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BEHAVIOR AND NEUROBIOLOGY

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Experimental Evolution: Concepts, Methods, and Applications of Selection Experiments, edited by Theodore Garland, Jr., and Michael R. Rose.

The tree of life is decorated with an extraordinary diversity of animal behavior (figure 11.1). Such behaviors as foraging, reproducing, moving through the environment, and avoiding predators are all clearly major determinants of survival and reproductive success and hence are thought to be under relatively strong natural and sexual selection. Although some behaviors are culturally transmitted, the vast majority evolve by genetic mechanisms. One of the earliest pieces of direct evidence that behavior can be shaped by evolutionary processes was domestication of wolves into dogs, which is thought to have

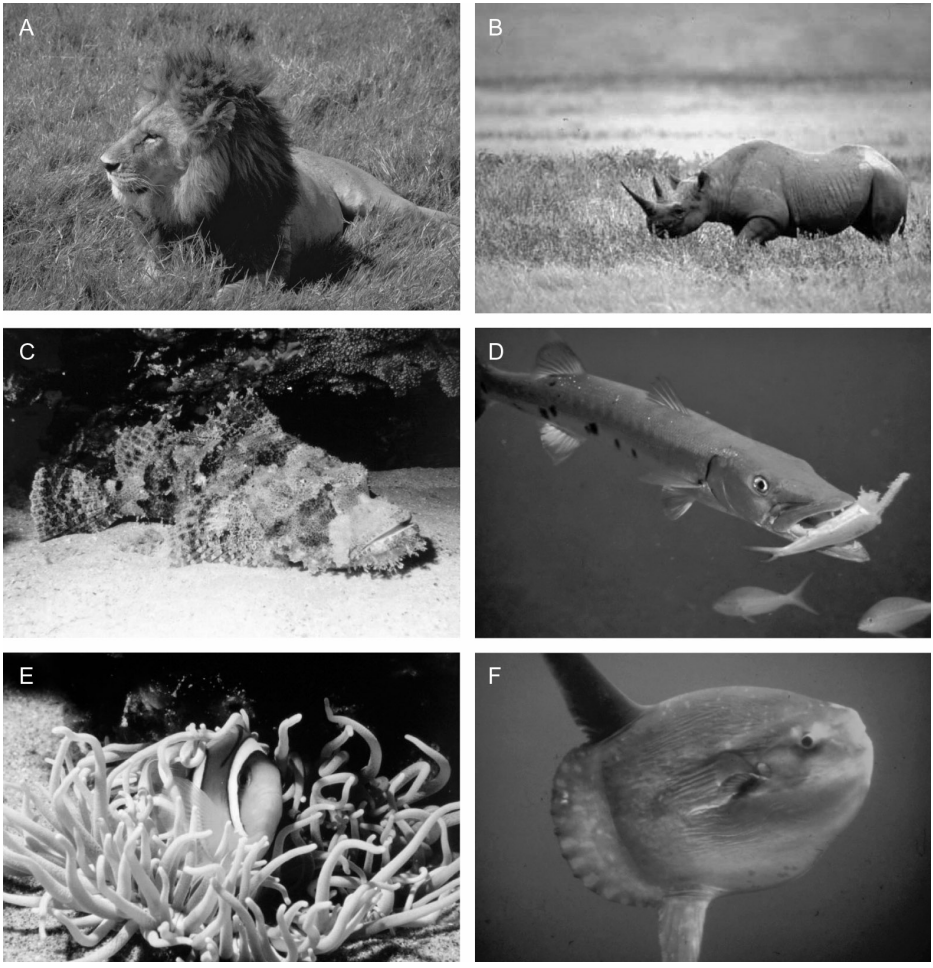

FIGURE 11.1

Massive behavioral diversity in feeding and home range size among vertebrate species. Some species stalk, attack, and kill other animals for food (A), whereas others forage entirely on plant material (B). Among the predators, some sit and wait for their prey to come to them (C), whereas others actively pursue their prey (D). Some animals spend their entire life within few square meters of space (E), whereas others roam for miles in the open ocean (F). Presumably these behavioral shifts are mediated by changes in the brain that evolved through structural modifications of the genome.

occurred as far back as fifteen thousand years ago (Savolainen et al. 2002). Since then, a variety of animals have been domesticated, thus providing ample evidence that selective breeding can alter behavior (see also Barnett and Smart 1975; Simões et al. this volume). Domestication also demonstrates that genes can influence behavior and that behavior can evolve rapidly (Garland 2003; Greenspan 2003; Robinson 2004). In recent decades, a number of natural genetic polymorphisms that affect behavior have been identified, and some progress has been made toward understanding how changes in DNA alter gene expression and/or protein structure, nervous system development, and neural physiology to produce differences in behavior (Ross and Keller 1998; Keller and Parker 2002; Greenspan 2004; Fitzpatrick et al. 2005).

Selection experiments and experimental evolution approaches offer powerful tools for elucidating the origin and mechanisms of behavioral diversity. The discipline is useful to establish basic knowledge about nature, but it also has powerful applications for biomedicine. For example, advances in understanding how genes influence behavior could provide insights into the etiology of drug addiction, obesity, or attention-deficit/hyperactivity disorder (ADHD) (Rhodes et al. 2005).

Here, we review some of the methods in experimental evolution that can be used to study the evolution of behavior (see also Fry this volume; Swallow et al. this volume; Zera and Harshman this volume). We illustrate how these methods can be applied toward understanding the origin and mechanisms of behavioral diversity using examples from our own work and from the literature.

BEHAVIOR EVOLVES FIRST

A long-standing idea in evolutionary biology is that "behavior evolves first" (Mayr 1958; Blomberg et al. 2003). For example, natural selection will only favor physiological adaptations to a novel host plant species in herbivorous insects after the females have begun utilizing the new host for oviposition. Similarly, before the ancestors of whales evolved fins, they probably evolved a brain that made them want to spend time in the water. Providing support for this idea are fossil whales without fins (Gingerich et al. 2001) and living species that display evidence of a recent behavioral transition but without corresponding morphological adaptations (Fryer and Iles 1972). For example, the speciose family of freshwater tropical fish, cichlids, display an extraordinary diversity in feeding habits with clear evidence for genetic adaptations in dentition (Ruber and Adams 2001). However, a few species show no evidence for specialized dentition. *Cyrtocara moorii*, for instance, has large, irregularly shaped teeth that are inserted on the jaws in an uneven fashion. This cichlid feeds on food particles stirred up by other fish that do not require large dentition. Therefore, it has been proposed that recent ancestors of this fish displayed a different feeding behavior and that at some point in the recent past these fish changed their feeding habits, which relaxed selection on the original form of dentition and allowed the dentition to become irregular. Another cichlid species, *Haplochromis*

acidens, displays large teeth that are typically seen in piscivorous fish, but this species feeds nearly entirely on plants. It has been proposed that *H. acidens* recently changed its feeding habits from a carnivore to an herbivore, and that the piscivorous dentition has apparently not greatly impaired the ability of the animals to eat plants and hence has yet remained unchanged (Fryer and Iles 1972).

The implication of "behavior evolves first" is that many adaptations in morphology or whole-animal physiology originate from or are constrained by behavioral shifts (see also the later section on "Testing Adaptive Hypotheses"). Thus, in the whale example, genetic changes in the physiology of the brain were necessary for the appearance of such nonbehavior morphological or physiological traits as fins, blubber, cardiovascular, and breath-holding abilities. In the cichlid example, genetic changes in feeding behavior lead the way for adaptations in dentition.

Hence, behaviors have a unique ecological significance. They often allow the animal to compensate for deficiencies in morphology or physiology (see also Oufiero and Garland 2007; Gibbs and Gefen this volume), facilitating invasion of novel habitats and opening new adaptive zones. Behavior can also be a key aspect of speciation (Fry this volume). The evolution of behavior, especially the higher forms associated with learning and intelligence, also has a unique relevance for understanding human nature because a major difference between human beings and the rest of animals is intelligent behavior.

THE EVOLUTION OF BEHAVIOR

One goal of studying the evolution of behavior is to understand the underlying changes at different levels of biological organization, such as neural physiology, cell biology, or molecular biology (e.g., molecules and genes). At the level of behavioral phenotypes, one can also explore how a specific behavior changes as a consequence of selection on another behavior (or on organismal traits that have at least some behavioral component; see, e.g., Fry this volume; Gibbs and Gefen this volume; Huey and Rosenzweig this volume; Swallow et al. this volume; Zera and Harshman this volume). For example, selective breeding for high levels of voluntary wheel running in house mice *(Mus domesticus)* resulted in a corresponding decrease in thermoregulatory nest-building behavior (Carter et al. 2000). The reverse was also true: selection for small nests resulted in increased voluntary wheel running (Bult et al. 1993). This consistency implies that nest building and wheel running share a common genetic and/or neural basis (and hence are genetically correlated; e.g., see Rauser et al. this volume; Roff and Fairbairn this volume), even though the specific genes and neural circuits are not yet known.

The prospect of understanding how the brain changes at the level of neural physiology to produce a corresponding shift in behavior is intriguing and has the potential for making important contributions in biomedicine as well as evolutionary biology (discussed later). The nervous system is composed of an extraordinary number of components, any or all of which might play important roles in the evolution of behavior. For example,

changes in the density of neurotransmitter receptor proteins, synthesis of neurotransmitters, and/or the structure or quantity of signaling molecules or transcription factors downstream of receptors might underlie evolutionary changes in behavior. Alternatively, changes in the development or connectivity of neurons, neuron numbers, morphology of neurons, electrical properties of neurons, properties of glial cells, or blood vessels in the brain might be subject to change by the evolutionary processes of selection and random genetic drift. We provide some examples of neurophysiological changes associated with genetic adaptation of behavior. In each case, presumably a change in DNA sequence somewhere in the genome underlies the physiological changes that lead to alterations in behavior. However, the genetic architecture of the natural heritable variation underlying evolutionary change in behavior has rarely been traced all the way to the structure of DNA. One rare example where great progress has been made in vertebrates is pair-bonding behavior in vole species. In these animals, investigators have identified a specific pattern of DNA (microsatellite) upstream of a gene that causes differential expression and neuroanatomical distribution of a neuropeptide (arginine vasopressin) receptor protein that appears to have a strong influence over pair-bonding behavior (Young et al. 1999; Hammock and Young 2005) (see later discussion).

The paucity of such examples will likely change soon, owing to recent technological advances for rapid genotyping and profiling of gene expression. The obvious question to ask is, Which genes change to facilitate the evolution of a specific behavior? But that may be somewhat misleading because the changes in DNA that underlie behavioral evolution may not occur directly in sequences of protein-coding genes (exons or introns), but rather in regulatory regions, as in the vole example (Young et al. 1999; Hammock and Young 2005). Such changes may indirectly affect gene expression by altering the folding properties of DNA, patterns of methylation, binding sites for transcription factors, and/or noncoding RNA products that regulate transcription (Lindahl 1981; Castillo-Davis 2005). Thus, rather than say the search is for "the genes," it might be conceptually more appropriate to search for "the structural variation in the genome" that leads to behavioral evolution. Whether those changes happen to occur in protein-coding sequences or not is an empirical question of considerable interest in the study of evolution, and it has enormous biomedical importance. It has been argued that changes in the genome that affect gene expression as opposed to those that affect the structure of proteins are particularly important for the evolution of behavior, though this issue is far from resolved (Whitehead and Crawford 2006).

Other interesting questions to be addressed at the level of genes include testing whether the type of genetic variation that is important for behavioral evolution consists of single nucleotide polymorphisms (SNPs), as compared with microsatellites or tandem repeats upstream of genes (Hammock and Young 2005), and whether behavioral evolution occurs from many small structural changes in DNA that cause many small changes in gene expression or from a few large structural changes or a few large changes in gene expression. Research in these areas has only begun, and the field is wide open for exploration.

Compared with morphology or life history (see Zera and Harshman this volume), behavior displays some unique features that bring specific challenges (see also Boake 1994). For example, behavior is highly sensitive to small and often uncontrollable environmental influences, as well as the animal's physiological and motivational states. For example, the response of parasitoid wasps to plant volatile chemicals is strongly affected by atmospheric pressure (Steinberg et al. 1992). Furthermore, behavior of most animals may be influenced by learning—that is, by the memory of past experience, which will depend on past environments. But which environments have been encountered in the past may in turn be affected by past behaviors. Thus, behaviors are expected to show complex patterns of genotype-by-environment interactions. The low repeatability of behavior and its dependence on past experience makes reliable measurements of behavior particularly challenging. On the one hand, the low repeatability would require multiple assays on the same individuals; on the other, the experience of being assayed once is likely to affect the animal's behavior in subsequent assays. For example, see Dohm et al. (1996) on differential heritability of sequential measures of sprint-running speed in mice.

