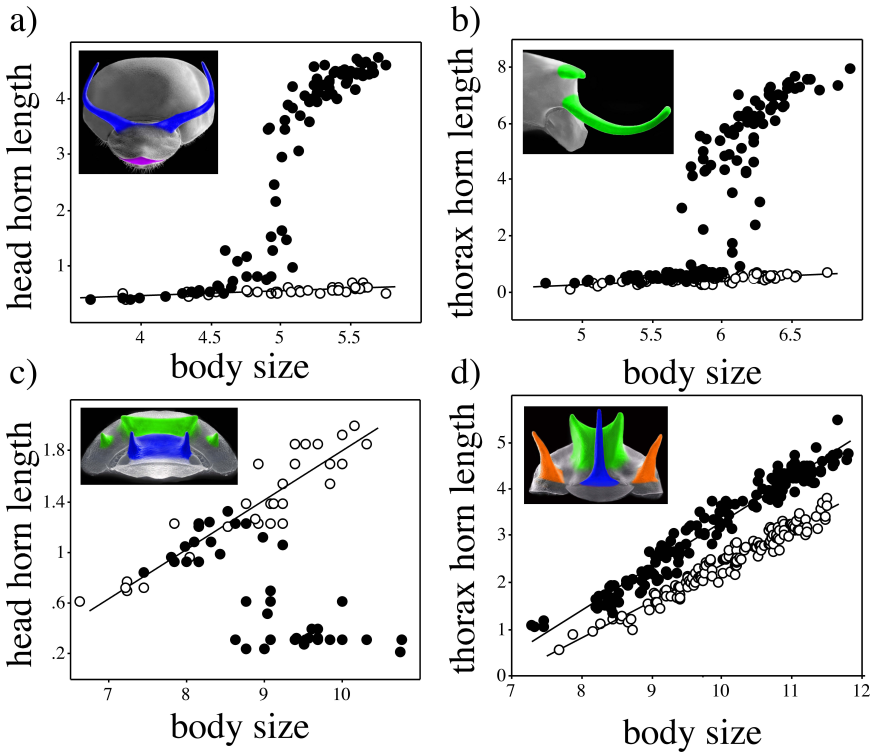

FIGURE 14.5 Evolution of horn dimorphism. Species differ in the presence and nature of dimorphism in horn expression (males = closed circles; females = open circles). In *Onthophagus taurus* (**a**) and *O. nigriventris* (**b**), large adult males produce either a head (*O. taurus*) or a thoracic (*O. nigriventris*) horn that is not present in small males (male dimorphism) or females (sexual dimorphism). In *O. sloanei* (**c**) the patterns of horn dimorphism are reversed: small male and female adults produce a head horn that is not present in large males. In *O. pentacanthus* (**d**) all individuals of both sexes produce the horn, but the relative sizes of these horns differ between males and females. Insets are SEM photographs of the head and thorax of male (**a,b,d**) or female (**c**) individuals, with horns colorized to indicate their physical location. Reprinted with permission from Emlen et al. (2005a).

The major adult structures in metamorphic insects form from isolated "pockets" of cells called imaginal discs, analogous in many ways to the limb buds of vertebrates (Truman and Riddiford, 2002). In *Drosophila*, these discs have been especially thoroughly studied (Kojima, 2004; Weihe *et al.*, 2005), but discs occur in all metamorphic insects, and arguably, disc-like patterns of growth occur in nonmetamorphic insects and other

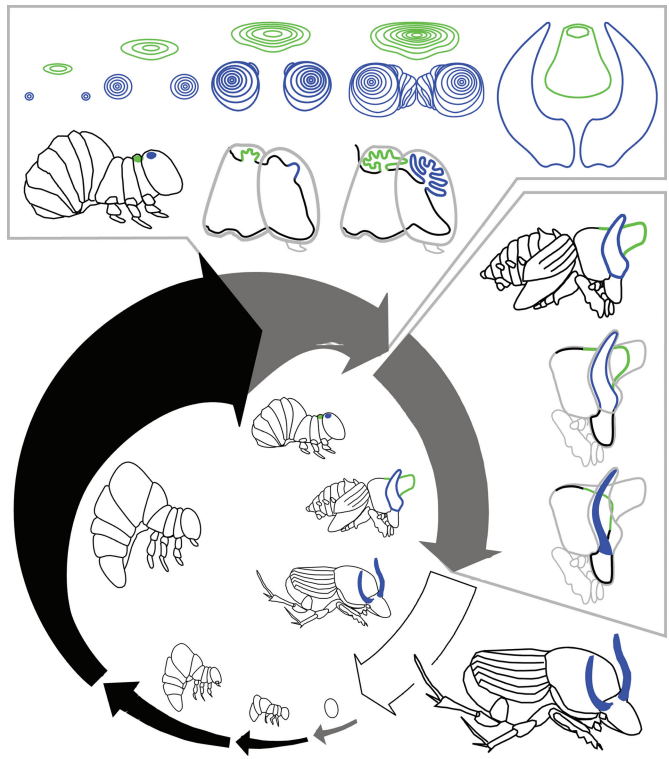

FIGURE 14.6 Development of beetle horns. Life cycle shown for the dung beetle *Onthophagus taurus*. After hatching, beetles pass through three larval instars before molting first into a pupa and then into an adult. Black arrows indicate feeding periods; gray arrows indicate nonfeeding periods. Arrow thickness approximates overall animal body size, and gaps between arrows indicate molting events. The final (third) larval instar can be divided into a feeding period and a nonfeeding prepupal period. Drawings inside the arrows illustrate egg, first through third larval instars, prepupa, pupa, and adult. Horn development can be divided into two stages: a period of horn growth when horn cell proliferation occurs, and a period of horn remodeling. The top box shows horn growth. Front view of thoracic and head horn discs are shown, along with two profile views of the prepupal head and thorax during this stage. The side box shows horn remodeling. The drawings illustrate the profile of a large male just after pupation and the head and thorax of the same male at two later stages during the pupal period. The head horns are remodeled slightly, to form a pair of curved and slender adult horns. The pupal thoracic horn is removed completely, and is not present in adults. Close-up profiles of prepupa and pupa are adapted from figure 2 of Moczek (2006a).

arthropods as well (Truman and Riddiford, 2002). These clusters of cells behave as remarkably autonomous units, and even when removed (*in vitro*) or transplanted, these discs are able to complete most of their growth and development, the result of a complex series of molecular and genetic interactions that unfolds within the disc (''patterning''; see Serrano and O'Farrell, 1997; Held, 2002; Johnston and Gallant, 2002; Kojima, 2004; Weihe *et al.,* 2005).

The full process of appendage patterning can be functionally subdivided into at least four hierarchical and relatively dissociable modules, each entailing the deployment of a specific and largely self-contained network of genetic interactions [specification of appendage identity (leg, antenna, wing, etc.), formation of an axis of outgrowth (proximal–distal), subdivision of the appendage into segments, and localization and growth of sensory structures, bristles, and hairs (Carroll *et al.,* 2001; Held, 2002)]. The portion of this patterning process that is most relevant to beetle horn development is the formation of an axis of outgrowth. Beetle horns do not have segments or joints, but they do have an axis of outgrowth. It now appears that horns form by deploying the outgrowth portion of the patterning cascade (Moczek and Nagy, 2005; Emlen *et al.,* 2006; Moczek, 2006a,b). We do not describe the details of this pathway here (for reviews, see Serrano and O'Farrell, 1997; Johnston and Gallant, 2002; Kojima, 2004; Weihe *et al.,* 2005). Instead, we highlight a few properties of this pathway that are especially relevant for understanding how beetle horns develop.

In an insect appendage, such as a *Drosophila* leg, the expression of patterning genes is confined to specific domains within the imaginal disc. These expression domains overlap partially, but not completely, with the domains of expression of other genes in the network, and the result is a spatially explicit mosaic of molecular signals defined by the boundaries of expression of the patterning genes. Cells physically located at the intersection of two of these boundaries, because of their position, come into contact with high concentrations of several different signals, including proteins of the patterning genes *hedgehog* (*hh*), *wingless* (*wg*), and *decapentaplegic* (*dpp*), and this critical combination of molecular signals causes these cells to become active organizers of the rest of the disc. These focal cells will give rise to the eventual distal/outermost tip of the new appendage.

Once their fate has been established, these focal cells begin expressing a new suite of patterning genes. The proteins of many of these genes diffuse outwards into the surrounding cells of the disc, activating additional tiers of patterning gene expression. This process both stimulates and coordinates cell proliferation within the disc such that there is a burst of localized growth concentrated around the focal cells. The result is a folded tube of epidermis that will subsequently unfurl to form the appendage.

Localized activation of this portion of the patterning pathway stimulates and coordinates the formation of a new body outgrowth. These molecular interactions define the precise location of a structure (which cells will form the distal tip of the structure) and the signals released from these focal cells direct the subsequent behavior of neighboring cells.

Like the patterning process as a whole, the outgrowth portion of the pathway is a cascade of molecular interactions that once started, unfolds to completion relatively autonomously. This means that exposing cells to the appropriate combination of signals can activate the entire module of the patterning cascade, and result in the formation of a complete (and new) body outgrowth. For example, juxtaposition of *wg* and *dpp* signals in an inappropriate region of a developing *Drosophila* wing disc initiates formation of a second axis of outgrowth: a new distal tip that subsequently generates a new wing (Campbell *et al.,* 1993; Zecca *et al.,* 1995). This results in the formation of a bifurcated double wing blade, one wing blade that is the default outgrowth, and a second wing blade that is an aberrant outgrowth generated by activating this pathway in a second region of the disc. Similar juxtaposition of these same two signals in a *Drosophila* leg disc can generate a second fully formed leg attached to the original leg, again resulting in a bifurcated final structure (Diaz-Benjumea *et al.,* 1994; Gibson and Schubiger, 1999). Although these outgrowths are generated artificially in the laboratory, they beautifully illustrate the autonomous property of this pathway, and the potential for this pathway to underlie the evolution of novel morphological structures.

Although the molecular details of this process have been especially well studied in *Drosophila* leg discs, the basic elements of this outgrowth portion of the patterning pathway appear to be highly conserved across different imaginal discs within a species and across taxa; indeed, all arthropod body outgrowths that have been studied to date appear to use some form of this process in their development (e.g., Panganiban *et al.,* 1994; Jockusch *et al.,* 2004). Thus, the outgrowth portion of the patterning pathway is an evolutionarily conserved developmental module that leads to the formation of body outgrowths in diverse taxa, including horns in beetles.