To illustrate the complexity of this issue, consider the following example. One of us is currently in the process of establishing pilot data for a large-scale selective breeding experiment for increased physical activity in the home cage of house mice and has encountered an interesting problem. We are using sixteen video cameras mounted to the ceiling that feed into two computers to track the movement of singly-housed animals. Under the cameras, sixty-four mice can be housed simultaneously in modified cages (with food and water delivered from the side, and clear tops). Data are collected continuously in the light and dark (under red light) and are analyzed online with software (TopScan from Clever Sys., Inc., Reston, VA) designed to track the movement of the center of mass of the animals. This gives very precise measurement of total distance traveled at any desired increment, seconds, minutes, days, weeks, and so forth.

We have begun examining mice from the outbred stock Hsd:ICR strain (Harlan-Sprague-Dawley, Indianapolis, IN), the same strain used in another selection experiment for increased levels of voluntary wheel-running behavior (Swallow et al. 1998; Swallow et al. this volume). After measuring hundreds of animals over periods up to four weeks, what we have discovered is that the distance covered by an individual animal in their home cage is extremely repeatable between consecutive days: R^2 values are on the order of 0.75 or higher, with lower values occurring occasionally because of outliers. However, the correlation between daily values separated by two weeks is much lower, on the order of 0.25, and after four weeks is near zero! This poses a problem because we were hoping to be able to measure each animal over a period of six days and use the total distance on days 5 and 6 (as in Swallow et al. 1998) to capture a feature of the individual related to their physical activity level. But, now it would seem that the value for an individual would strongly depend on which week that individual happened to be measured.

It does not seem to be related to the age of the animals or their level of habituation to a cage, because some animals at the same age start out inactive the first two weeks and then become active, while others do the reverse. It seems to be related to some spontaneous property within the animal, or perhaps it is influenced by the other animals around them.

The home cages described here were designed so that adjacent animals can interact with each other (lick, smell, or groom each other) through wire mesh separating their cages in one corner. We have also experimented with housing animals without physical contact, using clear plastic inserts instead of the mesh, and have preliminary evidence that repeatability is higher with the plastic inserts. This suggests that the level of physical activity displayed by one animal depends on who its partners are in adjacent cages and that this effect is more potent when animals can interact via smell and touch.

In accord with this discussion, our estimates of narrow-sense heritability from mid-parent offspring regression of home cage activity (distance traveled on days 5 and 6 in the home cages that allow social contact via the wire grid) was zero, and no response to within-family selection was observed in one line (composed of twelve male-female pairs of mice) after four generations of selection. The take-home message from this example is that repeatability of behavior is difficult to assess and may be lower than predicted because it can change dramatically on different time scales and can be influenced by spontaneous events or events that are not easily measurable.

Another challenge is determining an appropriate assay for quantifying behaviors. Such assays usually aim to reduce the (presumably) high complexity of an animal's behavior under natural conditions to a manageable number of aspects that are measurable under standard conditions. This is illustrated, for example, by the controversy about how to measure host preference in herbivorous insects (e.g., Singer 2000). If the goal is to carry out an evolutionary experiment and breed for a particular behavior, then choosing an appropriate selection criterion is often difficult, especially if the target is a complex, high-level, cognitive behavior, such as locomotor activity, aggression, or learning ability, which can be measured in a variety of ways. One strategy is to screen animals on a variety of tests and then use some form of index selection (e.g., based on the first principal component) (Falconer and Mackay 1996). To our knowledge, this has rarely been done. Index selection may have its own problems if factor loadings change over generations. Moreover, it is not clear if sufficient selection could ever be levied against any single component variable to effect changes in allele frequencies. The problem with choosing one measurement is that the response may not be as generalizable as desired. For example, in the 1940s, in one of the most famous selective breeding experiments in psychology, Robert Tryon and Edward Tolman bred two separate lines of rats based on their performance on a maze-learning task. The selection criterion was total number of errors on a maze. Their goal was to breed "bright" and "dull" rats. However, later it was discovered

that the "bright" rats were actually quite limited in their cognitive approach to solving mazes in that they had a strong preference for using spatial strategies (remembering where to move relative to distal landmarks in the room) rather than response strategies (e.g., turn left twice, then right twice, etc.; no need for remembering distal landmarks) (Innis 1992). Similarly, another line of rats selected to learn to avoid auditory and visual stimuli with electric shock turned out to be not better in learning but more fearful (Brush 2003).

Another feature of behavior and of life-history (Zera and Harshman this volume) and whole-organism performance traits (Swallow et al. this volume) is that the underlying genetic architecture is expected to be extremely complex (e.g., Leamy et al. 2008). For example, whereas a difference in coat color may be a (relatively) simple consequence of differences in the activity of an enzyme involved in pigment synthesis (e.g., Hoekstra et al. 2006), for behavior, the pathway from genes to phenotype is likely to be much longer. Changes at a variety of levels, including biochemistry of intracellular signaling and cell-cell communication, development and physiology of nervous and sensory system, endocrine regulation, and so forth, may all underlie an evolutionary change in behavior. Consider, for example, the complexity in how natural polymorphism at a single locus affects foraging behavior in *Drosophila*. It appears to influence behavior by affecting biology at a number of different of levels of organization, including biochemical activity of the enzyme it encodes (Osborne et al. 1997), physiology of synapses at neuromuscular junction (Renger et al. 1999), larval and adult foraging behavior (Pereira and Sokolowski 1993; Osborne et al. 1997), responsiveness to sugar (Scheiner et al. 2004), food intake (Kaun et al. 2007), associative learning (Mery et al. 2007), and success in competition (Fitzpatrick et al. 2007). Similarly, selection for a tonic response to threat (feigning death) in a beetle indicated that this antipredator behavior is negatively genetically correlated with locomotor activity, the correlation apparently being mediated by the levels of dopamine (Miyatake et al. 2008). In another such example, selection for increased voluntary wheel running in mice (Swallow et al. 1998) entailed changes in dopamine signaling (Rhodes et al. 2005), and these changes may in turn account for increased predatory aggression (toward crickets) in the selected lines (Gammie et al. 2003).

Depending on the question of interest, the study organism, the focal behavior, and technical limitations, a researcher intending to do an evolutionary experiment on a behavior faces a choice between three basic approaches (with the distinctions somewhat blurred), which we refer to here as artificial selection, mass selection, and laboratory natural selection (see also Rose and Garland this volume; Futuyma and Bennett this volume). These methods allow the investigator to produce an evolutionary change in a behavior. The result is an animal model that can be used to explore the genetic architecture of the response and the physiological bases for the behavioral shift by use of such additional tools as line-cross analysis, gene mapping (e.g., QTL analysis), and genetic engineering (discussed later).

In artificial selection, a target behavior is quantified for a number of individuals, and some top or bottom fraction is selected as breeders to produce the next generation (Garland 2003). For example, when selecting for preference for a particular odor, each individual may be repeatedly given a choice between the focal odor and a number of other odors; those choosing most consistently the focal odor would then be selected. Obtaining reliable individual measurements reduces noise, increases repeatability, and thus effectively increases the narrow-sense heritability of the focal behavior (the proportion of phenotypic variance in the trait that is due to additive effects of genes). This is because when noise is reduced, total variance is reduced without changing the genetic contribution, so the proportion that is genetic is larger. This is important because larger values for narrow-sense heritability increase the rate of response to selection. The effectiveness of selection can be further increased by controlling the mating by pairing specific individuals among the selected cohort (Falconer and Mackay 1996). Artificial selection also allows a direct measurement of selection differential or intensity, as well as realized heritability (Falconer and Mackay 1996).

Artificial selection is the approach used in most evolutionary studies of behavior, including virtually all experiments on vertebrates (see also Garland 2003; Eisen 2005; Swallow and Garland 2005; Swallow et al. this volume), where the population size and the number of generations are limited by other considerations (e.g., cost, low reproductive rate, long generation time). Breeding for desired behavioral characteristics has also been practiced for millennia in the process of domestication of animals, long before being applied to scientifically motivated study of the evolution of a behavior. One interesting observation from domestication is that unintentional changes (i.e., correlated responses) in morphological characters are remarkably similar across domestication events and across species of vertebrates (Belyaev 1979). For example, selection for tameness in the Russian fox, *Vulpes vulpes*, found patterned changes in pigment in the skin and fur in the shape of a star on the face (common in dogs, *Canis lupus familiaris*), floppy ears (common to dogs, goats, and sheep), and rolled tails (common in dogs and pigs) (Belyaev 1979; Belyaev et al. 1981; Trut 1999). Many other changes were noted in behavioral and physiological traits, such as the onset of hormonally driven fear and aggression responses during early postnatal development, and changes in serotonin metabolism in the brain (Hare et al. 2005). Although a great deal has been learned from these domestication events, they are not scientific experiments (e.g., variables are not always controlled, lines are not replicated). (For a review of experimental domestication studies in *Drosophila*, see Simoes et al. this volume.) Here, we restrict our attention to artificial selection applied to behaviors in the scientific context. A comprehensive review is beyond the scope of this chapter, but we mention a number of examples to illustrate the variety of experiments that have been performed.

Dietary and Other Preference Traits For example, many different lines of mice and rats have been bred for their preference for ethanol (Mardones and Segovia-Riquelme 1983; Hilakivi et al. 1984; Crabbe et al. 1994; Grahame et al. 1999; Murphy et al. 2002). Honeybees were selected to specialize in foraging for pollen versus nectar, resulting in sixfold difference in the amount of pollen hoarded between colonies of the high and low line after only five generations of selection (Page et al. 1995). Outside of the dietary context, quail were selected for color preference (Kovach et al. 1981).

Activity Amount and Patterns For example, mice were selected for increased levels of voluntary wheel running (Swallow et al. 1998), Japanese quail for dust-bathing activity (Gerken and Petersen 1992), and *Drosophila* for emergence from the pupa at a particular time of the day (Clayton and Paietta 1972). In another avian example, blackcaps showed a strong response in both directions to selection on migratory restlessness, a behavior thought to indicate motivation for seasonal migration (Berthold et al. 1990).

Courtship and Mating Traits For instance, in *Drosophila*, selective breeding was imposed on the interval between courtship song pulses (Ritchie and Kyriacou 1996) and the rate at which males lick female genitalia during courtship (Welbergen and Vandijken 1992). Interestingly, in both cases the response occurred only in the direction of lower performance (i.e., greater interpulse interval and lower licking rate), suggesting that sexual selection keeps these traits at their maxima. Readiness to mate was successfully targeted by artificial selection in *Drosophila* (Manning 1961; Spuhler et al. 1978) and in stalk-eyed flies (Rogers et al. 2005).

Anxiety and Aggression Mice were selectively bred for high and low activity in an open-field arena, which is considered a measure of exploratory behavior, response to novelty, and/or anxiety (DeFries et al. 1978). Mice have also been selected for various types of aggression (e.g., Gammie et al. 2006; references therein). More recently, genetic analysis of *Drosophila* lines bred for high intermale aggression led to the discovery of several genes that affect aggression (Dierick and Greenspan 2006; Edwards et al. 2006).

Higher Cognitive Abilities, Such as Learning Ability and Memory Rats were selected for their learning performance in mazes (Tryon 1940) and for moving to a different compartment in response to an acoustic or visual signal previously associated with electric shock (Brush and Sakellaris 1973). Several species of insects (*Drosophila*, blowflies, honeybees) were selected for performance in various versions of the so-called proboscis extension reflex (i.e., the tendency to extend the mouthparts in response to a stimulus associated with a food reward) (McGuire and Tully 1987; Brandes et al. 1988; Lofdahl et al. 1992).

The main aim of most artificial selection experiments has been to demonstrate heritable variation for a particular behavioral response and to obtain genetically diverged lines. These lines are then used to discover the underlying genetic bases of the response

through analysis of line crosses, quantitative trait loci (QTL) mapping, and verification of candidate genes with quantitative complementation tests or genetic engineering (e.g., McGuire and Tully 1987; Chandra et al. 2001; Dierick and Greenspan 2006; Edwards et al. 2006). They can also be used to study the underlying physiological, neural, and molecular mechanisms of the differences in behavior between selected and control lines, or between divergently selected lines.

However, reliable assays of individual behavior are time-consuming and labor-intensive, so artificial selection experiments on behavior face an acute trade-off between the precision of individual measurements and the number of individuals assayed. The latter limits the population sizes. For example, when selectively breeding *Drosophila* for a characteristic of the courtship song (interpulse interval), Ritchie and Kyriacou (1996) were constrained by the workload to a population size of four pairs per selection line, with only one line selected in each direction. Even assuming (rather generously) an effective population size equal to the census size, the selection lines would have lost to drift about one-third of their original heterozygosity within six generations of selection (Hartl and Clark 1997). It is thus not surprising that the response decelerated sharply within just six generations (Ritchie and Kyriacou 1996). With small population sizes, the response to selection will usually underestimate the evolutionary potential of the original base population and may be confounded by drift and inbreeding. This is particularly important for interpreting correlated responses to selection. Rather than being due to pleiotropic effects of alleles favored by selection, they can reflect fortuitous fixation of alleles at loci unrelated to the targeted phenotype. As we discuss later, one way to deal with this problem is to increase the scale of the experiment. Large artificial selection experiments have been performed on behavior, where multiple lines and reasonable population sizes are maintained. This reduces inbreeding depression, and the replicate selected and control lines allow a way to account for drift as an alternative explanation for correlated responses.

To summarize, artificial selection is a powerful tool with which to explore the question of how behavior evolves (i.e., the underlying proximate mechanisms), but it is less informative concerning the adaptive significance (i.e., costs and benefits) of behavior. However, consider the following unusual artificial selection experiment conducted on largemouth bass that has direct adaptive significance because it was conducted in the wild. Between 1977 and 1998 in experimental ponds in central Illinois, bass were caught and released by sport fishers, except the fish were tagged and records were kept about how many times the fish were caught in a season. At the end of a season, the ponds were drained and lines were transplanted into new ponds based on whether they were caught four or more times in a single season or fewer than one time. After, three generations of this type of selection, bass from high- and low-vulnerability lines were stocked into a common-garden pond and permitted to grow for three years. The authors discovered that fish from high-vulnerability lines displayed higher metabolic rates, greater food consumption, and greater investment in parental care as compared with the low lines. The authors concluded that if fish are selectively harvested based on vulnerability, then the

remaining fish in the population may be less effective in providing parental care and potentially reduce reproductive output (Cooke et al. 2007). For a recent review of the evolutionary consequences of fishing with respect to salmonids, see Hard et al. (2008).

MASS SELECTION

A second approach, mass selection, relies on an experimental setup that sorts individuals into groups depending on a particular behavior. For example, mass selection on odor preference could be applied by running large numbers of individuals through a Y-maze with the focal odor coming from one arm and another odor from the other arm, and breeding the next generation en masse from those that chose the arm with the focal odor. This approach allows for greater population sizes, thus alleviating the problem of inbreeding. However, such a binary behavioral response will usually have a large random component—even an indifferent individual will have a 50 percent chance of being selected, and so selection imposed this way will be rather weak. Furthermore, the distribution of the underlying preference trait (which could be defined as the probability of choosing the focal odor) remains unknown, so the selection differential cannot be estimated. This problem is less acute if the individuals are sorted into multiple categories that reflect a degree of a particular behavioral tendency. For example, in probably the longest experimental evolution study on behavior in a eukaryote (see also Travisano this volume), *Drosophila* were selected for over five hundred generations for geotaxis, using a simple but ingenious setup that sorted flies according to their geotaxis score on the scale from 1 to 9 (figure 11.2; Ricker and Hirsch 1988). Unfortunately, not all behaviors are amenable to such automatic sorting.

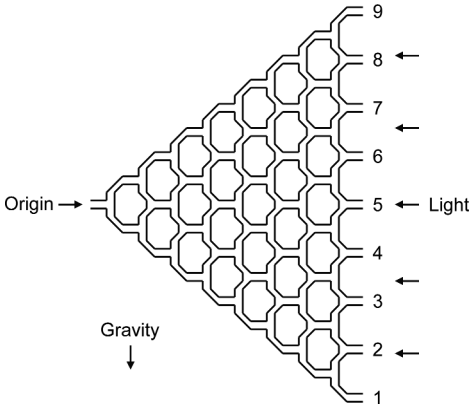

FIGURE 11.2
The apparatus for fractionating flies according to geotaxis used in the five-hundred-generation mass selection experiment of Hirsch and coworkers (Hirsch 1959; Ricker and Hirsch 1988). The flies are released at the origin and move to the right attracted by light, having to choose at each fork whether they move up or down. Traps at the end collect flies according to their geotaxis score, from 1 (strong positive geotaxis) to 9 (strong negative geotaxis). From Toma et al. (2002).

The power of mass selection on behavior is maybe best illustrated by an experiment in which *Drosophila* were selected for increased flying speed in a wind tunnel (Weber 1996). An automatic setup sorted fifteen thousand flies in a single run, according to how far they were able to travel in a wind tunnel with increasing wind speeds from the beginning of the tunnel to the end. The two thousand fastest adults out of about fifty thousand were chosen as breeders each generation. Within one hundred generations, the average speed increased almost 100-fold, from about 0.02 meter/second to over 1.7 meters/second (Weber 1996). In other experiments, *Drosophila* have been successfully selected for the tendency to move along or against a moving striped pattern ("optomotor behavior") (Gotz 1970), or for the height of the pupation site above the medium (Singh and Pandey 1993). However, not all mass selection experiments on behavior were successful. For example, a parasitoid wasp did not respond to selection for a shift in preference of plants used by potential hosts (Rutledge and Wiedenmann 2003). Similarly, in one of our labs, we failed to obtain a response to mass selection on the ability of *Drosophila* to avoid an odor previously associated with mechanical shock, a form of associative learning (Kolss and Kawecki 2008).

To summarize, mass selection provides a way to implement selective breeding with large population sizes. This reduces the problem of inbreeding, but the types of behaviors amenable for this method require creative designs for sorting individuals en masse, such as the geotaxis device or wind tunnel. Mass selection is also less informative than laboratory natural selection or field experiments (Irschick and Reznick this volume) concerning the adaptive significance (i.e., costs and benefits) of behavior.

LABORATORY NATURAL SELECTION

In both approaches just described, selection is imposed by allowing only a defined subset of individuals to breed. This aspect is absent from a third approach to experimental evolution of behavior, which we refer to as *laboratory natural selection* (see Huey and Rosenzweig this volume; Garland 2003). Here, rather than explicitly excluding some individuals from breeding, experimental populations are maintained under a husbandry regimen in which individuals with particular characteristics are expected to contribute more offspring to the next generations. To the best of our knowledge, behavior has never been the intentional "target" of any field introduction or laboratory natural selection experiment. However, field experiments have been carried out with guppies (see Irschick and Reznick this volume), where guppies are introduced between sections of streams in Trinidad below or above waterfalls with or without predatory fish, and scientists interested in the evolution of behavior have taken advantage of these experiments. For example, Magurran et al. (1992) examined schooling behavior and reaction to the presence of a mock predator in guppies from low- and high-predation sites in the laboratory. Fish were born and reared under the same conditions, and still those from high predation sites displayed stronger schooling behavior and greater avoidance of the mock predator.

Another interesting experiment that has yet to be done would be to introduce fish into replicate streams or aquaria with varying flow speeds to study the evolution of swimming behavior and performance.

To summarize, similar to artificial and mass selection experiments, such natural selection experiments can be used to obtain lines with genetically divergent behavior for genetic analysis or to study constraints on evolution. However, the response is expected to be slower because effective selection on any intended focal trait will be weaker than would be possible under direct artificial selection. Moreover, the results will be less predictable because no particular trait is being selected directly by the investigator. Depending on the goals of the experiment (e.g., if one wishes to discover "multiple solutions"), this may be an advantage or a disadvantage (Garland 2003). The main general advantage of natural selection experiments is that they allow testing adaptive hypotheses about ecological factors thought to favor particular phenotypes (Garland and Carter 1994; Gibbs and Gefen this volume; Huey and Rosenzweig this volume). We return to this application toward the end of the chapter.

METHODOLOGICAL CONSIDERATIONS IN EVOLUTIONARY EXPERIMENTS

Choice of Species Obviously, the experimental question will, to some extent, dictate the species (e.g., swimming in fish vs. walking in land animals). As a general rule, though, it is desirable to choose a model species with a short generation time so that results can be acquired in a reasonable amount of time. Consider that it might take five or more generations to observe a response, depending on narrow-sense heritability and the intensity of selection. For example, if the goal is to study the behavior of mammals, small-bodied rodents are a good choice because generation time is on the order of three months. In contrast, chimpanzees have a generation time of approximately seventeen years, so it would take more than the lifetime of the researcher to observe a response to selection. Other desirable features are that the animals are easy to breed, produce large litters, are small in body size, and are easy to keep alive (and healthy) in the laboratory. Another consideration if the goal is to carry out an artificial selection experiment in vertebrates where individuals are paired for breeding is that the species must be amenable to investigator-imposed matchmaking. For example, zebra finches do not always pair bond when placed in the same cage, even though they seem to meet all the other criteria nicely.

Replication In a selective breeding experiment, the unit of replication is the line, not the individual (Henderson 1997). This is because even in the absence of selection, lines of finite population size will diverge in gene frequencies, and hence in the mean value for various traits, because of random genetic drift. Thus, without replication (e.g., if only two lines are maintained, one high-selected line and one nonselected line as a control), then it is impossible to know whether the difference in a trait between the two lines is

due to selection or drift. Note that the degrees of freedom for the statistical test of the effect of selection on a phenotype are based on the number of replicate lines, not the number of individuals. In a two-line comparison, degrees of freedom are zero. In an eight-line comparison (four high and four control), degrees of freedom are six (Swallow et al. 1998; Garland 2003). This argument has been described in detail elsewhere and in other chapters of this book (e.g., Swallow et al. this volume), so we will not elaborate further here. An empirical example from our own work demonstrating the importance of replication is shown in figure 11.3.

Line Types In addition to the selected lines, it is important to maintain unselected lines to serve as controls. This can be done with a random or quasi-random breeding design where pairing of siblings or close relatives is avoided. Sometimes it may be of interest to include lines bred for both high and low values for the trait. However, even when this is done, it is important to maintain control lines. It should not be assumed that the same genes (i.e., different alleles at the same locus) underlie behavioral shifts up versus down because different suites of genes may be involved. For example, high levels of locomotor activity may involve genes that make the organisms perceive the physical activity as pleasurable or rewarding, whereas low levels of activity might involve genes that promote fear or shyness. Figure 11.4 illustrates this point with an empirical example taken from the literature.

Base Population For a response to selection to occur, the population must be composed of genetically variable individuals. Depending on the species, this could be accomplished by collecting individuals from a wild population or by use of a deliberately outbred captive stock. If a wild-derived population is to be used as the base for beginning a selection experiment, then one must consider whether it is better to use a population that has experienced only a few generations of potential "domestication" or has had substantial time to adapt to the new (artificial) environment (see Fry this volume; Rauser et al. this volume; Simões et al. this volume). Outbred stocks are relatively large populations that include hundreds or thousands of individuals in each generation. Various outbred strains of rodents are maintained by commercial vendors (e.g., Harlan-Sprague-Dawley), and typically individuals are bred randomly except that siblings are never mated together. The wheel-running experiment of Swallow et al. (1998) began with outbred Hsd:ICR mice. Another option is to start with a population derived from a cross of inbred strains that has subsequently been outbred for two or more generations. This option is commonly used in mouse behavior genetics (Crabbe and Phillips 1993). For example, Lynch (1980, 1994) used HS/Ibg mice, originally created by an eight-way cross of inbred strains.