All evidence to date suggests that beetle horns form their axis of outgrowth using this same basic patterning pathway. Eight patterning genes are already known to be expressed in horn discs during the period of disc cell proliferation, and most (but interestingly, not all) of these have domains of expression consistent with their putative role in the formation of the axis of outgrowth [dung beetle (*Onthophagus*) horns: *wingless*, *decapentaplegic*, *distal-less*, *daschshund*, *aristaless*, *epidermal growth factor receptor*, *homothorax*, *extradenticle* (Moczek and Nagy, 2005; Moczek, 2006a; Moczek *et al.,* 2006b; and L.C.L. and D.J.E., unpublished data); rhinoceros beetle (Dynastinae) horns: *wingless*, *decapentaplegic* (L.C.L. and

D.J.E. unpublished data)]. Ongoing research involves experiments that test for functional roles for these genes (e.g., by knocking down transcript abundance, using RNA interference methods), and more comprehensive examinations of the patterns of expression of these genes in individuals that differ in horn size and in species that differ in horn form. Future studies are likely to resolve this process in greater detail, but it is probably safe to conclude that the first stage of building a beetle horn involves the deployment of the outgrowth module of the appendage patterning process.

Step 2: Modulation of Horn Growth in Response to Nutrition

The patterning of insect imaginal discs is not the whole story. Anyone who has reared insects in captivity knows that trait sizes are almost always phenotypically plastic. In particular, they are sensitive to nutrition. Somehow, the basic patterning and growth of structures must be modified in response to the conditions animals encounter as they develop, including and especially the larval nutritional environment.

Beetle horn growth depends critically on larval access to nutrition (Emlen, 1994; Iguchi, 1998; Moczek and Emlen, 1999; Hunt and Simmons, 2000; Karino *et al.,* 2004). Both horn size and body size are sensitive to variation in nutrition, with the consequence that there is a coupling of the amount of horn growth with overall body size. Iterated across a number of different individuals developing under a range of nutritive conditions and environments, the result of this phenotypic plasticity is allometry, the scaling of body parts with body size (Fig. 14.7). This highlights an important, but slightly counterintuitive point: nutrition-dependent phenotypic plasticity and allometry are related. In insects at least, they both result from physiological mechanisms that modulate the amount of trait growth in response to nutrition (Stern and Emlen, 1999; Emlen and Nijhout, 2000; Emlen and Allen, 2004).

We illustrate this relationship with data from a diet-manipulation experiment, in which individuals from a number of different maternal lines were divided among either a high (large food amount) or a low (reduced food amount) nutrition environment. Larvae given poor nutrition emerged into adults with small body sizes that had short horns and legs and tiny wings (Fig. 14.7, closed circles). From the same families, siblings given large food amounts matured into adults with much larger body sizes that had much longer horns, legs, and wings (open circles in Fig. 14.7). Each of these traits is phenotypically plastic, because trait size is sensitive to the larval nutritional environment. In all cases, the magnitude of the plastic developmental response is reflected in the resulting population-level trait size versus body size allometries. Traits that

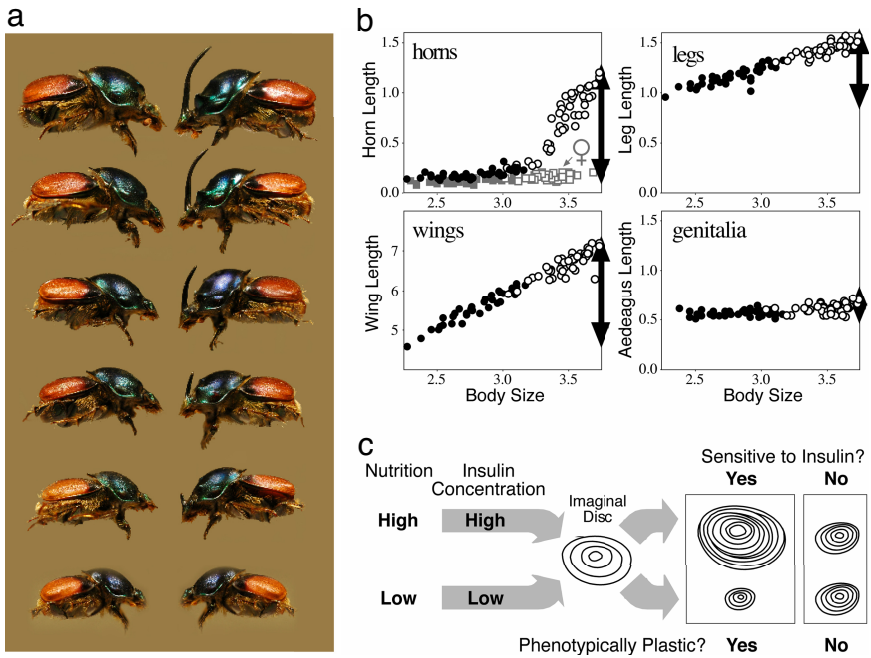

FIGURE 14.7 Nutrition-dependent phenotypic plasticity and allometry in insects. (**a**) Female (left) and male (right) *Proagoderus* (*Onthophagus*) *lanista*, showing among-individual variation in body size and, in males, horn size. (**b**) Scaling relationships (allometries) for four morphological traits in the beetle *O. acuminatus*. Individuals reared with access to large food amounts (high nutrition) (open symbols) emerged at larger adult body sizes than full-sibling individuals reared with smaller food amounts (low nutrition) (closed symbols). Traits differed in how sensitive (plastic) their growth was to this variation in nutrition. Male horns were the most sensitive, and horn lengths were >10-fold longer in the largest individuals than they were in the smallest individuals (females of this species do not produce enlarged horns, and the height of the corresponding head region is indicated by the gray squares). Leg and wing development was also sensitive to nutrition, but legs were less plastic than wings or horns. Male genitalia were almost entirely insensitive to nutrition, and the size of the aedeagus was largely body size invariant. Horns, legs, and genitalia are plotted on the same scale to illustrate the relative plasticity (horns, legs) or canalization (male genitalia, female horns) of their development. Wings were much larger and are shown on their own scale. In all cases, the degree of plasticity/canalization (black arrows) is reflected in the steepness of the trait size–body size allometries. (**c**) Model for one developmental mechanism of allometry in insects. Larval nutritional state is reflected in circulating levels of insulins (and growth factors; data not shown), which modulate the rate of growth of each of the trait imaginal discs. Traits whose disc cells are sensitive to these signals exhibit greater nutrition-dependent phenotypic plasticity and steeper allometry slopes than other traits whose disc cells are less sensitive to these signals.

are exquisitely sensitive to nutrition, such as horns, have the steepest allometry slopes; traits that are less sensitive to nutrition, such as legs, have shallower allometry slopes. Genitalia in these beetles are essentially not plastic at all (their growth is *in*sensitive to larval nutrition), which is reflected in their respective trait allometry (Fig. 14.7*b*). Thus, traits vary in their sensitivity to the nutrition environment (plasticity), which is manifest across individuals as trait differences in allometry.

Any developmental mechanism of allometry is likely to involve whole-animal circulating signals whose levels (*i*) are sensitive to larval nutrition and (*ii*) modulate the growth of the different imaginal discs in accordance with the actual nutritional environment experienced by a larva. Several physiological pathways meet these criteria (reviewed in Emlen and Allen, 2004; Shingleton *et al.*, 2007), and we briefly describe the best studied of these, the insulin receptor (InR) pathway.

Cell proliferation requires high levels of protein synthesis, and in both insects and vertebrates, this process is regulated by the InR pathway (Weinkove and Leevers, 2000; Johnston and Gallant, 2002). In insects, insulin-like peptides secreted primarily by the brain, and probably in cooperation with growth factors secreted by the fat bodies, act as whole-animal circulating signals. When these signals reach the imaginal discs, they bind to InRs and activate a signal transduction cascade that controls the rate of cell proliferation within that disc (Weinkove and Leevers, 2000; Brogiolo *et al.*, 2001; Claeys *et al.*, 2002). Both insulin and growth factor signal levels are sensitive to larval nutrition, and concentrations of these signals affect the rate of cell proliferation in the imaginal discs (Kawamura *et al.*, 1999; Goberdhan and Wilson, 2002; Ikeya *et al.*, 2002). Thus, cell proliferation should occur at a faster rate in large well-fed individuals, increasing the sizes of their traits relative to those of smaller or poorly fed individuals. Recent evidence suggests that insulin and the InR pathway comprise at least one of the developmental mechanisms modulating the amount of trait growth in response to nutrition in insects (Goberdhan and Wilson, 2002; Nijhout, 2003; Shingleton *et al.*, 2005).

Understanding how growing insects respond to variations in their nutritional environment has been a focus of considerable recent research, and several important patterns have emerged from these studies (reviewed in Edgar, 2006; Shingleton *et al.*, 2007). Insects store nutrients in dispersed organs collectively called fat bodies, which may act as nutrient sensors that signal to the brain and other tissues information pertaining to the nutritional state of the animal (Colombani *et al.*, 2003). Body size appears to be assessed from the relative growth of the prothoracic gland. This endocrine organ communicates size information in the form of the secreted steroid hormone ecdysone (Colombani *et al.*, 2005; Mirth *et al.*, 2005). Interactions between circulating levels of ecdysone and juvenile hormone, which is

also sensitive to larval nutrition (Tu and Tatar, 2003), coordinate the timing of many developmental events, including molting and metamorphosis (Nijhout, 1994; Gilbert *et al.*, 1996), as well as both the onset and cessation of cell proliferation in the different imaginal discs (Truman and Riddiford, 2002; Emlen and Allen, 2004). All these signals influence the insulin-producing cells in the brain and coordinate circulating levels of insect insulins (Nijhout, 2003; Tu and Tatar, 2003; Colombani *et al.*, 2005).

By the time that the cells in a horn disc (or any of the traditional imaginal discs) initiate the outgrowth portion of the patterning cascade and begin their burst of proliferative growth, they are bathed in a milieu of circulating whole-animal physiological signals whose levels depend critically on the nutritional state of the animal. Several of these signals have been shown to modulate the rate of cell proliferation in these growing tissues in a way that couples their growth with nutrition. The result of this process is a beetle horn of the appropriate length relative to the final body size attained by that individual.

Step 3: Remodeling of Horns During the Pupal Period

Both of the above developmental processes (steps 1 and 2) combine to stimulate cell proliferation and growth within developing horn discs. Together, they specify the total amount of growth that occurs. By the time the animal sheds its larval cuticle and molts into a pupa, these growth processes appear to be largely completed and the densely folded discs of horn tissue unfurl to form the fully extended fluid-filled tubes visible in pupae (Figs. 14.6 and 14.8).

One of the most exciting recent discoveries regarding beetle horn development was the observation by Moczek and colleagues (Moczek, 2006b; Moczek *et al.*, 2006a) that these pupal horns often undergo extensive remodeling during the pupal period. The regions of pupal horns that subsequently are removed by local apoptosis map to domains of expression of some of the same patterning genes that presumably were involved in the initial formation of the horn outgrowth (Moczek, 2006b; Moczek *et al.*, 2006b). Pupal remodeling of horns is only just beginning to be explored, but already it is clear that during this process, the final shape of the adult structure can be modified (e.g., by producing a narrower and/or more curved final horn; see Fig. 14.6), and it can also lead to the complete loss of the horn from the adult phenotype in some species. Many scarab species produce a thoracic pupal horn that is not retained in the adult, and in these species this horn is completely reabsorbed by all individuals during the pupal period (Moczek, 2006b). However, in other species with thoracic pupal horns, the horns are initially grown by all individuals but then selectively reabsorbed by only a subset (e.g., females, small males),

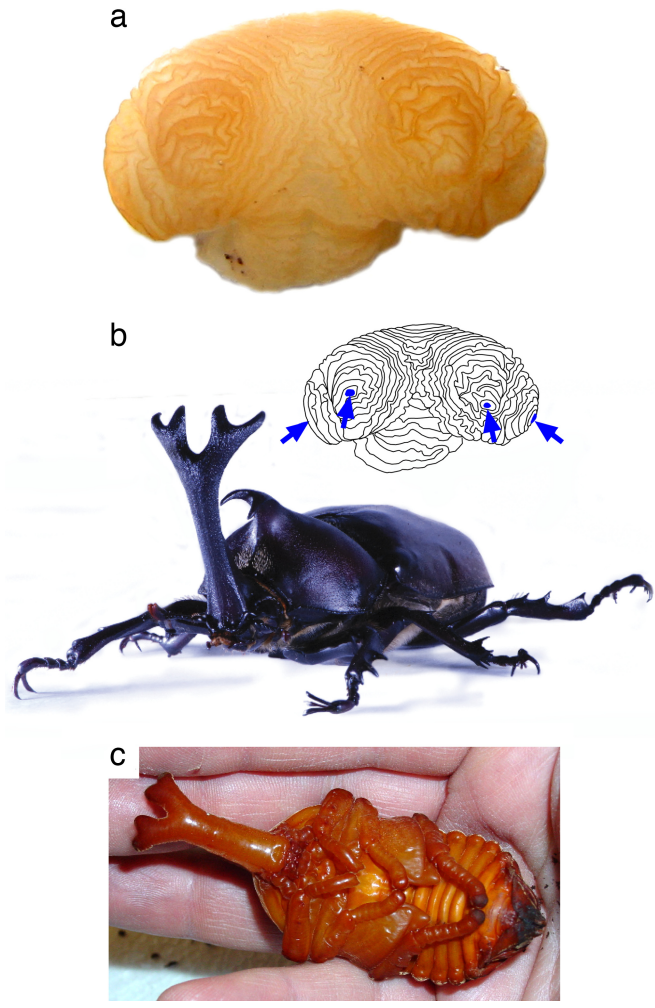

FIGURE 14.8 Development of a branched beetle horn. Horn disc from a late-stage prepupa of a male rhinoceros beetle (*Trypoxylus [Allomyrina] dichotoma*, Dynastinae) showing the folded tubes of epidermis (**a**) that, once unfolded, will comprise the branched adult horn (**b**). Four distinct axes of proximal–distal outgrowth are visible in this disc (arrows, inset) corresponding to each of the distal branch tips of the final horn. All branches are already formed by the time the animal pupates (**a** and **c**), suggesting that the evolution of a branched horn shape in this lineage resulted primarily from genetic modifications to the patterning processes that control cell proliferation (horn growth). However, the grooves between horn branches are more pronounced in the adult than in the pupa (**b** and **c** show the same individual), suggesting that some remodeling of horn shape also occurs during the pupal period.

resulting in either sexual dimorphism or male dimorphism in expression of the adult weapon (Moczek, 2006b).

In summary, the three steps to building a beetle horn involve separate and relatively dissociable developmental mechanisms. Deployment of a patterning gene network (mechanism 1), combined with permissive responses to nutrition-sensitive signals (mechanism 2), activates and coordinates a burst of cell proliferation that results in a densely folded disk of epidermal tissue (horn growth). As the animal pupates, this disk unfolds into an extended, fluid-filled tube that is visible in pupae. Subsequent remodeling by selective and domain-specific reabsorbtion of horn tissue during the pupal period (mechanism 3) specifies the final shape and size of the adult horns (horn remodeling). In the next section, we use these developmental mechanisms as a framework for beginning to predict how diversity in beetle horns may have been generated.

A DEVELOPMENTAL MODEL FOR THE ORIGIN AND EVOLUTIONARY DIVERSIFICATION OF BEETLE HORNS

New Developmental Axes of Outgrowth: Origin of the First Beetle Horns?

Beetle horns arose as novel morphological structures. Although their development shares many similarities with the development of other "traditional" insect appendage imaginal discs, the horns do not themselves derive from these discs (at least so far as we know). Instead, they appear to have arisen as novel discs: new regions of epidermal tissue that at some point in the history of the scarabs began to behave like imaginal discs. Specifically, they began to form an axis of outgrowth. This strongly suggests that the evolution of the first beetle horns entailed the deployment of the outgrowth portion of the limb-patterning pathway in novel regions of the larval epidermis.

It is already clear that many (indeed most) of the genes involved with this portion of the patterning process are expressed in developing beetle horns, and this pathway is sufficiently autonomous that activation of the outgrowth module of the limb-patterning pathway may be sufficient to form an entire structure. We suspect that activation of this pathway alone could stimulate the growth of a full beetle horn.

The evolution of beetle horns most likely resulted from the localized cooption of the outgrowth module of an ancient and existing limb-patterning process (e.g., Moczek, 2006a; Moczek *et al.*, 2006b). This would have generated new axes of outgrowth and new morphological structures that project outwards from the body surface. We suggest that this pathway was deployed independently at least twice, giving rise to the two most

common horn locations: the center of the thoracic pronotum and the dorsal surface of the head. We also suspect that this occurred early in the history of the scarabs, possibly in the common ancestor to all of the scarabs. This Jurassic beetle is thought to have lived in burrows and could have used these early horns in an ecological context not unlike what we observe in extant taxa. Today, there are five recognizable body regions with horns, raising the possibility that the patterning pathway was independently deployed additional times as well. It is not yet clear how readily the horn foci migrate across body segment boundaries. For example, could an evolutionary shift in horn location from the back of the head (the vertex) to the center (frons) or front (clypeus) of the head result from a gradual migration of the position of the focal cells activating horn growth? Or must these involve new cooptions of the patterning process, combined with suppression of expression of an earlier horn? Species exist with all possible combinations of these five horn locations, which may indicate that each of these horn types arose *de novo*; but convincing answers to these questions will have to wait until additional studies of horn patterning have been conducted. Regardless of how many times this pathway was coopted, it is likely that once expression was initiated, this patterning process provided a viable mechanism for subsequent evolutionary modifications to horn form.

Changes in the Expression of Patterning Genes: Evolution of Horn Location and Shape?

If the patterning of beetle horns works in the same way that it does in other insect appendages such as *Drosophila* legs, then the patterning genes will have precise domains of expression that map to specific parts of the final structure. Critically, the process of patterning will be inextricably coupled with cell proliferation and disc growth. In *Drosophila*, many of the same signal interactions that specify the domains within a disc also stimulate and coordinate cell proliferation within those domains. This means that altered levels of expression of these patterning genes changes the final sizes of structures (Serrano and O'Farrell, 1997; Campbell, 2002; Johnston and Gallant, 2002). Furthermore, because these genes control proliferation within specific subsets of the disc that map precisely to corresponding parts of the final appendage, altered expression of these genes is predicted to change the shape of the developing structure (e.g., the size of the tibia relative to the femur in an insect leg, or the size of the dorsal surface of the tibia relative to the ventral surface).

These two critical features of patterning (the explicit spatial map generated by these molecular signals and the local nature of their effects on cell proliferation) link this developmental process with morphological

evolution. Even subtle genetic changes to the levels of expression of these patterning genes can have significant and predictable consequences for the shapes and sizes of adult insect appendages. For these reasons, the limb-patterning pathway has been a major focus for studies of the developmental basis for morphological evolution in arthropods. In the case of beetle horns, we think that subtle changes in the levels of expression of these patterning genes may underlie at least two types of changes in horn form, the evolution of horn location and horn shape (Emlen *et al.,* 2006).

Because cells exposed to high levels of *hh*, *wg*, and *dpp* signals become the distal tip of an appendage, their domains of expression determine the precise physical location of a horn. Genetic changes to any of these domains (e.g., an increase in the expression of *wg*) would shift the relative location of the domain boundaries, changing the respective point of intersection. Thus, a different cluster of cells would be induced to become the distal tip, and there would be a shift in the precise physical location of the outgrowth. Consequently, genetic modifications to the domains of expression of these patterning genes comprise a plausible mechanism for this trajectory of horn evolution, for example, the migration of a horn from the center to the sides of the head (Fig. 14.2 *Lower*).

In addition, because changes in the domains of expression of these same genes can duplicate or bifurcate appendages, this same process could give rise to a multitude of evolutionary changes in horn shape. One horn could be split into 2 or even 3, as in *Onthophagus fuliginosus* (Fig. 14.3). This mechanism could even account for the addition of forks or branches to horns (Fig. 14.8). Comparative studies of horn patterning are still in their infancy, but the behavior of this pathway in the appendages of other insects, combined with existing evidence for species differences in horn patterning (e.g., Moczek and Nagy, 2005; Moczek *et al.,* 2006b) suggest that this mechanism underlies at least some of the evolutionary diversification of horn form.