One advantage of crossing inbred strains is that a population can be produced in which all the alleles at a given locus are represented with approximately equal frequency. This reduces the chance that a given allele in the starting population will be lost due to random genetic drift (Falconer and Mackay 1996). Moreover, distantly related progenitor strains can be crossed, potentially including those derived from separate subspecies,

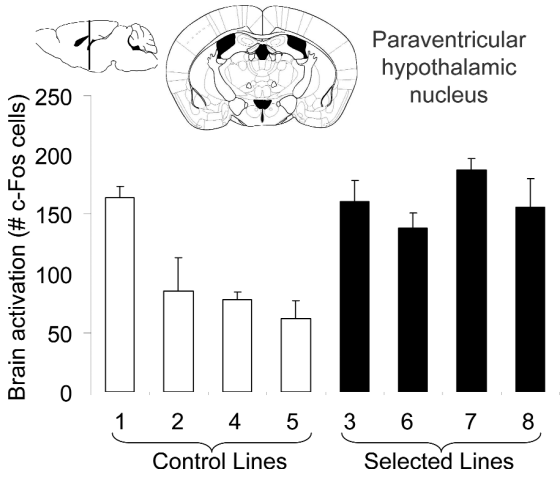

FIGURE 11.3

Statistical evidence for correlated evolution of neurophysiology and behavior requires replication (data from Rhodes et al. 2003). Data from eight separate lines of mice are shown. After initial establishment, four lines were exposed to twenty-nine generations of selective breeding for increased voluntary wheel-running behavior. These are referred to as the "Selected Lines" in the figure and are numbered 3, 6, 7, and 8, based on initial random assignment of the eight lines before selection was applied (Swallow et al. 1998). Four other lines were bred randomly with respect to wheel running for the same number of generations, and they serve as the controls. These are referred to as the "Control Lines" and are numbered 1, 2, 4, and 5. Each bar represents the mean (± SEM) value of the trait (described later) measured in three separate animals (i.e., $n = 3$ per bar). Each animal was placed with access to a running wheel for six days. On day 7, access to wheels was blocked by placing a slate between the wheel-access tunnel and the cage. Animals were sacrificed on day 7 at a time when they would normally be running at peak levels. The purpose was to sample the animals at a time when they would be in a psychological state of high motivation to run without actually running. The brains were removed, sectioned, and stained for c-Fos protein, which is a transcription factor that is transiently expressed in the nucleus of brain cells after they are stimulated. The number of nuclei that stained positive for c-Fos were counted in the paraventricular hypothalamic nucleus (PVN), a brain region whose projecting neurons secrete corticotrophin-releasing hormone into the blood and hence initiate a pathway that results in secretion of corticosterone from the adrenal glands. A comparison of the trait values for control lines 1 and 2 shows that sampling error and/or the random evolutionary processes of genetic drift (and, to a lesser extent, founder effects) are capable of producing large differences in this trait that are of a similar magnitude as those produced by selection. Only when all the replicate lines are considered together is it possible to infer, with a reasonable level of statistical significance, that this trait is associated with past selection for high running ($F_{1,6} = 14.7$, $p = 0.009$; see table 2 in Rhodes et al. 2003). The implication with respect to the mechanism of how the behavior evolved is that increased activation in an area of the brain that initiates the stress response appears necessary for the evolution of increased physical activity, but that this activation alone is not sufficient for causing the high wheel running, as suggested by control line 1 (i.e., other mechanisms are also involved).

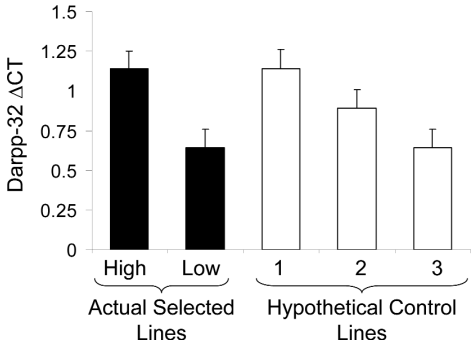

FIGURE 11.4

Control lines are necessary for interpreting results of a selection experiment. Data above the label "Actual Selected Lines" were redrawn from figure 2 in Palmer et al. (2005). Their experiment consisted of two lines of mice. One line was selectively bred for increased locomotor stimulation following administration of methamphetamine (High) and the other was bred for low stimulation in response to methamphetamine (Low) (Palmer et al. 2005). DARPP-32 gene expression was higher in the Low line than in the High line in the nucleus accumbens (a key brain region in the natural reward circuit; see figure 11.5). Note that delta CT stands for change in cycle time and is inversely proportional to transcript abundance. DARPP-32 is an important signaling molecule that responds to dopamine and other neurotransmitters when they bind to their receptors. This result is intriguing because methamphetamine increases dopamine in extracellular spaces and, hence, DARPP-32 seems like a plausible mechanism for the differential response to methamphetamine. However, as no control line was measured, it is impossible to know whether DARPP-32 was associated with low stimulation or high stimulation or both. The hypothetical control lines (1–3) illustrate how the interpretation would change for three possible outcomes: (1) DARPP-32 is associated with low stimulation but not high stimulation; (2) DARPP-32 is associated with both low and high stimulation; (3) DARPP-32 is associated with high but not low stimulation.

such as *Mus spretus*, *Mus domesticus*, and *Mus musculus*, which increases the number of polymorphic loci. On the other hand, populations composed in this way are derived from strains that were each formerly stripped of their genetic diversity through the process of inbreeding (and some may have been directionally selected for one or more traits prior to the intentional inbreeding). Thus, the number of possible alleles at a given locus is limited to the number of inbred strains in the cross. This may be an important consideration, especially if highly polymorphic regions of the genome, such as microsatellites or tandem repeats, are important for the evolution of behavior, which appears to be the case (see later discussion Hammock and Young 2005). Moreover, the process of removing genetic variation by inbreeding and then piecing it back together by crossing inbred strains introduces an artificiality that may not be desirable if the goal is to develop a model for the evolution of behavior in nature. Although it could be argued that any model derived from laboratory-adapted animals has limited relevance for the natural world, the structure of genetic variation in outbred laboratory mice has been

empirically shown to be similar to wild populations of mice (Carter et al. 1999 and references therein). Hence, outbred laboratory stocks may respond to selection and drift in a manner more similar to wild populations than would populations derived from crosses of inbred strains.

Using a cross of inbred strains has some practical advantages, however. One is that progenitor strains can be used that have already been genotyped or entirely sequenced. This makes it easy to find genetic polymorphisms (such as SNPs) that are needed for QTL mapping. Note, however, that this is less of an advantage today than it was a few years ago due to the recent advances in genotyping, which makes it relatively easy to genotype large numbers of animals over a short period of time. In fact, databases are now publicly available that provide information on genetic polymorphisms for a variety of commercially available outbred stocks (see the Mouse SNP detector: http://gscan.well.ox.ac.uk/gs/strains.cgi).

Another practical advantage of using a cross of inbred strains is that once a QTL has been identified, it is possible to develop congenic strains for fine mapping (Silver 2005). Congenic strains are produced by taking two inbred strains that differ for the trait of interest, and backcrossing one on the other many times until a small region of the genome (near the QTL) from one inbred strain is placed on an entire background of the other. This requires genotyping individuals at each generation in the region of the QTL and only backcrossing those that contain the donor DNA in the region. The congenic strains can then be tested for the trait of interest. If they show a trait value similar to the donor strain whose small region was transferred to the background strain, then that constitutes strong evidence that the QTL is located in the small transferred region (Crabbe et al. 1994). However, fine mapping to the level of finding the gene or genes underlying the QTL with congenic strains takes a very long time (ten years or more; see Shirley et al. 2004). Recent advances in molecular genetics offer a new approach that promises to be much faster (see later discussion).

LINE-CROSS ANALYSIS

A response to selection certifies that the trait targeted by selection has heritable variation. This variation can be quantified in terms of realized heritability or additive genetic variance (Falconer and Mackay 1996). However, additional methods are required to characterize the genetic architecture of the experimental evolutionary change. Some insights into the nature of genetic variation underlying the response to selection (e.g., the prevailing patterns of dominance, overall magnitude of epistatic effects, and the number of loci involved) can be gained from the analysis of crosses of directionally selected lines. For example, line-cross analysis of the response of *Drosophila* to selection on interpulse interval of the courtship song indicated that it was based on many loci spread throughout genome, with no strong pattern of dominance or epistasis (Ritchie and Kyriacou 1996). In turn, an analysis of blowfly lines *(Fromia regina)* subjected to fourteen generations

of selective breeding for appetitive learning using the proboscis extension reflex showed a strong contribution of epistasis (McGuire and Tully 1987). It is also useful to examine crosses between parallel (replicate) selection lines within a selection regime. This may help detect confounding effects due to inbreeding as well as additional novel insights. For example, Kawecki and Mery (2006) analyzed crosses between pairs of replicate lines selected for improved olfactory learning ability. While F1 crosses between some pairs of lines performed as well as the parents, F1 crosses between other pairs of lines lost all their response (i.e., they performed as poorly as the unselected controls; the performance of F2 and backcrosses was intermediate). This indicates that the response of replicate lines to selection had different genetic bases, even though the lines were derived from the same base population (Kawecki and Mery 2006).

GENE MAPPING: NEW TECHNOLOGY

Identification of genes responsible for the response to selection can be a starting point for trying to understand how the behavioral change is mediated at the molecular, physiological, and neural level (Wehner et al. 2001). Gene mapping has historically been difficult and time-consuming, with only a few successful attempts at identifying the gene or genes underlying a QTL (Flint and Mott 2001). However, state-of-the-art technology brings new promise. Now it is possible to rapidly genotype and monitor expression of nearly every gene in the genome using microarray technology. If the same animals that are measured for a behavioral trait of interest are also genotyped throughout the genome and assessed for gene expression, then this puts the investigator in an excellent position to find evidence that particular genes are involved in a behavior. This is because the microarray data will likely identify genes whose expression is correlated with the behavior. If these genes happen to be physically located in the QTL interval, then that constitutes strong evidence that those genes probably underlie the QTL (Jansen and Nap 2001; Chesler et al. 2005). Their involvement can be verified with quantitative complementation tests (Mackay and Fry 1996) or with an analysis of mutants or transgenics (e.g., Toma et al. 2002). The complementation test is a tool developed in *Drosophila* genetics where a mutant "null" allele (which does not transcribe the hypothesized candidate gene) is crossed onto a variety of backgrounds with different naturally occurring alleles in the QTL interval. If the heterozygote shows the mutant phenotype, then it is said to have failed the complementation test (because the alternative allele was not able to compensate for the mutation), and it provides evidence that natural variation in the candidate gene underlies the QTL. This strategy has recently been applied for the first time in mammals, to identify genes underlying anxiety in outbred mice (Yalcin et al. 2004).

Another advantage of the new technology is that it is possible to treat expression of each gene on the microarray as a quantitative trait for QTL mapping. A QTL for expression of a gene is referred to as an *eQTL* (with this terminology, *bQTL* is used to specify that the QTL refers to the behavior). Thus, if expression of a gene is correlated with the

behavioral trait, and the eQTL for that gene maps to the same location as the bQTL, then that constitutes strong evidence for involvement of the gene in the behavior, even if the physical location of the gene is not in the eQTL interval (i.e., if the gene is *trans* regulated as opposed to *cis* regulated). In the case of *trans* regulation, the structural variation in the genome that underlies evolution of the behavior may be located far from the genes it influences (Doerge 2002; Schliekelman 2008). Whether evolution of behavior tends to involve more *trans*-regulated genes than *cis*-regulated genes is not known (Hoekstra and Coyne 2007). This gene-mapping technology is very new, and to the best of our knowledge, nothing has been published using this method on any vertebrate model of behavioral evolution. However, the tool is currently being used to explore the genetic architecture underlying the evolution of increased voluntary wheel running in house mice (Swallow et al. 1998) and has been used successfully to implicate particular genes in underlying behavioral variation in biomedical animal models of alcoholism, obesity, and hypertension (Dumas et al. 2000; Chesler et al. 2005; Schadt et al. 2005).

Several factors limit the usefulness of expression profiling to study responses to selection. First, the method assumes that changes in gene expression underlie the changes in behavior and ignores the possibility that different alleles might also, or instead, affect the structure of proteins rather than quantity of message RNA. Second, because analysis of microarray data amounts to thousands of statistical comparisons, it faces an unavoidable trade-off between a large number of false positives and false negatives. Third, because of hitchhiking and genetic drift, changes in allele frequencies are likely to occur in selection lines at loci unrelated to the phenotype under selection (although this limitation can be overcome by studying replicate selected and control lines). Nonetheless, microarray expression data are useful to identify candidate loci and metabolic pathways, which can then be verified with other means. For example, Toma et al. (2002) combined microarray technology with quantitative complementation tests to identify a gene involved in the response to selection on geotaxis in *Drosophila*. Hence, empirical data generally support the idea that heritable variation in gene expression is strongly associated with behavioral variation (Chesler et al. 2004; Mery et al. 2007).

We conclude this section by noting an additional tool that has been used widely by behavior geneticists in the biomedical sciences, but not as often by biologists interested in evolutionary mechanisms (but see Hughes and Leips 2006). The approach is to use panels of isogenic strains for gene discovery. Isogenic strains are strains in which same-sex members are genetically identical. These strains are typically produced by repeated inbreeding (breeding of siblings). Two different inbred strains can also be crossed to produce an F1 that is also isogenic but different from an inbred strain in that it is heterozygous (as opposed to homozygous) at all loci where the two strains differ. Examples of useful isogenic strains include sets of standard or recombinant inbred strains of mice (Chesler et al. 2004) or flies (Jordan et al. 2006). Recombinant inbred strains are panels of inbred strains derived from the same outbred population, usually an F2 or higher cross of two or more inbred strains (Silver 2005). The advantage of using isogenic

strains is that because same-sex individuals within a strain are all genetically identical, data on each genotype can be collected in different individuals over a period of many years by different investigators, and the data are all cumulative. Hence, once the animals are genotyped at a large number of loci and measured for gene expression in a variety of tissues and time points after different experimental manipulations, the data can be cataloged so that all the investigator needs to do is measure the trait of interest in the panel, and automatically, access is available to a dense genetic map along with gene expression values. Such information is publicly available in the form of the Mouse Phenome Database (http://phenome.jax.org/pub-cgi/phenome/mpdcgi?rtn=docs/home) for approximately one hundred standard inbred strains and WebQTL (www.genenetwork.org/) for approximately eighty-eight recombinant inbred lines derived from a cross of two standard inbred strains, C57BL/6J and DBA/2J. WebQTL also includes tools for gene mapping online. A large effort, entitled the Collaborative Cross, is now underway to produce hundreds of recombinant inbred mouse lines derived from a cross of eight inbred strains that were chosen for biomedical relevance and for genetic diversity (three different subspecies are represented, *Mus musculus*, *Mus domesticus*, and *Mus castaneus*) (Churchill et al. 2004). Note that, at the present time, it is unclear how relevant genes discovered via the Collaborative Cross will prove to be for understanding phenotypic evolution in any one species of *Mus*, whether in experimental settings or in the wild.

Because of inbreeding depression (i.e., low fecundity, low performance), some have questioned the use of inbred strains for studying the evolution of behavior (Fonio et al. 2006). One way to address this issue is to study F1 hybrids of inbred strains, which are isogenic but also heterozygous at all loci that differ between the parental strains. We view the Mouse Phenome Database, WebQTL, and the Collaborative Cross as powerful resources for exploring molecular mechanisms of behavioral evolution. We wish to remind skeptical readers that panels of inbred strains or F1 hybrids are regarded as animal models, and although they may lack certain ecological relevance, they may still be useful to explore features of behavioral evolution—namely, molecular genetic mechanisms.

GENETIC ENGINEERING

Great advances have been made in recent years in genetic engineering. It is now possible to render a gene nonfunctional (null mutant, or knockout), engineer a gene so that it is over- or underexpressed, and even change the anatomical pattern of expression of the gene in the brain (Wells and Carter 2001). Among the null mutants, it is now possible to control expression of the gene depending on the presence of a chemical (inducible knockout) (Olausson et al. 2006). These tools can be used to directly test whether a gene is necessary for a behavior, but each gene must be evaluated separately. This poses a substantial limitation in the context of studying the evolution of behavior—or, indeed, any complex trait—which presumably involves changes in many genes that interact with

each other to coordinate complex changes in physiological pathways (Swallow and Garland 2005). Nonetheless, genetic engineering has its place. For example, later we describe a research program in which transgenic engineering technology was used in an extremely creative way to dissect the genetic mechanism underlying the evolution of pair bonding in voles.

EXAMPLES OF CORRELATED RESPONSES TO SELECTION ON BEHAVIOR

One of the most interesting aspects of the evolution of voluntary behavior is that presumably it requires changes in the brain related to motivation. This is because voluntary behaviors require motivation (an emotional state of urgency to engage in the behavior, as opposed to doing something else), and motivation is expected to vary among individuals depending on experience, personality, and genetics (Horn et al. 1976). Hence, by studying the evolution of a voluntary behavior, we may gain insight into how the brain processes decisions related to wanting or liking a particular experience. One of the best examples of this phenomenon involves an artificial selection experiment for increased voluntary wheel running in house mice (Rhodes et al. 2005). The experiment is currently in its fifty-first generation. The model consists of four replicate high-runner (HR) lines and four control lines. The base population was derived from an outbred stock (Hsd:ICR strain; Harlan-Sprague-Dawley, Indianapolis, IN. Each generation, mice were given access to running wheels for six days. In the selected lines, mice were chosen as breeders for the next generation based on their total number of revolutions run on days 5 and 6. In the control lines, mice were bred without regard to their wheel running. By generation 15, selected lines ran approximately 13 kilometers/day, whereas control lines ran 5 kilometers/day. The differential (expressed as a ratio) has remained relatively stable since then, although large fluctuations in actual mean distances run by both control and selected lines have occurred from one generation to the next (Garland 2003).

Selection on wheel running has resulted in the correlated evolution of other behaviors. For example, when the animals are placed in an operant situation where a lever press is required to unlock a wheel and allow it to freely rotate for a set duration, the HR mice showed fewer lever presses than control mice when running duration was short, set to ninety seconds, but similar when duration was lengthened to thirty minutes (Belke and Garland 2007). This led Belke and Garland (2007) to hypothesize that there may be an inherent trade-off in the motivational system for activities of short versus long duration. The HR mice are also more active in their cages without wheels (Rhodes et al. 2001; Malisch et al. 2008), build smaller thermoregulatory nests (Carter et al. 2000), and show greater predatory aggression toward crickets (Gammie et al. 2003). Note, however, that HR mice are not more active than control mice when placed in an open-field arena for a three-minute test (Bronikowski et al. 2001). This suggests that locomotor activity in a habituated environment is a different trait (i.e., influenced by different genes and

neural pathways) than locomotor activity in a novel environment. Locomotor activity in the open-field test is probably more related to novelty seeking, exploratory behavior or anxiety-related emotions, rather than motivation or physiological capacity for exercise (Kliethermes and Crabbe 2006).

Some of the most striking discoveries in the mice bred for increased wheel running have been changes in the brain that appear to underlie increased motivation to run. For example, dopamine is widely known for its role in motivation, reward, and reinforcement, and the HR lines respond entirely differently than controls to psychoactive drugs that increase dopamine signaling, such as apomorphine, cocaine, methylphenidate, and GBR 12909 (Rhodes et al. 2001; Rhodes and Garland 2003). Moreover, areas of the brain that comprise the natural reward circuit (figure 11.5) show differential levels of activation in HR versus control lines when the animals are prevented from running and presumably are in a state of high motivation or "withdrawal" from running (see figure 11.3) (Rhodes et al. 2003). Presumably, structural changes in the genome underlie these differences in neurophysiology and pharmacology, but the identity and location of those changes in the genome are not known at present. An eQTL project is underway.

The discovery of the involvement of the natural reward circuit in the evolution of running behavior may have implications for how voluntary behaviors evolve, in general. It seems intuitive that motivation should need to evolve to shift levels of a voluntary behavior, whatever the behavior. Given that motivation is influenced by neural activity in the natural reward circuit, it seems likely that this will be a target of change. Consistent with this hypothesis is the evolution of an entirely different behavior, pair bonding in voles, which appears to involve a change in the distribution of arginine vasopressin receptors in the natural reward circuit (discussed later) (Young and Wang 2004). Thus, perhaps increasing motivation for one behavior versus another requires adaptations in specific aspects of the natural reward circuit. So, for wheel running in mice, a signaling molecule downstream of a dopamine receptor may be a target (Rhodes et al. 2005); whereas for pair bonding in voles, distribution of arginine vasopression receptors may be a mechanism (Young and Wang 2004).

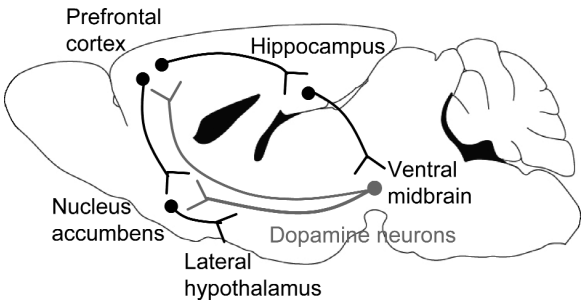

FIGURE 11.5
The natural reward circuit. A drawing of a mouse brain (saggital view) showing connections among a few key brain regions involved in motivation and reinforcement of appetitive behaviors.

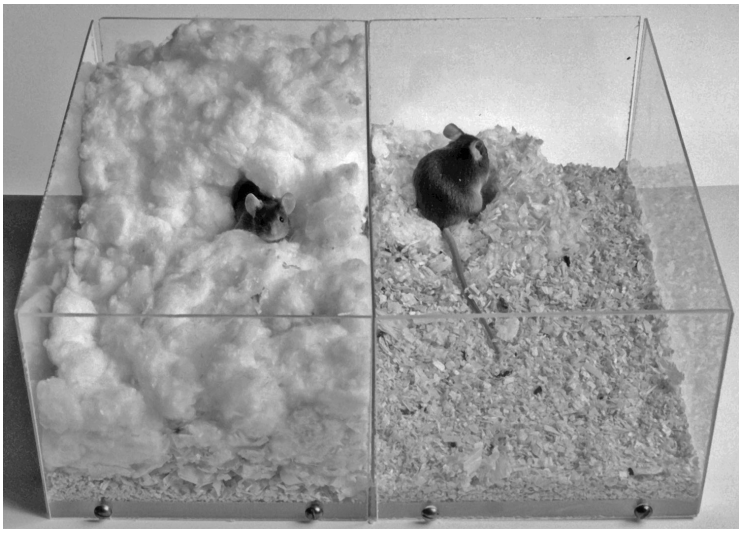

FIGURE 11.6
Dramatic evolution of nest size has been accomplished using bidirectional selection for thermoregulatory nest building behavior. A representative mouse from a high line (left) versus a low line (right). The experiment included six lines, two high-selected, two low-selected, and two maintained as non-selected control lines (Lynch 1980, 1994). Photo by Abel Bult-Ito.

A replicated selective breeding experiment for thermoregulatory nest-building behavior has provided another intriguing model with which to explore the evolution of motivation for behavior (Lynch 1980, 1994). This model consists of two high, two low, and two replicate control lines. The selection criterion was the total mass of cotton used to build a nest over four consecutive daily trials. The starting population was derived from a cross of eight standard inbred strains. By generation 15, the high-selected lines were building nests with approximately fifty grams of cotton, whereas the low lines were using only five grams and control lines, fifteen (figure 11.6). Recall from the experiment on wheel running that nest building evolved as a correlated response (Carter et al. 2000). The nest-building experiment provides further support for the hypothesis that these seemingly different behaviors are jointly affected by some of the same genes because the small-nest builders ran more than the controls or high-nest builders (Bult et al. 1993).

Surprisingly little work explored how the brain has changed in the divergent nest-building lines. Two reports found an increase in the number of arginine-vasopressin neurons in the suprachiasmatic hypothalamic nucleus of a low line relative to a high or a control line, but results for the other replicate lines were not reported, and to the best of our knowledge, no one has looked to see whether alterations have taken place in the natural reward circuit (Bult et al. 1992; Bult et al. 1993).

THE EVOLUTION OF MATING SYSTEMS IN VOLES: FROM GENES TO BEHAVIOR

One limitation of experimental evolution is that it is unclear whether behavioral performance scores targeted by artificial selection will correspond to traits shaped by natural selection. An alternative approach for identifying mechanisms of behavioral evolution is to use experimental methods to explore genetics and physiology of real behavioral shifts that occurred among populations or species in nature. In this section, we describe an interesting example of how laboratory experimental tools, such as genetic engineering and pharmacology, were used to discover the evolution of mating systems in voles. It is one of the most complete stories of discovery of a real evolutionary shift all the way from the genes to physiology to behavior in a vertebrate.

Voles of the genus *Microtus* are one of the most speciose genera of mammals (Jaarola et al. 2004). They also display an extraordinary diversity in social behavior. Some species, such as *M. orchrogaster* (prairie vole) and *M. pinetorum* are socially monogamous (i.e., they develop pair bonds for life and display biparental care); whereas others, such as *M. montanus* and *M. pennsylvanicus*, are solitary and nonmonogamous and do not typically display biparental care (figure 11.7). Thus, the *Microtus* genus provides a useful model with which to explore the neurophysiology and molecular-genetic mechanisms underlying the evolution of pair bonding (Young and Wang 2004).