Changes in the Sensitivity of Horn Cells to Insulin: Evolution of Horn Allometry?

Insulin signaling couples trait growth with nutrition, and for this reason, this pathway comprises another likely mechanism for horn evolution. The InR pathway is activated within each of the imaginal discs, and the sensitivity of each disc to these insulin signals will determine to a large extent how that particular structure will grow. Consequently, genetic changes in the expression or activity of elements in the InR pathway could cause specific traits to become more or less sensitive to insulin signals,with profound consequences for subsequent patterns of growth of that trait. Increased sensitivity of horn disc cells to insulin is predicted to increase

the rate of growth of that trait overall and enhance the sensitivity of that trait to nutrition. Thus, it should lead to increased nutrition-dependent phenotypic plasticity in horn growth, a steeper population-level horn allometry slope, and, in the best-fed individuals, a disproportionately larger final horn size.

It is noteworthy that all of these properties (enhanced nutrition-sensitivity, steep allometry slopes, and disproportionately large final trait sizes) are characteristic of the most extreme and exaggerated morphological structures in insects (Emlen and Nijhout, 2000) and of the enlarged ornaments and weapons of sexual selection in general (Andersson, 1994; Cotton *et al.,* 2004). It is tempting to speculate that the evolution of extreme sizes in these charismatic traits resulted from something as simple as genetic changes to the sensitivity of their cells to insulin or other nutrition-dependent physiological signals.

One way to begin to test these ideas involves a comparison of insulin sensitivity across the different traits within a species. We predict that traits that have steep and positive allometry slopes should be exquisitely sensitive to insulin signals (e.g., wings, horns). Other traits that are insensitive to nutrition and have shallow allometry slopes should be relatively insensitive to insulin signals (e.g., the genitalia). What we are suggesting is that the degree of phenotypic plasticity or canalization of trait expression could result from disc-specific differences in their sensitivity to circulating insulin signals (Shingleton *et al.,* 2007).

In *Drosophila* trait differences in nutrition-dependent plasticity and allometry result at least in part from disc-specific differences in their responsiveness to insulin signals. Recent experiments by Shingleton *et al.* (2005) showed that traits like wings were sensitive to both insulin and to perturbations to the InR, whereas the genitalia were not. Growth of the genitalia was almost entirely unaffected by perturbations to the InR. This important study confirmed that activity of the InR pathway does affect trait allometry. Results from several other studies where genetic perturbations to elements of this pathway were examined show this as well (e.g., Goberdhan and Wilson, 2002; Mirth *et al.,* 2005).

We have used quantitation of relative transcript abundances of the InR gene as our first measure of insulin pathway activity in beetle imaginal discs, and our preliminary results indicate that horn, leg, wing, and genital discs differ predictably in their relative activities of this pathway during the period of disk growth (L.C.L. and D.J.E., unpublished results). Reduced activity of this pathway also appears to be one of the mechanisms used by scarabs to truncate horn growth, in this case, in the horn discs of small males and females of the species *Onthophagus nigriventris* (Emlen *et al.,* 2006; and L.C.L. and D.J.E., unpublished data). Consequently, the insulin pathway now appears a likely candidate mechanism for the development

and evolution of trait plasticity and trait allometry in insects generally and in beetle horns specifically. Although we initially illustrated this point by examining how the different traits within a species have diverged (e.g., horns versus wings versus genitalia), it is important to recognize that this same process can also account for population and species differences in the expression of a single trait (Fig. 14.4). This process could explain evolutionary shifts in both the relative size of a trait and the evolution of extreme or exaggerated trait sizes.

Changes in the Amount of Horn Resorption During the Pupal Period: Evolution of Horn Shape?

Programmed cell death is an integral part of animal development and can lead to significant remodeling of appendages. Cell death is responsible for generating interdigital spaces in tetrapod limb buds (Rodriguez-Leon *et al.,* 1999; Zuzarte-Luis and Hurle, 2002) and for creating cavities in the developing inner ear (Fekete *et al.,* 1997). In insects, programmed cell death sculpts head morphology in flies (Lohmann *et al.,* 2002) and remodels the outer margins of butterfly wings (Kodama *et al.,* 1995). Interestingly, programmed cell death has also been shown to underlie sexual dimorphism and caste differences in insect wing morphology (Lobia *et al.,* 2003; Sameshima *et al.,* 2004; Nardi *et al.,* 2005), a situation analogous in many respects to what Moczek *et al.* (Moczek, 2006b; Moczek *et al.,* 2006b) have observed with beetle horns.

The preliminary findings of Moczek and colleagues (Moczek, 2006b; Moczek *et al.,* 2006a) suggest that variation in the spatial domains of expression of the patterning gene *dll* during the prepupal (horn growth) period map to subsequent variation observed in the amount of resorption of horn tissue. The involvement of patterning signals in this process of pupal horn remodeling would be exciting, because it would suggest parallels with the molecular mechanisms involved with tissue remodeling in other taxa (e.g., Rodriguez-Leon *et al.,* 1999; Rusconi *et al.,* 2000; Adachi-Yamada and O'Connor, 2002), and because it would illustrate yet another route to beetle horn evolution. Genetic changes to the spatial domains of expression of patterning genes could underlie evolutionary changes in horn shape through their effects on the relative locations and amounts of cell death in pupal horns, rather than (or in addition to) any effects that they may have on proliferation. Pupal remodeling appears to be widespread, at least within the genus *Onthophagus* (Moczek, 2006b), and this process could lead to the carving of spaces between horns (analogous to the spaces between vertebrate limb digits) and to fine-scale sculpting of horn barbs, branches, or curves.

The Many Routes to Horn Dimorphism

In this article, we have focused on the developmental mechanisms underlying the evolutionary origin of horns and the subsequent diversification of horn forms. For space reasons, we have not elaborated on the mechanisms generating dimorphism in horn expression. However, it is already clear from the few species that have been studied to date that scarabs use a variety of means to shut off horn growth. Indeed, they appear to be remarkably good at it. Both the patterning and insulin pathways are required for horn growth, and a disruption or truncation in the activities of either pathway could halt the proliferation of horns and result in a hornless adult phenotype. Our studies measuring transcript abundances for the patterning gene *wg* and the InR gene in the species *O. nigriventris* suggest that both pathways may be involved. Both pathways showed reduced activities in the horn discs of small males and females (which grow only minimal horns) compared with same-stage horn discs from large males (which grow full horns). In addition, Moczek (2006b) showed that differential amounts of pupal remodeling also contribute to horn dimorphism in this same species: females and small males reabsorb greater amounts of horn tissue than large males. Thus, developmental studies from just this one species implicate three possible mechanistic routes to the suppression of horn growth and to the evolution of horn dimorphism. Other studies by Moczek and colleagues (Moczek and Nagy, 2005; Moczek, 2006a,b; Moczek *et al.,* 2006b) have begun to relate domains of expression of patterning genes and relative amounts of pupal remodeling with horn dimorphism in additional *Onthophagus* species. These studies also reveal a variety of mechanisms for shutting off horn growth.

CONCLUSIONS

Even this preliminary examination of the mechanisms of beetle horn development reveals a great deal about their capacity for evolution. The conclusions from these studies of development are remarkably similar to the ones we get by mapping horns onto a phylogeny: it may not be hard to gain a horn. The outgrowth portion of the limb-patterning pathway is sufficiently autonomous that initiating this cascade may be all that is needed to get a fully formed horn. This might be possible in a single step or within a single beetle generation, as suggested by the sporadic appearance of mutant individuals that emerge with fully formed horns from species that are otherwise entirely hornless. It certainly could account for the numerous irregular appearances of horned taxa securely nested within clades of otherwise hornless species.

It also does not appear to be difficult to lose horns. To truncate horn growth, scarabs employ numerous mechanisms, any of which could lead

to the sudden loss of horns from a lineage. Subsequent breakdowns in these suppressive mechanisms could just as easily lead to sudden regains of horns. There is no question that the history of beetle horns is a story of repeated gains, losses, and re-gains of these weapons. We suggest that comparative studies of horn evolution and developmental studies of horn growth both attest to the relative ease with which growth of these structures can be turned on or off.

Finally, it does not appear to be difficult to change horn morphology. All three of the mechanisms now thought to be involved with horn development are likely candidates for genetic changes in horn form. We now suspect that subtle genetic changes in just a few elements within these mechanisms might be sufficient to generate all four of the principal trajectories of horn evolution: changes in horn location, shape, allometry and dimorphism.

One hundred and thirty five years ago, Darwin noted that sexual selection appeared to have acted "especially effectively" in scarab beetles (Darwin, 1871, p. 371), and 55 years ago, Gilbert Arrow, then curator of the British Museum, noted that these beetles appeared to have a "special tendency toward the acquisition of horns" (Arrow, 1951, p. 94). Today, we are finally able to elucidate the mechanisms underlying these observations. We now understand a lot about how beetles make a complex structure like a horn, and we are beginning to visualize how these horns might change in form. In essence, we are starting to elucidate what that "special tendency" of the scarabs was, and these insights from development are transforming how we think about the patterns of horn evolution.

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Part V

CONCLUDING ESSAY

15

Biological Design in Science Classrooms

EUGENIE C. SCOTT and NICHOLAS J. MATZKE

Although evolutionary biology is replete with explanations for complex biological structures, scientists concerned about evolution education have been forced to confront "intelligent design" (ID), which rejects a natural origin for biological complexity. The content of ID is a subset of the claims made by the older "creation science" movement. Both creationist views contend that highly complex biological adaptations and even organisms categorically cannot result from natural causes but require a supernatural creative agent. Historically, ID arose from efforts to produce a form of creationism that would be less vulnerable to legal challenges and that would not overtly rely upon biblical literalism. Scientists do not use ID to explain nature, but because it has support from outside the scientific community, ID is nonetheless contributing substantially to a long-standing assault on the integrity of science education.