One of the first hypotheses for the mechanism of the evolution of pair bonding was generated from the discovery that central administration of arginine vasopressin increases social behavior in voles and other animals, especially in males (Winslow et al. 1993). Hence, it was hypothesized that the evolution of monogamy would involve alterations in expression of the arginine vasopressin receptor in the brain. Consistent with this hypothesis, a comparative study of the four species of voles described here showed striking differences in the pattern of distribution of the arginine vasopressin receptor (V1aR) in the brain (of both sexes), depending on whether the species was monogamous. For example, male monogamous voles showed more V1aR in the ventral pallidum and less V1aR in the lateral septum than nonmonogamous species (Young et al. 1999). This result has been replicated numerous times (figure 11.7) (for a review, see Young and Wang 2004).

On the surface, this seems like strong evidence that V1aR plays a role in pair boding in voles, but note the severe limitation of this four-species comparison in light of the phylogeny (figure 11.7). Differences in social behavior are completely confounded with genetic relatedness. Hence, this analysis gets dangerously close to a two-species comparison with zero degrees of freedom (Garland and Adolph 1994). Even in the absence of a role in pair bonding, V1aR would be expected to show similarities within and differences between groups *orchrogaster-pinetorum* versus *montanus-pennsylvanicus* because of phylogenetic relatedness.

It is interesting that despite weaknesses in the comparative data, direct evidence supports the hypothesis that V1aR receptor distribution is associated with social behavior in

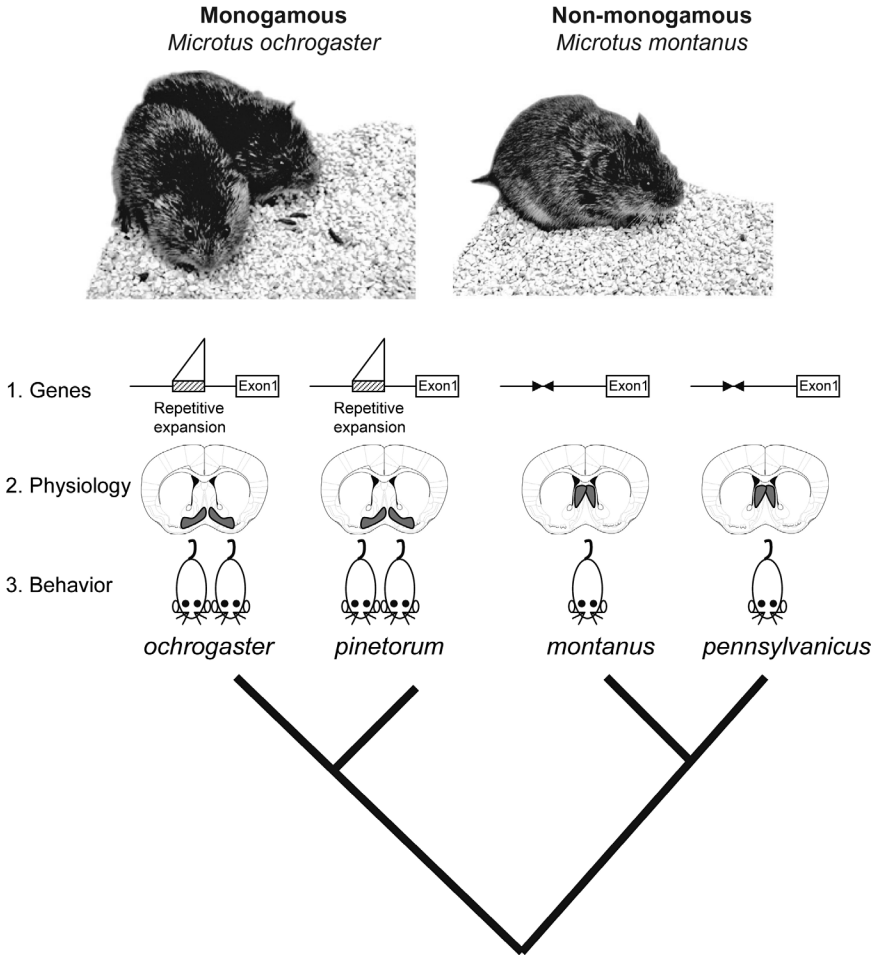

FIGURE 11.7

Strong evidence for the evolution of pair bonding in voles from genes to behavior in spite of weaknesses in the comparative data. In the speciose genus *Microtus*, some species are monogamous, whereas others are nonmonogamous. In the four species shown, a repetitive expansion in a regulatory region upstream of the gene for arginine vasopressin receptor (V1aR) is associated with a change in distribution of this receptor in the brain that parallels behavior (Young et al. 1999). Note the weakness in these data in light of the phylogeny. An association between genes, physiology, and behavior is expected even in the absence of pair bonding because the animals are related to each other in a hierarchical way. Nonetheless, extensive direct evidence has established a causal role for the repetitive expansion on V1aR distribution and behavior using a combination of state-of-the-art transgenic technology, viral gene transfer, and pharmacology (see text). Photos by Larry Young.

these animals. First, in an impressive feat of genetic engineering, a small segment of DNA containing the V1aR promoter region from the monogamous prairie vole was inserted into the genome of a mouse. Remarkably, the neuroanatomical distribution of V1aR in the genetically engineered mice was similar to that in the prairie vole. The mice were then injected with arginine vasopressin. Male transgenic mice expressing the prairie vole V1aR promoter displayed more affiliative behavior (as measured by olfactory investigation and grooming) than control mice (Young et al. 1999).

The authors later provided additional support for the V1aR hypothesis. They overexpressed V1aR in the ventral pallidum of the polygamous meadow vole, *Microtus pennsylvanicus*, using locally administered viral gene transfer, and discovered that overexpression of this single gene substantially increased partner preference formation. Then they blocked V1aR signaling by injecting V1aR antagonists into the ventral pallidum in male prairie voles, and they discovered that this inhibited partner preference formation. Taken together, these data provide strong support for the hypothesis that V1aR signaling in the ventral pallidum (along with other regions of the natural reward circuit) mediates pair-bond formation in *M. orchrogaster* (prairie vole) (Lim et al. 2004).

A molecular-genetic mechanism underlying the differential pattern of V1aR expression in the brains of monogamous and polygamous voles was hypothesized to involve an expansion and contraction of a microsatellite in the *cis*-regulatory region of the V1aR gene. This microsatellite is greatly expanded in the two monogamous species shown in figure 11.7 as compared with the nonmonogamous species (Young et al. 1999). The mechanism for the expansion was identified as several repeat blocks interspersed with nonrepetitive sequences. This expansion was later determined to have a functional effect on gene expression in cell culture (Hammock and Young 2004).

Despite all the positive evidence, a recent phylogenetic analysis failed to find the predicted association between expansion in this microsatellite region and affiliative behavior in a larger sample of twenty-three species of voles (Fink et al. 2006). All twenty-three species showed the expanded version of the microsatellite except for the two species chosen as representative nonmonogamous voles (figure 11.7). Because many vole species are nonmonogamous, these data demonstrate that expansion of the microsatellite is not sufficient to produce monogamy, but it still might be necessary in the monogamous species shown in figure 11.7.

At least in the prairie vole, the evidence is strong that expansion of the microsatellite region increases affiliative behavior. Within prairie voles, individual alleles of the microsatellite predict both individual differences in receptor distribution patterns and affiliative behavior. Moreover, in three independent cell culture experiments, the longer alleles significantly changed transcriptional activity relative to the shorter alleles (Hammock and Young 2005).

Taken together, these data provide strong evidence that an evolutionary shift in affiliative behavior in voles was caused, in part, by a change in a regulatory region of a gene

that resulted in altered distribution and expression of a neurotransmitter receptor in the brain, and that this alteration is important for the behavior. To the best of our knowledge, no one has attempted to test this hypothesis using a selection experiment to increase affiliative behavior in voles (or any other animal), and then see whether the microsatellite locus responds in the same way. This would constitute a very strong alternative test of the primary hypothesis.

TESTING ADAPTIVE HYPOTHESES

Much research on behavior in recent decades has been done within the framework of an adaptationist research program, looking at behavior as a product of evolution by natural selection and aiming to explain how and when particular forms of behavior contribute to Darwinian fitness. Although adaptive hypotheses about behavior may be easy to formulate, testing them is often difficult (e.g., see Garland and Adolph 1994; Garland and Carter 1994; Garland et al. 2005). Experimental evolution under experimentally imposed natural selection is a direct way to test hypotheses about factors (environmental, social, etc.) thought to favor the evolution of particular behaviors. For example, Joshi and Mueller (1993) showed that high (low) larval density result in natural selection for fast (slow) feeding in *Drosophila* larvae, but does not impose differential selection on pupation height (see also Mueller this volume). In another *Drosophila* study, experimental removal of sexual selection (by enforcing monogamy) led to the evolution of lower courtship rate by males, in addition to having various effects on the physiological aspects of male-female conflict (Holland and Rice 1999).

Experimental natural selection may also help to clarify which traits have the greatest potential to respond to a selection regime that favors several complementary or alternative adaptations. For example, by maintaining bean weevil populations on a mixture of two host seeds (A and B) either alone or together with a competitor specializing on host A, Taper (1990) tested the prediction of ecological character displacement. As predicted, the target species became physiologically better adapted to host B in the presence of specialized competitor but, surprisingly, did not evolve a greater preference for host B. Thus, behavioral traits do not always evolve faster than physiological ones.

Finally, experimental evolution can be used to test hypotheses about the consequences of behavioral evolution for evolutionary processes in general. For example, Mery and Kawecki (2004) used experimental evolution in *Drosophila* to show that, depending on circumstances, an opportunity to learn may either accelerate or slow down behavioral adaptation to a novel environment. This was the first direct experimental test of a century-old idea (known as the Baldwin effect) that learning may accelerate evolution (Baldwin 1896). In a similar vein, Rice and Salt (1987) showed that ecological reproductive isolation can evolve as a by-product of divergent selection on behavioral ("habitat") preferences (a combination of geotaxis, phototaxis, and odor preference). These studies illustrate the potential of experimental evolution for testing hypotheses of evolutionary adaptations.

RELEVANCE FOR PUBLIC HEALTH

The vast majority of molecular-genetic and neurobiological analyses of behavioral traits has been directed by biomedical interests where the goal is to understand the etiology of mental illness, how to improve the health of the mind, or, more generally, to understand human behavior. Experimental methods in evolution can be useful for studying the etiology of mental illness to the extent that mental disease can be modeled as extreme forms of behavior or neurobiology, symptomatic of the disorder. For example, a great deal of work has explored the neurobiological and genetic bases of motivation for drugs of abuse using animals genetically predisposed to drink intoxicating quantities of ethanol (Mardones and Segovia-Riquelme 1983; Hilakivi et al. 1984; Crabbe et al. 1994; Grahame et al. 1999; Murphy et al. 2002; Kamdar et al. 2007). In principle, the methods described herein hold promise for exploring such mental disorders as addiction, obesity, ADHD (e.g., see Rhodes et al. 2005), mania, learning disorders, stress-related disorders, and depression. It is important to note here that the real challenge is not how to apply the genetic and neurobiological methods, but rather how to represent these illnesses in animal models via behavioral assays that are actually relevant to the human phenomenon (Dole and Gentry 1984; Kamdar et al. 2007).

CONCLUSIONS AND FUTURE DIRECTIONS

Experimental evolution offers powerful tools to identify the origin and mechanisms of behavioral variation at different levels of biological organization, from structural changes in DNA to expression of genes to physiological pathways to behavior. However, some important practical issues should be considered before embarking on an evolutionary experiment on behavior, such as the difficulty in developing an assay to capture the essence of a complex behavior and the sensitivity to subtle, and often uncontrollable, environmental factors. Behaviors are often influenced by multiple genes with complex gene-by-gene, gene-by-environment, and environment-by-environment interactions. This is one reason, for example, that single-gene mutants are relatively uninformative (see also Rauser et al. this volume), though we described a case in which such mutants were useful for exploring mechanisms underlying the evolution of mating systems in voles.

One feature consistent across many different vertebrate models of behavioral evolution is the involvement of the natural reward circuit in the brain. This circuit has many components that play a role in motivation and reinforcement. Specific alterations in signaling between particular regions could change motivation for one behavior versus another. We discussed an example with V1aR in the ventral pallidum of voles that caused increased affiliative social behavior (Young and Wang 2004), and another example in which dopamine signaling is altered in mice bred for increased wheel running (Rhodes et al. 2005). Exactly which substrates are necessary and how they alter motivation to shift behavior has not been worked out for any vertebrate species.

Technology in genotyping, measuring expression of genes (microarray), gene mapping, genetic engineering, and an understanding of how to combine multiple sources of information (bioinformatics) have greatly advanced recently. These tools offer promise for finding genes or regulatory sites on chromosomes, and for testing mechanisms of behavioral evolution.