Nature is full of complex biological adaptations such as the camera eye, the bird wing, the bacterial flagellum, the mammalian immune system, or the complex traps of orchid flowers. Evolutionary biology continues to make progress in explaining such fascinating structures through the scientific process of positing natural explanations

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and testing them against the natural world. Nevertheless, in recent years scientists have been forced to confront a resurgence of opposition to evolution in the political realm of public education. This new antievolutionism is called "intelligent design" (ID). Its proponents allege that it is a revolutionary new scientific explanation for complex adaptations, that it is purely secular and definitely not creationism, and that it is therefore pedagogically and legally appropriate for public school biology classrooms. However, an analysis of ID shows that in both content and history, it is a subset of an earlier antievolution movement known as creation science.

HISTORICAL BACKGROUND: FUNDAMENTALIST OPPOSITION TO EVOLUTION EDUCATION IN THE UNITED STATES

The creationism/evolution battle began in the 1920s as a by-product of the acrimonious split of American Protestantism into "fundamentalist" and "modernist" camps. Fundamentalism arose in the early 20th century in reaction to issues such as modern historical criticism of the Bible, technological and social progress, and evolution (Marsden, 1991; Armstrong, 2000). Modernists moved toward viewing the Bible as allegorical and as a product of human history, whereas fundamentalists tried to defend what they viewed as "the fundamentals" of the Christian faith by adopting a strict doctrine of biblical inerrancy, wherein the entire text of the Bible was considered to be divinely inspired truth and without error (and usually, but not always, to be interpreted literally).

Open conflict between modernists and fundamentalists was suppressed by the drive for Prohibition and by World War I. But after the war, the populist politician William Jennings Bryan decided that "Darwinism" had been the cause of German militarism as well as a threat to traditional religion and morality (Marsden, 1991). In the early 1920s, he spearheaded a national crusade against the teaching of evolution in the public schools, which in the previous decades had become common in textbooks and thus in the curriculum (Grabiner and Miller, 1974). Bans on teaching evolution were passed in several states (Larson, 2003).

Bryan's campaign peaked in the 1925 Scopes Monkey Trial in Dayton, TN, where he was humiliated on the stand by Clarence Darrow; he died a few days later. But although fundamentalism was discredited in the eyes of the media, Tennessee's ban on teaching evolution was not overturned. Other states and many local jurisdictions enacted laws or policies that discouraged or forbade the teaching of evolution, and evolution rapidly disappeared from high school textbooks.

Evolution was not part of the precollege curriculum for 40 years, until fears of technologically falling behind the Soviet Union led in the late 1950s to federal money for new science textbooks—unusually for the time,

written by scientists (Larson, 2003). The Biological Sciences Curriculum Study's series of biology textbooks reintroduced evolution. The Arkansas Education Association, concerned about teachers being caught between a state ban on evolution and district requirements to use textbooks that included evolution, challenged the state's antievolution law. This suit resulted in the 1968 Supreme Court decision *Epperson v. Arkansas* (1968), which ruled that bans on teaching evolution were an unconstitutional favoring of the fundamentalist religious view.

"Creation science" arose on the national scene in the late 1960s as a counter to the reintroduction of evolution into the curriculum. The person largely responsible for its invention was Henry M. Morris, who declared, "Creationism is on the way back, this time not primarily as a religious belief, but as an alternative scientific explanation of the world in which we live" (Numbers, 2006). Morris's creation science was his literal interpretation of Genesis (including a young Earth, global flood, and special creation of plants and animals) expressed in scientific terminology. Explicit references to the Bible were optional: Morris's 1974 book *Scientific Creationism* (Morris, 1974) came in two versions, one with Bible quotes, and one without.

In 1972, Morris founded the best-known creation science organization, the Institute for Creation Research (ICR), now in Santee, CA, and served as its president until his retirement in 1996. Even after retirement, Morris continued to promote creation science until his death in 2006 at the age of 87. Morris and the ICR have spun-off or inspired other organizations promoting creation science, the most important of which is the Kentucky-based ministry Answers in Genesis. Answers in Genesis rivals ICR in size and influence, and plans to open in 2007 a 50,000-square-foot museum promoting a literal Genesis creation about 10,000 years ago. Dozens of smaller institutions and active independent creation science ministries, fundamentalist churches, and several television evangelists also contribute to the movement (Scott, 2004).

Despite its scientific veneer, creation science was ruled to be clearly religious and therefore unconstitutional to advocate in the public schools in the district court decision *McLean v. Arkansas* (1982) and the Supreme Court decision *Edwards v. Aguillard* (1987). As will be shown below, ID arose as a direct response to these defeats. However, even though ID recently has attracted more national media attention, partially as a result of the *Kitzmiller v. Dover* case (2005) where it too was ruled unconstitutional, creation science remains the larger of the two movements and generates much grass-roots activity.

INTELLIGENT DESIGN ARGUMENTS

The ID movement has its de facto headquarters at the Discovery Institute, a Seattle-based, policy-oriented think tank founded in 1990. In 1996, the Discovery Institute added ID to its agenda by opening the Center for Renewal of Science and Culture. In 2002, the words "Renewal of" were deleted from the name, producing the Center for Science and Culture, probably to appear more secular. As documented by Forrest and Gross's examination of the "Wedge Document," a fund-raising proposal prepared by staff at the Center for Science and Culture, Christian cultural renewal is precisely the goal of the ID movement (Forrest and Gross, 2004). Although the Discovery Institute has vociferously claimed that ID is a scientific research program and "not creationism," in reality, many of the movement's claims are derived directly from creation science with no modification. However, the ID movement promotes a few phrases and concepts that at first glance seem to be novel.

As defined by William Dembski (1999), the most prolific ID proponent:

> Intelligent design is three things: a scientific research program that investigates the effects of intelligent causes, an intellectual movement that challenges Darwinism and its naturalistic legacy, and a way of understanding divine action.

Challenging the alleged "naturalistic legacy" of "Darwinism" and "understanding divine action" are not scientific endeavors, so the scientific component of ID defaults to the investigation of "the effects of intelligent causes." It might seem, then, as though ID is intended to be a contribution to psychology, ethnology, or archaeology, all scientific fields that involve the effects of uncontentiously intelligent causes. Not so: ID is conspicuously absent from the scientific literature of those fields, as indeed from the scientific literature in general. In any case, ID's proclaimed goal is significantly more ambitious: to detect intelligent design in nature.

ID proponents claim to be able to distinguish complex things that are the result of unintelligent causes and those that are the result of intelligent causes. The differentiation is supposedly accomplished through a variety of approaches; the two most popular being "irreducible complexity," promoted by biochemist Michael Behe in *Darwin's Black Box* (1996), and Dembski's "specified complexity," which leads to a "design inference" (Dembski, 1998).

Behe defines an irreducibly complex structure as one with many components, all of which must be in place for the structure to function. He typically illustrates the concept with a mousetrap, which requires the simultaneous presence of a spring, bar, platform, and some other parts to

catch a mouse, but his favorite biological example is the bacterial flagellum. Behe contends that all of the more than 40 different proteins that make up the flagellum must be present for the flagellum to function. He then infers that the incremental process of mutation and selection, requiring a selective benefit at each step of construction, cannot (or is extraordinarily unlikely to) produce such a system, ''because any precursor to an irreducibly complex system that is missing a part is by definition nonfunctional'' (Behe, 1996). Instead, the functional system must have been produced all at once, as a ''purposeful arrangement of parts,'' much like a watch or any other human-designed machine. Hence, irreducibly complex structures, like human machines, are the product of an intelligent agent, not natural selection.

Dembski's ''design inference'' resembles Behe's ID criterion, but Dembski's arguments tend to be conducted at a high level of abstraction, ornamented with mathematical notation of dubious utility. In brief, Dembski contends that if a given event or object has a low probability of occurrence on all of the nondesign hypotheses available, then it exhibits what he calls specified complexity; specified complexity is, he argues, a reliable indicator of design. The only biological structure to which Dembski attempts to apply his method is the bacterial flagellum (Dembski, 2002). Noting that the chance of its parts assembling at random is astronomically low, and relying on Behe's argument to exclude gradual evolutionary assembly, Dembski concludes that it was intelligently designed. Like Behe, however, he asserts that science is incapable of proceeding further to determine the nature of the designer or the means by which the design was instantiated.

The design inference and irreducible complexity consist of two components: an extensive negative argument against the plausibility of evolutionary explanations, and then a brief attempt at a positive argument relying on an analogy between biological adaptations and human artifacts. Behe's negative argument against stepwise assembly of ''irreducible'' systems fails because it mistakenly assumes that evolution proceeds only by improvement of an extant function, whereas evolutionary theory extending back to Darwin has always emphasized the importance of changes of function in the origin of complex adaptations (Darwin, 1859b, 1862; Mayr, 1960; Jacob, 1977; Ganfornina and Sanchez, 1999; True and Carroll, 2002). The flagellum, although elucidated long after Darwin, is a useful case to examine. Contrary to the assertions of Behe and Dembski, a survey has shown that only 23 of the 42 proteins of the *Salmonella typhimurium* flagellum are universally required in bacterial flagella; and of those, 21 have already been found to have homologous related proteins that function in other, simpler biochemical systems (Pallen and Matzke, 2006). It is therefore not true that simpler precursors would be nonfunctional; they clearly

could have had different functions, just like the related systems in existence today. Deleting parts from a modern system does not simulate evolution in reverse, any more than decapitating modern vertebrates provides information about the origin of cephalization in early invertebrates.

The scientific criticisms of ID's objections to evolution will not be treated in depth here, but it is important for scientists to be aware of and have ready reference to the most detailed scholarly critiques of the ID movement's claims. These include rebuttals to the movement's claims about the philosophy of science and the nature of science (Fitelson *et al.,* 1999; Pennock, 1999; Smith, 2001; Peterson, 2002). Dembski's argument inferring design from specified complexity, besides relying entirely on Behe's argument for its application to biology, has been shown to rely on misconstruals of probability and information theory (Wilkins and Elsberry, 2001; Sober, 2002; Perakh, 2004; Shallit and Elsberry, 2004). The ID movement's common claim that evolution cannot produce "new genetic information" is contradicted by numerous papers documenting the origin of new genes (e.g., Long *et al.,* 2003) or even entire multiprotein catabolic pathways for artificial compounds that humans have released into the environment in recent decades (Copley, 2000; Johnson and Spain, 2003). Behe's claim has been rebutted in general (Miller, 1999; Thornhill and Ussery, 2000; Matzke and Gross, 2006) and for specific complex systems such as bird wings (Gishlick, 2004), the vertebrate blood clotting cascade (Davidson et al., 2003), the vertebrate immune system (Bottaro *et al.,* 2006), and the ID movement's favorite system, the bacterial flagellum (Miller, 2003; Musgrave, 2004; Pallen and Matzke, 2006). Faced with such rebuttals, Behe and Dembski typically make the unsupported assertion that indirect pathways are highly improbable or, ironically, given the absence of any detail in their own explanation, complain that the proffered explanations lack sufficient detail to be empirically tested.