When the question changes from trying to understand broad correlations across species to testing individual mechanisms within species or among a handful of species, experimental evolution offers a strategy that nicely complements the comparative approach. The time is right for evolutionary biologists interested in behavior and neurobiology to take full advantage of the new and old technology that the molecular and behavior geneticists have brought to the table.

SUMMARY

Animal behavior (e.g., foraging, mating, parental care, aggression, territorial defense, moving through the environment, learning, escaping from predators) has obvious evolutionary significance and is amenable for experimental evolution, provided some creative thought is given to surmount certain methodological obstacles. One unique feature about behavior, as compared with morphological or life-history traits, is that behavior is highly sensitive to small and often uncontrollable environmental influences, as well as the animal's motivational states. This sensitivity reduces reliability or repeatability, and it makes measuring or quantifying behavioral traits relatively difficult. Nonetheless, many successful selection experiments have targeted behavior in species ranging from flies to mice, including behaviors ranging from geotaxis to nest building. This chapter reviews some of the methods used and problems encountered. Several conclusions can be drawn. One is that behavior often evolves rapidly and that the changes in DNA that lead to variation in behavior occur in both coding and noncoding, regulatory regions. These changes ultimately lead to developmental changes in the nervous system at all levels of biological organization (molecules, cells, physiology, morphology). In vertebrates, the natural reward circuit in the brain appears to be a major target where alterations take place to shift motivation that underlies voluntary behavior. For example, both the evolution of increased wheel-running behavior in house mice and increased pair-bonding behavior in voles appears to have resulted from specific changes in this circuit. For wheel running, altered dopamine signaling from the ventral midbrain to forebrain areas was implicated in increased motivation for physical activity; whereas for pair bonding, distribution of arginine vasopressin and oxytocin receptors in various regions of the circuit affected reinforcement of social contact. The technology for genotyping, measuring global expression of genes, and mapping location of genes that influence behavioral traits is rapidly advancing, and it is expected that within the next ten to twenty years, the connection between specific polymorphisms in DNA and variation in behavior will be elucidated for many behavioral traits in a variety of organisms. Results of such studies hold

great promise for biomedicine because finding genes and pathways that regulate behavior can provide useful targets for therapeutic manipulation of maladaptive behavior and mental illness.

REFERENCES

Baldwin, J. M. 1896. A new factor in evolution. *American Naturalist* 30:441–451, 536–553.

Barnett, S. A., and J. L. Smart. 1975. The movements of wild and domestic house mice in an artificial environment. *Behavioral Biology* 15:85–93.

Belke, T. W., and T. Garland, Jr. 2007. A brief opportunity to run does not function as a reinforcer for mice selected for high daily wheel-running rates. *Journal of the Experimental Analysis of Behavior* 88:199–213.

Belyaev, D. K. 1979. Destabilizing selection as a factor in domestication. *Journal of Heredity* 70:301–308.

Belyaev, D. K., A. O. Ruvinsky, and L. N. Trut. 1981. Inherited activation-inactivation of the star gene in foxes. *Journal of Heredity* 72:267–274.

Berthold, P., G. Mohr, and U. Querner. 1990. Control and evolutionary potential of obligate partial migration: Results of a 2-way selective breeding experiment with the blackcap *(Sylvia atricapilla)*. *Journal für Ornithologie* 131:33–45.

Blomberg, S. P., T. Garland, Jr., and A. R. Ives. 2003. Testing for phylogenetic signal in comparative data: Behavioral traits are more labile. *Evolution* 57:717–745.

Boake, C. R. B., ed. 1994. *Quantitative Genetic Studies of Behavioral Evolution*. Chicago: University of Chicago Press.

Brandes, C., B. Frisch, and R. Menzel. 1988. Time-course of memory formation differs in honey bee lines selected for good and poor learning. *Animal Behaviour* 36:981–985.

Bronikowski, A. M., P. A. Carter, J. G. Swallow, I. A. Girard, J. S. Rhodes, and T. Garland, Jr. 2001. Open-field behavior of house mice selectively bred for high voluntary wheel-running. *Behavior Genetics* 31:309–316.

Brush, F. R. 2003. Selection for differences in avoidance learning: The Syracuse strains differ in anxiety, not learning ability. *Behavior Genetics* 33:677–696.

Brush, F. R., and P. C. Sakellaris. 1973. Bidirectional genetic selection for shuttlebox avoidance learning in rat. *Behavior Genetics* 3:396–397.

Bult, A., L. Hiestand, E. A. Van der Zee, and C. B. Lynch. 1993. Circadian rhythms differ between selected mouse lines: A model to study the role of vasopressin neurons in the suprachiasmatic nuclei. *Brain Research Bulletin* 32:623–627.

Bult, A., E. A. van der Zee, J. C. Compaan, and C. B. Lynch. 1992. Differences in the number of arginine-vasopressin-immunoreactive neurons exist in the suprachiasmatic nuclei of house mice selected for differences in nest-building behavior. *Brain Research* 578:335–338.

Carter, P. A., T. Garland, Jr., M. R. Dohm, and J. P. Hayes. 1999. Genetic variation and correlations between genotype and locomotor physiology in outbred laboratory house mice *(Mus domesticus)*. *Comparative Biochemistry and Physiology A Molecular and Integrative Physiology* 123:155–162.

Carter, P. A., J. G. Swallow, S. J. Davis, and T. Garland, Jr. 2000. Nesting behavior of house mice *(Mus domesticus)* selected for increased wheel-running activity. *Behavior Genetics* 30:85–94.

Castillo-Davis, C. I. 2005. The evolution of noncoding DNA: How much junk, how much func? *Trends in Genetics* 21:533–536.

Chandra, S. B. C., G. J. Hunt, S. Cobey, and B. H. Smith. 2001. Quantitative trait loci associated with reversal learning and latent inhibition in honeybees *(Apis mellifera)*. *Behavior Genetics* 31:275–285.

Chesler, E. J., L. Lu, S. Shou, Y. Qu, J. Gu, J. Wang, H. C. Hsu, et al. 2005. Complex trait analysis of gene expression uncovers polygenic and pleiotropic networks that modulate nervous system function. *Nature Genetics* 37:233–242.

Chesler, E. J., L. Lu, J. Wang, R. W. Williams, and K. F. Manly. 2004. WebQTL: Rapid exploratory analysis of gene expression and genetic networks for brain and behavior. *Nature Neuroscience* 7:485–486.

Churchill, G. A., D. C. Airey, H. Allayee, J. M. Angel, A. D. Attie, J. Beatty, W. D. Beavis, et al. 2004. The Collaborative Cross, a community resource for the genetic analysis of complex traits. *Nature Genetics* 36:1133–1137.

Clayton, D. L., and J. V. Paietta. 1972. Selection for circadian eclosion time in *Drosophila melanogaster*. *Science* 178:994.

Cooke, S. J., C. D. Suski, K. G. Ostrand, D. H. Wahl, and D. P. Philipp. 2007. Physiological and behavioral consequences of long-term artificial selection for vulnerability to recreational angling in a teleost fish. *Physiological and Biochemical Zoology* 80:480–490.

Crabbe, J. C., J. K. Belknap, and K. J. Buck. 1994. Genetic animal models of alcohol and drug abuse. *Science* 264:1715–1723.

Crabbe, J. C., and T. J. Phillips. 1993. Selective breeding for alcohol withdrawal severity. *Behavior Genetics* 23:171–177.

DeFries, J. C., M. C. Gervais, and E. A. Thomas. 1978. Response to 30 generations of selection for open-field activity in laboratory mice. *Behavior Genetics* 8:3–13.

Dierick, H. A., and R. J. Greenspan. 2006. Molecular analysis of flies selected for aggressive behavior. *Nature Genetics* 38:1023–1031.

Doerge, R. W. 2002. Mapping and analysis of quantitative trait loci in experimental populations. *Nature Reviews Genetics* 3:43–52.

Dohm, M. R., J. P. Hayes, and T. Garland, Jr. 1996. Quantitative genetics of sprint running speed and swimming endurance in laboratory house mice *(Mus domesticus)*. *Evolution* 50:1688–1701.

Dole, V. P., and R. T. Gentry. 1984. Toward an analogue of alcoholism in mice: Scale factors in the model. *Proceedings of the National Academy of Sciences of the USA* 81:3543–3546.

Dumas, P., Y. Sun, G. Corbeil, S. Tremblay, Z. Pausova, V. Kren, D. Krenova, et al. 2000. Mapping of quantitative trait loci (QTL) of differential stress gene expression in rat recombinant inbred strains. *Journal of Hypertension* 18:545–551.

Edwards, A. C., S. M. Rollmann, T. J. Morgan, and T. F. C. Mackay. 2006. Quantitative genomics of aggressive behavior in *Drosophila melanogaster*. *PLoS Genetics* 2:1386–1395.

Eisen, E. J., ed. 2005. *The Mouse in Animal Genetics and Breeding Research*. London: Imperial College Press.

Falconer, D. S., and T. F. C. Mackay. 1996. *Introduction to Quantitative Genetics*. 4th ed. Essex, U.K.: Longman.

Fink, S., L. Excoffier, and G. Heckel. 2006. Mammalian monogamy is not controlled by a single gene. *Proceedings of the National Academy of Sciences of the USA* 103:10956–10960.

Fitzpatrick, M. J., Y. Ben-Shahar, H. M. Smid, L. E. M. Vet, G. E. Robinson, and M. B. Sokolowski. 2005. Candidate genes for behavioral ecology. *Trends in Ecology & Evolution* 20:96–104.

Fitzpatrick, M. J., E. Feder, L. Rowe, and M. B. Sokolowski. 2007. Maintaining a behaviour polymorphism by frequency-dependent selection on a single gene. *Nature* 447:210–212.

Flint, J., and R. Mott. 2001. Finding the molecular basis of quantitative traits: Successes and pitfalls. *Nature Reviews Genetics* 2:437–445.

Fonio, E., Y. Benjamini, A. Sakov, and I. Golani. 2006. Wild mouse open field behavior is embedded within the multidimensional data space spanned by laboratory inbred strains. *Genes, Brain, and Behavior* 5:380–388.

Fryer, G., and T. D. Iles. 1972. *The Chichlid Fishes of the Great Lakes of Africa*. Neptune City, NJ: t.f.h. publications.

Gammie, S. C., T. Garland, Jr., and S. A. Stevenson. 2006. Artificial selection for increased maternal defense behavior in mice. *Behavior Genetics* 36:713–722.

Gammie, S. C., N. S. Hasen, J. S. Rhodes, I. Girard, and T. Garland, Jr. 2003. Predatory aggression, but not maternal or intermale aggression, is associated with high voluntary wheel-running behavior in mice. *Hormones and Behavior* 44:209–221.

Garland, T., Jr. 2003. Selection experiments: An underutilized tool in biomechanics and organismal biology. Pages 23–56 *in* V. Bels, J. Gasc, and A. Casinos, eds. *Biomechanics and Evolution*. Oxford: BIOS Scientific.

Garland, T., Jr., and S. C. Adolph. 1994. Why not to do two-species comparative studies: Limitations on inferring adaptation. *Physiological Zoology* 67:797–828.

Garland, T., Jr., and P. A. Carter. 1994. Evolutionary physiology. *Annual Review of Physiology* 56:579–621.

Garland, T., Jr., A. F. Bennett, and E. L. Rezende. 2005. Phylogenetic approaches in comparative physiology. *Journal of Experimental Biology* 208:3015–3035.

Gerken, M., and J. Petersen. 1992. Direct and correlated responses to bidirectional selection for dustbathing activity in Japanese quail *(Coturnix coturnix japonica)*. *Behavior Genetics* 22:601–612.

Gingerich, P. D., M. Haq, I. S. Zalmout, I. H. Khan, and M. S. Malkani. 2001. Origin of whales from early artiodactyls: Hands and feet of Eocene Protocetidae from Pakistan. *Science* 293:2239–2242.

Gotz, K. G. 1970. Fractionation of *Drosophila* populations according to optomotor traits. *Journal of Experimental Biology* 52:419.

Grahame, N. J., T. K. Li, and L. Lumeng. 1999. Selective breeding for high and low alcohol preference in mice. *Behavior Genetics* 29:47–57.

Greenspan, R. J. 2003. The varieties of selectional experience in behavioral genetics. *Journal of Neurogenetics* 17:241–270.

———. 2004. *E pluribus unum, ex uno plura*: Quantitative and single-gene perspectives on the study of behavior. *Annual Review of Neuroscience* 27:79–105.