THE ARTIFACT ANALOGY AND DESIGN

The ID movement's negative arguments against evolution are numerous, but its positive argument for design consists of variations on an analogy between biological systems and human artifacts. Behe and other ID proponents will often analogize the recognition of design in biology to the recognition of design in human-made artifacts, for example, Mount Rushmore. Behe writes, "unintelligent physical forces like plate tectonics and erosion seem quite sufficient to account for the origin of the Rocky Mountains. Yet they are not enough to explain Mount Rushmore" (Behe, 2005a). Intelligence is required to explain the purposeful arrangement of stone surfaces into the faces of four presidents. Similarly, ID proponents argue, when a biological structure exhibits a complex and purposeful

arrangement of parts, ''intelligence'' is the obvious explanation once evolutionary processes have been eliminated.

As a scientific explanation, this argument is exceedingly vague. The artifact analogy proposes that a structure like the flagellum ''looks'' designed, evolution cannot explain it, and therefore it is designed. The indicators of design are complexity and/or a ''purposeful arrangement of parts.'' But complexity is not a reliable marker of intelligent agency: A paperclip is also the product of an intelligent agent, but it is certainly not complex. The only observable designers, humans, seem to favor simplicity as often as complexity in their designs; simple designs are often more efficient to manufacture and use, and less prone to breakage and user error. On the other hand, even outside of biology any number of physical forces can produce complexity so extreme that it is far beyond the capacity of any known or even theoretical computer to precisely model; chaotic systems such as weather are examples.

ID proponents often present archaeology as an example of how scientists search for complexity, and thus by analogy, ID is a scientific field. Yet archaeologists are not seeking complexity in the discovery of human artifacts. When a stone implement is discovered, it may be highly complex, with many facets removed to produce a specific shape, such as an intricate, fluted Native American Folsom point. But a human artifact may also be quite simple—such as a unifacially flaked chopper from Olduvai Gorge made by early humans. What distinguishes both a Folsom point and an Oldowan chopper from unworked stone is not complexity but the different chipping patterns produced by human manufacture versus natural weathering, and perhaps most importantly, the context of the discovery. As Hurd explains, ''We [archaeologists] have three sources of information: practical experience with the materials used, evaluation of objects in their context, and the commonality between contemporary behaviors and ancient behaviors'' (Hurd, 2004).

The ''purposeful arrangement of parts'' criterion invites the question of just what the purposes are supposed to be for a given system. Human purposes are well known, and are reflected in the kinds of artifacts they design: cutting implements are devised for cutting, etc. But what is the purpose of the bacterial flagellum? When queried about this on the stand in *Kitzmiller*, Behe gave the unhelpful reply that the only purpose that could be inferred was that the designer wanted to make a bacterial flagellum. The artifact analogy, then, fails even at the first, definitional stage.

In actual scientific research, inferences of design are not made by using the vague criteria put forward by ID advocates. Archaeological artifacts or constructions like Mount Rushmore are recognized as having been designed (by humans) because a great deal of background knowledge is available about human design, including methods and motives. The man-

ufacture of stone tools has been observed and replicated, as has the design and manufacture of sculptures such as Mount Rushmore. In archaeology, real design events are reconstructed in detail, including the time, location, materials, tools, techniques, motivation, and culture that produced an artifact, and these, in addition to basic physical laws that humans must follow such as conservation of mass, result in a highly constrained explanatory hypothesis that is readily testable with additional data. ID offers none of this. It invokes an unidentified, unconstrained agent (the intelligent designer) who makes complex biological structures such as the bacterial flagellum for an unknown purpose, using unknown techniques and unknown materials. Even questions such as the time of origin and whether or not mass and energy were conserved remain unanswered. ID provides none of the information that we have about human artifacts and their creators that allow us to make the decision that a given object is artificial rather than occurring naturally (Wilkins and Elsberry, 2001).

ID proponents regularly analogize machines (truly ''purposeful arrangements of parts'') with multipart molecular structures and processes. Yet, on inspection, such analogies break down. The differences between biological phenomena and human-built machines easily outweigh the superficial similarities. Machines and other artifacts serve human purposes, whereas biological designs serve only the ultimate ''purpose'' of self-replication. Machines made by humans consist of parts designed for the task; complex biological ''machines'' are always, upon investigation, found to be cobbled together from preexisting modules with other functions. Biological designs are not really ''purposeful arrangements of parts,'' they are really adaptations of parts originally used for some other purpose. Some differences are even more fundamental. As Woese (2004) notes, ''The machine metaphor certainly provides insights, but these come at the price of overlooking much of what biology is. Machines are not made of parts that continually turn over, renew. The organism is.'' Woese suggests that organisms are like eddies in a current, ''resilient patterns in a turbulent flow—patterns in an energy flow'' (Woese, 2004).

ID proponents contend that scientists reject ID for religious/philosophical reasons, allegedly to promote a materialistic worldview (Johnson, 1991). But as this discussion shows, ID has been rejected for its scientific failings. Its negative arguments against evolution are based on a strawman version of evolutionary theory and ignorance about the data and the literature. Its positive argument approaches the problem of biological design from an erroneous premise of an inaccurate analogy. Living things may be composed of individual parts, and may be highly complex, but they are not artifact-like in any way that would help explain their origins. Scientists who have examined claims of ID reject it because ID does not adequately explain the natural world. Significantly, these

scientific criticisms of ID come both from scientists who believe in God, such as Kenneth R. Miller (1999), and those who do not, such as Richard Dawkins (2006).

But if ID is flawed on so many levels, why does it exist at all? The answer is found in its historical origins.

DESIGN IN CREATION SCIENCE

Long before the ID movement arose, creation scientists constantly invoked design arguments. Some deny this connection (Ratzsch, 2005), but an extensive 1989 survey (McIver, 1989) of creationist literature notes the ubiquitous role of design:

> The venerable Argument from Design remains the chief weapon in creationist apologetics. Creationists consider it self-evident and incontrovertible. Although the theory of evolutionary adaptation stood the design argument completely on its head, creationists continue to appeal to Design without even a trace of defensiveness. It is featured in virtually every book or article promoting creation-science. "Actually," says John Morris [(1989)], Henry Morris's son, "any living thing gives such strong evidence for design by an intelligent designer that only a willful ignorance of the data (II Peter 3:5) could lead one to assign such intricacy to chance."

Design as an argument against evolution has historically been a constant theme in creationist periodicals such as the *Creation Science Research Quarterly*. A cursory search shows that design arguments are invoked for tetrapod limbs (Davis, 1965), the yucca and its moth (Clark, 1965), the hummingbird (Keithley, 1977), and long lists of adaptations from across biology (Shute, 1965a,b). All of these examples of design use some version of Behe's irreducible complexity argument, and even Behe's mousetrap is presaged by numerous articles claiming design for the traps of carnivorous plants (Keithley, 1972, 1982; Howe, 1978). Even the bacterial flagellum, the iconic example of the ID movement, is found in the creation science literature before Behe promoted it (Anonymous, 1992; Lumsden, 1994). In fact, creation science leaders have criticized the ID movement for stealing their arguments.

> Dembski often refers, for example, to the bacterial flagellum as a strong evidence for design (and indeed it is); but one of our ICR scientists (the late Dr. Dick Bliss) was using this example in his talks on creation a generation ago. And what about our monographs on the monarch butterfly, the bombardier beetle, and many other testimonies to divine design? Creationists have been documenting design for many years, going back to Paley's watchmaker and beyond (Morris, 2005).

The concept of design thus is central to both creation science and ID. Although ID claims to be agnostic on much of creation science, such as the age of the Earth, Noah's Flood, and the like, when it comes to design, creation science and ID speak in one language. This language is that of William Paley, whose argument from design in his 1802 *Natural Theology* proclaimed that structural complexity of biological organisms was evidence for the existence of God (Paley, 1802).

Like the irreducible complexity argument, the other prominent claims made by the ID movement, and often the specific terminology, trace back to creation science. "Specified complexity" entered the antievolution literature in Thaxton *et al.* (1984), in the midst of a chapter that attempted to repair the infamous creation science shibboleth, much ridiculed by scientists, that a decrease in entropy in biological systems contradicts the Second Law of Thermodynamics. The authors grudgingly conceded that local decreases in entropy were not prohibited in open systems like the earth, which experience a continuous energy flow, but claimed that genetic information exhibits specified complexity, and that thermodynamic limitations block any nonintelligent increase in information. More generic "no new information" arguments had been made by the European creation scientist A. E. Wilder-Smith, who has been repeatedly cited as an inspiration by many ID proponents (Touchstone, 2004). Other ID arguments, such as the claim that there are no transitional fossils in the fossil record or that "microevolution" is proven but "macroevolution" is dubious, are indistinguishable from those in the creation science literature (Matzke and Gross, 2006).

The microevolution/macroevolution distinction is particularly revealing. In evolutionary biology, microevolution refers to evolutionary processes operating within a species. Although scientists sometimes colloquially refer to macroevolution as "evolution above the species level," this definition does not do justice to the complexity of topics included within the concept. Macroevolution refers to patterns that emerge as species and lineages branch through time, including the rate and pace of evolutionary change, adaptive radiation, morphological trends in lineages, extinction or branching of a lineage, concepts such as species sorting, and the emergence of major new morphological features (such as segmentation, or shells, or the fusion or loss of bones). Decades ago, creationists began to use microevolution and macroevolution idiosyncratically. Creationists' use of "microevolution" is not dissimilar to that of evolutionary biologists, although they apply it not just to species but to evolution within the limits of a specially created "kind" of organism. When ID supporters and other creationists claim to accept some evolution, they generally mean it in this limited sense of evolution "within the kind." A larger distinction occurs in the creationist definition of macroevolution, which to them

refers to (unacceptable) common ancestry of different created kinds. It also refers to the acquisition of major morphological features or body plan changes, also considered impossible without the direct involvement of God. Both creation science and ID approach the micro/macro divide similarly: microevolution is accepted, and macroevolution (their definition) is rejected.

SPECIAL CREATION

The conservative Christian theological doctrine of special creation is central to creation science. Special creation insists on the creation of natural phenomena in their present form, although variations occur. The most extreme special creationists believe that the entire universe (galaxies, stars, the earth, and living things on the earth) was created essentially as we see it today, with only limited change since the Creation. Young-earth creationists such as Henry Morris accept such a view. Various schools of old-earth creationism accept cosmological evolution, but all reject biological evolution. For them, God specially creates organisms intermittently over the millions of years of the earth's history.

The idea of specially created ''kinds'' of organisms derives from the Book of Genesis:

> And God made the beast of the earth after his kind, and cattle after their kind, and everything that creepeth upon the earth after his kind: and God saw that it was good (Genesis 1:25, King James version).

For conservative Christians who believe that every word of the Bible is inerrant truth, biblical ''kinds'' are highly significant, because the language is plain and the phrase is repeated again and again in Genesis. ''Kinds'' have enough genetic variability to adapt to local conditions, but adaptation is strictly limited to the boundaries set by God; because kinds are specially created, common ancestry between created kinds is impossible by definition. Creationists have made efforts to discern the limits of the created kinds, but applying the doctrine to profligate biological diversity has proven difficult. According to Duane Gish, a biochemist who recently retired from the ICR, a ''kind'' might correspond to a whole phylum in the case of invertebrates, a family for some vertebrates, or a species in the case of humans (Gish, 1985).

The denial of common ancestry is unsurprising in creation science, but it is a common misconception that ID advocates accept common ancestry and ''macroevolution.'' In fact, the vast majority of ID proponents deny the common ancestry of humans and apes. Behe is the only significant exception, although he is much-touted by those who wish to

portray ID as a moderate position. Even Behe's support is lukewarm; in 2005, he wrote that "my Intelligent Design colleagues who disagree with me on common descent have greater familiarity with the relevant science than I do" (Behe, 2005b). Dembski's position is typical, accepting "some change in the course of natural history," but believing "that this change has occurred within strict limits and that human beings were specially created" (Dembski, 1995). This is the standard position of an ID advocate. In May 2005, ID supporters on the Kansas Board of Education held hearings to support ID-friendly science standards. Mainstream scientists boycotted the hearings, but a series of pro-ID witnesses, mostly teachers and academics (but few professional biologists) testified in support of the standards. During cross-examination, only 2 of 19 witnesses accepted the common ancestry of humans and apes. One was an independent scholar who clarified that although he supported the Kansas standards, he was not an ID advocate; and the other was Behe. The rejection of evolution by the vast majority of ID witnesses at the Kansas hearings parallels the rejection of evolution by ID proponents in general.

THE EMERGENCE OF INTELLIGENT DESIGN

Although the content of ID suggests that it is derived from creation science, the recently uncovered historical origin of ID illustrates this even more clearly.

The creation science movement reached its peak in the early 1980s. Equal time for evolution and creation science bills were proposed in at least 27 states in 1980 and 1981 (Scott, 2004). Arkansas and Louisiana passed laws mandating "equal time" for the "two models" of evolution and creation science. Arkansas Methodist minister Bill McLean and other plaintiffs, most of them professional clergy from various Christian denominations, brought suit against their state's equal time law in federal district court, and the trial was held in December 1981. *McLean v. Arkansas* pitted a team of plaintiffs' witnesses that included eminent scientists such as Francisco Ayala, Stephen Jay Gould, Harold Morowitz, and G. Brent Dalrymple against a team of creationist defense witnesses who were largely unknown in the world of science, and who had the impossible task of defending the scientific merits of a young earth and global flood.

McLean put creation science on trial, and creation science lost badly. In the January 1982 decision, the judge wrote that creation science was biblical literalist Christianity in disguise, and that to teach it would be to promote a sectarian religious view, which he held to be unconstitutional under the First Amendment of the Constitution (1982). The judge in *McLean* also noted a characteristic of creation science he termed a "contrived dualism" wherein evidence against evolution was considered to be

evidence supporting special creation. This was inadequate for a proper scientific explanation and "has no scientific factual basis or legitimate educational purpose."

Even conservative Christians recognized that creation science had been a legal disaster. *Christianity Today* editorialized, "Evangelicals are appalled at the adverse publicity given biblical faith by the public media as a result of the recent creation/evolution trial in Arkansas" (Kantzer, 1982). The fundamentalist *Moody Monthly* published a story asserting that Arkansas was "Where Creationism Lost its Shirt" and, despite being squarely behind creation science, concluded that the problem with the creation science witnesses had been the lack of published research supporting creation (Mawyer, 1982).

Despite their loss in Arkansas, the creationists had high hopes for the parallel bill enacted in Louisiana. The Louisiana bill was drafted with more deliberation and was more vague about the tenets of "creation science," leaving out explicit mention of the young earth and global flood. Furthermore, the state of Louisiana deputized the creationist lawyer Wendell Bird, ensuring that a highly motivated expert would defend the law from the inevitable American Civil Liberties Union challenge (Larson, 2003).

In the midst of the 1981 wave of creation science legislation and litigation came the first hints of the book that would later introduce the world to ID. The Fall 1981 issue of a creationist student newspaper carried the front-page headline, "Lawsuit prospects dim in Arkansas, bright in Louisiana." Below the main story was a short announcement for a "high school biology textbook" that would "present both evolution and creation while limiting discussion to scientific data" (Anonymous, 1981). Those interested in the project were urged to contact the Foundation for Thought and Ethics (FTE). FTE is a self-described "Christian think tank" located in Richardson, Texas. It was founded in December 1980 by Jon Buell, who had previously worked at the old-earth creationist Probe Ministries, also in Richardson. A document filed with the IRS in 1981 entitled "What is the Foundation for Thought and Ethics?" declared that:

> The Foundation for Thought and Ethics has been established to introduce biblical perspective into the mainstream of America's humanistic society, confronting the secular thought of modern man with the truth of God's Word.

FTE described two projects in the works to carry out its goals.

> [O]ur first project is a rigorous scientific critique of the theory of prebiotic evolution. Next, we will develop a two-model high school biology textbook that will fairly and impartially view the scientific evidences for

creation side by side with evolution. (In this case Scripture or even religious doctrine would violate the separation of church and state.)

The first project materialized as *The Mystery of Life's Origin* (Thaxton *et al.*, 1984), written by Charles B. Thaxton, Walter Bradley, and Roger B. Olsen, three conservative evangelicals who accepted an old earth but who were firmly against a natural origin of life or any substantial biological evolution. The book presented the problem of the origin of life as a scientifically unsolvable mystery and, in a postscript, endorsed divine creation as a better answer. Although ID proponents point to *The Mystery of Life's Origin* as being the foundational publication for the movement that came to be called intelligent design, it was just one of many books written in the early 1980s that represented attempts by believers in biblical inerrancy to develop a creationist science that avoided the pitfalls of more traditional creation science, such as hostility to an old earth (Pun, 1982; Wiester, 1983; Lester *et al.*, 1984; Pitman, 1984).

Much as had the creation scientists, the authors of *The Mystery of Life's Origin* proposed that the origin of life was not simply an extraordinarily difficult problem upon which the research community had not yet reached consensus. Instead, it was a problem that was categorically unsolvable by appeal to natural causes: The first cell was simply too complex to have been produced through natural—equated with "chance" or unguided—processes. Dean Kenyon, then a biologist at San Francisco State University, wrote in the introduction, ". . . it is fundamentally implausible that unassisted matter and energy organized themselves into living systems" (Thaxton *et al.*, 1984). The authors proposed that in the absence of any possible natural causes, the origin of life must therefore be the result of intelligent agency. The agent, they hastened to add, did not have to be God: it could be, perhaps, an intelligent alien.

Even before *The Mystery of Life's Origin* was published, FTE's Buell had begun work on the second project mentioned above: the "two-model high school biology textbook." This was published in 1989 as *Of Pandas and People*, later described by Buell as "the first place where the phrase 'intelligent design' appeared in its present use" (Buell, 2004). (Buell's remark occurs in his preface to the third edition of *Of Pandas and People*, temporarily available on Dembski's web site in 2004.) Credit for authorship was given to Percival William Davis and Dean Kenyon. Davis was described as a biology instructor, and Kenyon as a biology professor. These descriptions, while true, left unsaid the fact that both were traditional creation scientists. Davis was the coauthor of a creationist book (Frair and Davis, 1967) and articles in the *Creation Research Society Quarterly* (Davis, 1965; Howe and Davis, 1971). Kenyon in 1981 had been scheduled as a defense (i.e., creation science) witness in the *McLean* trial (although he

did not testify). He was to be Wendell Bird's lead expert in the Louisiana litigation, *Edwards v. Aguillard*. He also had authored several forewords for creationist books and stated in interviews that he believed there were "no errors in the Bible," that "10,000 to 20,000 years ago—the entire cosmos was brought into existence out of nothing at all by supernatural creation," (Salner, 1980) and that he had converted to scientific creationism after reading books by Wilder-Smith and Henry Morris (Pearcey, 1989). Two unacknowledged authors of *Of Pandas and People* are of interest: Nancy Pearcey, author of the Overview chapter, was another young-earth creationist and an editor at the *Bible-Science Newsletter*, and Michael Behe wrote much of the chapter on biochemistry for the second edition, published in 1993.

The history of the writing of *Of Pandas and People* illustrates the creationist roots of ID. During the course of the *Kitzmiller v. Dover* trial, early manuscript versions were subpoenaed by the plaintiffs' legal team and introduced into evidence as exhibits. By comparing these manuscripts, it was possible to document a transition from creationist to ID terminology during the preparation of the book. The titles of the manuscripts changed over time: in order, the early manuscripts (numbers in parentheses refer to court exhibits from the *Kitzmiller v. Dover* trial, on file at the National Center for Science Education) are titled *Creation Biology* (1983) (P-563), *Biology and Creation* (1986) (P-560), and *Biology and Origins* (1987) (P-561). In 1987, the title was changed to *Of Pandas and People*; there were two 1987 (1987-1: P-562; 1987-2: P-652) manuscripts with this title. In 1989, the first edition was published by a small Dallas publisher (Davis *et al.,* 1989), and in 1993, the second edition appeared (Davis *et al.,* 1993).

On June 19, 1987, the Supreme Court decided in *Edwards v. Aguillard* that teaching creation science was unconstitutional. Although Wendell Bird argued strenuously before the Court that Kenyon's expert witness affidavit showed that creation science was scientific and nonreligious, the justices voted 7–2 that supernatural creation was a religious view and that the Louisiana legislature had violated the Establishment Clause by promoting it in public schools. Creation science as a legal strategy was no longer viable.

The *Pandas* manuscripts reflect this important legal decision. During the *Kitzmiller* case, word counts for the terms "creationist" or cognates, and for the phrase "intelligent design" were compared across the manuscripts. When graphed, it becomes clear that one set of terminology was substituted for the other, with the change taking place between the two 1987 manuscripts (Fig. 15.1).

Another comparison, this time of a key sentence defining creationism, similarly illustrates the substitution of "intelligent design" for cognates

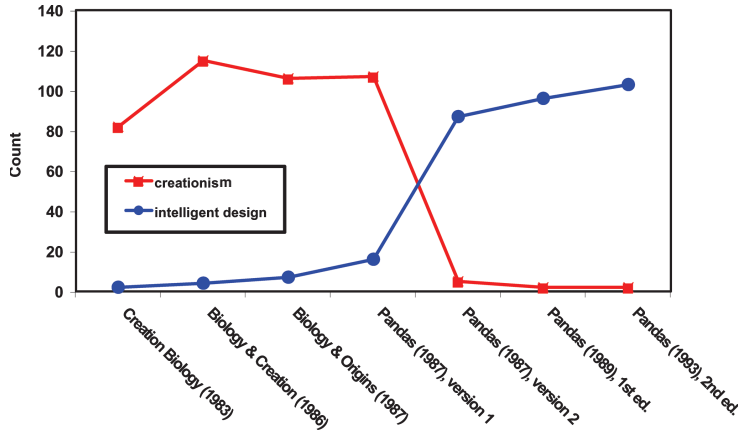

FIGURE 15.1 A comparison of phrasing in the prepublication manuscripts of the ID textbook *Of Pandas and People*. Early manuscripts freely used cognates of "creation" (creationism, creationist), but these terms were replaced by the phrase "intelligent design" after the mid-1987 *Edwards v. Aguillard* Supreme Court decision outlawing the teaching of creationism.

of creationism. In the 1986 manuscript *Biology and Creation*, a paragraph appears that reads:

> *Creation* means that the various forms of life began abruptly through the agency of an *intelligent creator* with their distinctive features already intact—fish with fins and scales, birds with feathers, beaks and wings, etc. (emphasis added) (p. 2-10)

This paragraph is repeated with only small changes (capitalization or punctuation) in early manuscripts, and appears also in the first of the two 1987 drafts titled *Of Pandas and People*:

> *Creation* means that various forms of life began abruptly through the agency of an *intelligent Creator* with their distinctive features already intact—fish with fins and scales, birds with feathers, beaks and wings, etc. (emphasis added) (pp. 2-14, 2-15)

In the second 1987 manuscript, the paragraph's wording has been changed:

> *Intelligent design* means that various forms of life began abruptly through an *intelligent agency* with their distinctive features already intact—fish with fins and scales, birds with feathers, beaks and wings, etc. (emphasis added) (p. 2-15)

Also introduced into evidence during the *Kitzmiller* case was chapter six of the manuscript for the third edition of *Of Pandas and People*, in preparation, also to be published by FTE, which will be given the new title, *The Design of Life* (P-775). This edition has new authors: William Dembski and fellow ID proponent Jonathan Wells. Perhaps considering that "intelligent agency" (or any creative source embodied by an agent) might imply creation by God, the newest version proposes an agent-free form of creationism:

> *Sudden emergence* holds that various forms of life began with their distinctive features already intact, fish with fins and scales, birds with feathers and wings, animals with fur and mammary glands. (emphasis added)

When these comparisons were presented during the *Kitzmiller* trial, they had a powerful effect. In his decision, the judge called this blatant switch of terminology "astonishing."

The manuscript drafts also preserved a further piece of evidence of the evolution of creationism into ID, although this was not presented in trial. A textual transitional fossil was discovered by *Kitzmiller* expert witness Barbara Forrest during her study of the trial exhibits. Shown in Fig. 15.2 for comparison are two excerpted passages from the two 1987 drafts. Evidently, the editor of the drafts was deleting the word "creationists" and inserting the phrase "design proponents" throughout the document. During the tedious procedure, the editor evidently forgot to delete the "c" and "ists" from the word "creationists."

a

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can sustain life?  Evolutionists think the former is correct,
creationists accept the latter view.  Creationists reason as
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b

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can sustain life?  Evolutionists think the former is correct,
cdesign proponentsists accept the latter view. Design proponents
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FIGURE 15.2 The missing link between creation science and intelligent design. In the early 1987 manuscript (*a*) of *Of Pandas and People*, the original wording of a sentence in chapter 3 reads, "Evolutionists think the former is correct, creationists accept the latter view." In the second 1987 manuscript (*b*), an incomplete (and uncorrected) block-and-paste of "design proponents" for the term "creationists" leaves "cdesign proponentsists" (sic), forming a missing link between creationism and ID.

In conclusion, examination of both the history and content of ID shows that it is a form of creationism, despite the persistent efforts of proponents to obscure this connection. Creation science was struck down because teaching it would be a form of religious advocacy. ID was invented as a way to circumvent the constitutional barrier to creation science, but when the constitutionality of ID was tested in *Kitzmiller*, it met the same fate (2005). However, unlike a Supreme Court decision, a district court decision such as *Kitzmiller* only sets a local precedent, and future attempts to incorporate ID in public school curricula are likely. However, because the *Kitzmiller* opinion was so thorough and powerful, it will undoubtedly discourage communities that may be contemplating ID policies. But just as creation science continued after *McLean v. Arkansas*, so ID will continue after *Kitzmiller*, even if in a reduced form.

INTELLIGENT DESIGN AND EDUCATIONAL POLICY

Despite its scientific shortcomings, the ID movement should be taken seriously because it has been disquietingly effective in reinforcing the sentiment, originally exploited by proponents of creation science, that evolution is inadequate science and that creationism is a valuable approach that students deserve to learn about in public school science classes. Recent survey data from the United States and foreign countries indicate that the United States is distinctive among developed nations for its unusually low level of acceptance of evolution (Miller *et al.,* 2006). Because evolution is rejected by so many, and because American education is highly decentralized and unusually politicized, it is not surprising to learn that evolution is under attack in many communities around the country. Such attacks take one or both of two forms: efforts to promote creation science or ID, and efforts to compromise or reduce the teaching of evolution.

The National Center for Science Education collects data on controversies over evolution education in the United States. Although these data are possibly incomplete, it is apparent that the country has experienced another wave of antievolutionism at the state level since the late 1990s. States have been revising their science education standards in response to the No Child Left Behind Act's mandate requiring students to be tested in science beginning in 2007. Between 2000 and 2006, the National Center for Science Education has monitored conflicts over the treatment of evolution in state science standards being developed or revised in Alaska, Arizona, Alabama, Georgia, Hawaii, Kansas, Minnesota, New Mexico, Nebraska, Ohio, Pennsylvania, North Carolina, South Carolina, and West Virginia.

The National Center for Science Education has also monitored attempts to undermine the teaching of evolution by state legislatures. In 2006 alone, legislation was introduced in nine states that would have either promoted

creationism/ID or inhibited the teaching of evolution. Most of these bills did not pass, due to action by citizens, including scientists, who persuaded legislators to vote against the bills. The states included (HB for House Bill, and SB for Senate Bill) Alabama (HB 106, SB 45), Indiana (HB 1388), Michigan (HB 5606, HB 5251), Missouri (HB 1266), Mississippi (HB 953, SB 2427, HB 214), Oklahoma (SB 1959, HB 2526, HB 2107, HB 1003), Utah (SB 96), New York (AB 8036), and South Carolina (SB 114). In Mississippi, a watered-down version of a bill appended to another bill did pass (HB 214, appended to SB 2427). This bill originally called for the teaching of "flaws or problems" in evolution and encouraged the teaching of ID. The final, reduced bill provided that "No local school board, school superintendent or school principal shall prohibit a public school classroom teacher from discussing and answering questions from individual students on the origin of life."

Although there are still rare attempts to promote creation science at the state level, most of the school board or legislative antievolutionism today is directed toward promoting ID and/or promoting the teaching of alleged "evidence against evolution." The latter strategy consists of taking the creationist objections to evolution and stripping them of any mention of a positive explanation of biology, such as creation or design. By avoiding explicit or implied reference to God or a Designer, creationists hope to survive constitutional challenges.

A number of phrases are being used to promote this "evidence against evolution" approach, including requiring students to "critically analyze evidence for evolution," to learn "both evidence for and evidence against evolution," to study "both the strengths and weaknesses of evolution," or to have evolution presented as "theory not fact." Teachers are also exhorted to "teach the full range of views about origins" and, in the slogan of the Discovery Institute, "teach the controversy." The vagueness of "teach the controversy" is its strength: The public is told by media sources that evolution is socially controversial and infers that evolution is also controversial among scientists. "Teach the controversy" does not mean that teachers should have students debate actual controversial scientific issues; it is rather an exhortation to teachers to instruct students that common ancestry (evolution) is a serious issue of contention among scientists.

Antievolutionists have also proposed policies and legislation that contend that it is the students' right to know and the teacher's right to teach creation science, intelligent design, or "evidence against evolution." Such "fairness" arguments resonate with the American public, which responds to the cultural attractiveness of hearing all sides to an issue but which by and large fails to understand that there is no serious scientific challenge to evolution.

ID therefore is making a serious challenge not in the world of science, but in the world of public educational policy. It aims to be a "big tent" presenting a minimalist form of creationism on which all creationists can agree (Scott, 2001), focusing on the supposed impossibility of the natural origin of biological complexity. In addition to its unsuitability for the public school classroom because of its promotion of a sectarian religious position, ID is also a failure as science and has not earned the right to be taught in precollege classrooms. For all its opportunistic use (and misuse) of recent biological discoveries, ID offers only a premodern and impoverished perspective to explain complex functional biological phenomena, a perspective different indeed from the fertile and unifying evolutionary principles underlying the field of evolutionary biology.

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