Hammock, E. A., and L. J. Young. 2004. Functional microsatellite polymorphism associated with divergent social structure in vole species. *Molecular Biology and Evolution* 21:1057–1063.

———. 2005. Microsatellite instability generates diversity in brain and sociobehavioral traits. *Science* 308:1630–1634.

Hard, J. J., M. R. Gross, M. Heino, R. Hilborn, R. G. Kope, R. Law, and J. D. Reynolds. 2008. Evolutionary consequences of fishing and their implications for salmon. *Evolutionary Applications* 1:388–408.

Hare, B., I. Plyusnina, N. Ignacio, O. Schepina, A. Stepika, R. Wrangham, and L. Trut. 2005. Social cognitive evolution in captive foxes is a correlated by-product of experimental domestication. *Current Biology* 15:226–230.

Hartl, D. L., and A. G. Clark. 1997. *Principles of Population Genetics*. Sunderland, MA: Sinauer.

Henderson, N. 1997. Spurious association in unreplicated selected lines. *Behavior Genetics* 27:145–154.

Hilakivi, L., C. J. Eriksson, M. Sarviharju, and J. D. Sinclair. 1984. Revitalization of the AA and ANA rat lines: Effects on some line characteristics. *Alcohol* 1:71–75.

Hirsch, J. 1959. Studies in experimental behavior genetics. *Journal of Comparative Physiology* 52:304–308.

Hoekstra, H. E., and J. A. Coyne. 2007. The locus of evolution: Evo devo and the genetics of adaptation. *Evolution* 61:995–1016.

Hoekstra, H. E., R. J. Hirschmann, R. A. Bundey, P. A. Insel, and J. P. Crossland. 2006. A single amino acid mutation contributes to adaptive beach mouse color pattern. *Science* 313:101–104.

Holland, B., and W. R. Rice. 1999. Experimental removal of sexual selection reverses intersexual antagonistic coevolution and removes a reproductive load. *Proceedings of the National Academy of Sciences of the USA* 96:5083–5088.

Horn, J. M., R. Plomin, and R. Rosenman. 1976. Heritability of personality traits in adult male twins. *Behavior Genetics* 6:17–30.

Hughes, K. A., and J. Leips. 2006. Quantitative trait locus analysis of male mating success and sperm competition in *Drosophila melanogaster*. *Evolution* 60:1427–1434.

Innis, N. K. 1992. Tolman and Tryon: Early research on the inheritance of the ability to learn. *American Psychologist* 47:190–197.

Jaarola, M., N. Martinkova, I. Gunduz, C. Brunhoff, J. Zima, A. Nadachowski, G. Amori, et al. 2004. Molecular phylogeny of the speciose vole genus *Microtus* (Arvicolinae, Rodentia) inferred from mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution* 33:647–663.

Jansen, R. C., and J. P. Nap. 2001. Genetical genomics: The added value from segregation. *Trends in Genetics* 17:388–391.

Jordan, K. W., T. J. Morgan, and T. F. Mackay. 2006. Quantitative trait loci for locomotor behavior in *Drosophila melanogaster*. *Genetics* 174:271–284.

Joshi, A., and L. D. Mueller. 1993. Directional and stabilizing density-dependent natural selection for pupation height in *Drosophila melanogaster*. *Evolution* 47:176–184.

Kamdar, N., S. Miller, Y. Syed, R. Bhayana, T. Gupta, and J. S. Rhodes. 2007. Acute effects of Naltrexone and GBR 12909 on ethanol drinking-in-the-dark in C57BL/6J mice. *Psychopharmacology* 192:207–217.

Kaun, K. R., T. Hendel, B. Gerber, and M. B. Sokolowski. 2007. Natural variation in *Drosophila* larval reward learning and memory due to a cGMP-dependent protein kinase. *Learning & Memory* 14:342–349.

Kawecki, T. J., and F. Mery. 2006. Genetically idiosyncratic responses of *Drosophila melanogaster* populations to selection for improved learning ability. *Journal of Evolutionary Biology* 19:1265–1274.

Keller, L., and J. D. Parker. 2002. Behavioral genetics: A gene for supersociality. *Current Biology* 12:R180–R181.

Kliethermes, C. L., and J. C. Crabbe. 2006. Genetic independence of mouse measures of some aspects of novelty seeking. *Proceedings of the National Academy of Sciences of the USA* 103:5018–5023.

Kolss, M., and T. J. Kawecki. 2008. Reduced learning ability as a consequence of evolutionary adaptation to nutritional stress in *Drosophila melanogaster*. *Ecological Entomology* 33:583–588.

Kovach, J. K., F. R. Yeatman, and G. Wilson. 1981. Perception or preference: Mediation of gene effects in the color choices of naive quail chicks *(C. coturnix japonica)*. *Animal Behaviour* 29:760–770.

Leamy, L. J., D. Pomp, and J. T. Lightfoot. 2008. An epistatic genetic basis for physical activity traits in mice. *Journal of Heredity* 99:639–646.

Lim, M. M., Z. Wang, D. E. Olazabal, X. Ren, E. F. Terwilliger, and L. J. Young. 2004. Enhanced partner preference in a promiscuous species by manipulating the expression of a single gene. *Nature* 429:754–757.

Lindahl, T. 1981. DNA methylation and control of gene expression. *Nature* 290:363–364.

Lofdahl, K. L., M. Holliday, and J. Hirsch. 1992. Selection for conditionability in *Drosophila melanogaster*. *Journal of Comparative Psychology* 106:172–183.

Lynch, C. B. 1980. Response to divergent selection for nesting behavior in *Mus musculus*. *Genetics* 96:757–765.

———. 1994. Evolutionary inferences from genetic analyses of cold adaptation in laboratory and wild populations of the house mouse. Pages 278–301 *in* C. R. B. Boake, ed. *Quantitative Genetic Studies of Behavioral Evolution*. Chicago: University of Chicago Press.

Mackay, T. F. C., and J. D. Fry. 1996. Polygenic mutation in *Drosophila melanogaster*: Genetic interactions between selection lines and candidate quantitative trait loci. *Genetics* 144:671–688.

Magurran, A., B. Seghers, G. Carvalho, and P. Shaw. 1992. Behavioral consequences of an artificial introduction of guppies *(Poecilia reticulata)* in Trinidad: Evidence for the evolution of anti-predator behavior in the wild. *Proceedings Biological Sciences* 248:117–122.

Malisch, J. L., C. W. Breuner, F. R. Gomes, M. A. Chappell, and T. Garland, Jr. 2008. Circadian pattern of total and free corticosterone concentrations, corticosteroid-binding globulin, and physical activity in mice selectively bred for high voluntary wheel-running behavior. *General and Comparative Endocrinology* 156:210–217.

Manning, A. 1961. The effect of artificial selection for mating speed in *Drosophila melanogaster*. *Animal Behaviour* 9:82–92.

Mardones, J., and N. Segovia-Riquelme. 1983. Thirty-two years of selection of rats by ethanol preference: UChA and UChB strains. *Neurobehavioral Toxicology and Teratology* 5:171–178.

Mayr, E. 1958. Behavior and systematics. *In* A. Roe and G. G. Simpson, eds. *Behavior and Evolution*. New Haven, CT: Yale University Press.

McGuire, T. R., and T. Tully. 1987. Characterisation of genes involved with classical conditioning that produces differences between bidirectionally selected strains of the blow fly *Phormia regina*. *Behavior Genetics* 17:97–107.

Mery, F., A. T. Belay, A. K. So, M. B. Sokolowski, and T. J. Kawecki. 2007. Natural polymorphism affecting learning and memory in *Drosophila*. *Proceedings of the National Academy of Sciences of the USA* 104:13051–13055.

Mery, F., and T. J. Kawecki. 2004. The effect of learning on experimental evolution of resource preference in *Drosophila melanogaster*. *Evolution* 58:757–767.

Miyatake, T., K. Tabuchi, S. K., K. Okada, K. Katayama, and S. Moriya. 2008. Pleiotropic antipredator strategies, fleeing and feigning death, correlated with dopamine levels in *Tribolium castaneum*. *Animal Behaviour* 75:113–121.

Murphy, J. M., R. B. Stewart, R. L. Bell, N. E. Badia-Elder, L. G. Carr, W. J. McBride, L. Lumeng, et al. 2002. Phenotypic and genotypic characterization of the Indiana University rat lines selectively bred for high and low alcohol preference. *Behavior Genetics* 32:363–388.

Olausson, P., J. D. Jentsch, N. Tronson, R. L. Neve, E. J. Nestler, and J. R. Taylor. 2006. DeltaFosB in the nucleus accumbens regulates food-reinforced instrumental behavior and motivation. *Journal of Neuroscience* 26:9196–9204.

Osborne, K. A., A. Robichon, E. Burgess, S. Butland, R. A. Shaw, A. Coulthard, H. S. Pereira, et al. 1997. Natural behavior polymorphism due to a cGMP-dependent protein kinase of *Drosophila*. *Science* 277:834–836.

Oufiero, C., and T. Garland, Jr. 2007. Evaluating performance costs of sexually selected traits. *Functional Ecology* 21:676–689.

Page, R. E., K. D. Waddington, G. J. Hunt, and M. K. Fondrk. 1995. Genetic determinants of honey bee foraging behaviour. *Animal Behaviour* 50:1617–1625.

Palmer, A. A., M. Verbitsky, R. Suresh, H. M. Kamens, C. L. Reed, N. Li, S. Burkhart-Kasch, et al. 2005. Gene expression differences in mice divergently selected for methamphetamine sensitivity. *Mammalian Genome* 16:291–305.

Pereira, H. S., and M. B. Sokolowski. 1993. Mutations in the larval foraging gene affect adult locomotory behavior after feeding in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the USA* 90:5044–5046.

Renger, J. J., W. D. Yao, M. B. Sokolowski, and C. F. Wu. 1999. Neuronal polymorphism among natural alleles of a cGMP-dependent kinase gene, foraging, in *Drosophila*. *Journal of Neuroscience* 19:RC28 (21–28).

Rhodes, J. S., S. C. Gammie, and T. Garland, Jr. 2005. Neurobiology of mice selected for high voluntary wheel running activity. *Integrative and Comparative Biology* 45:438–455.

Rhodes, J. S., and T. Garland, Jr. 2003. Differential sensitivity to acute administration of Ritalin, apomorphine, SCH 23390, but not raclopride in mice selectively bred for hyperactive wheel-running behavior. *Psychopharmacology* 167:242–250.

Rhodes, J. S., T. Garland, Jr., and S. C. Gammie. 2003. Patterns of brain activity associated with variation in voluntary wheel-running behavior. *Behavioral Neuroscience* 117:1243–1256.

Rhodes, J. S., G. R. Hosack, I. Girard, A. E. Kelley, G. S. Mitchell, and T. Garland, Jr. 2001. Differential sensitivity to acute administration of cocaine, GBR 12909, and fluoxetine in mice selectively bred for hyperactive wheel-running behavior. *Psychopharmacology* 158:120–131.

Rice, W. R. 1987. Speciation via habitat specialization: the evolution of reproductive isolation as a correlated character. *Evolutionary Ecology* 1:301–314.

Ricker, J. P., and J. Hirsch. 1988. Genetic changes occurring over 500 generations in lines of *Drosophila melanogaster* selected divergently for geotaxis. *Behavior Genetics* 18:13–25.

Ritchie, M. G., and C. P. Kyriacou. 1996. Artificial selection for a courtship signal in *Drosophila melanogaster*. *Animal Behaviour* 52:603–611.

Robinson, G. E. 2004. Genomics. Beyond nature and nurture. *Science* 304:397–399.

Rogers, D. W., R. H. Baker, T. Chapman, M. Denniff, A. Pomiankowski, and K. Fowler. 2005. Direct and correlated responses to artificial selection on male mating frequency in the stalk-eyed fly *Cyrtodiopsis dalmanni*. *Journal of Evolutionary Biology* 18:642–650.

Ross, K. G., and L. Keller. 1998. Genetic control of social organization in an ant. *Proceedings of the National Academy of Sciences of the USA* 95:14232–14237.

Ruber, L., and D. Adams. 2001. Evolutionary convergence of body shape and trophic morphology in cichlids from Lake Tanganyika. *Journal of Evolutionary Biology* 14:325–332.

Rutledge, C. E., and R. N. Wiedenmann. 2003. An attempt to change habitat preference of a parasitoid, *Cotesia sesamiae* (Hymenoptera: Braconidae), through artificial selection. *Journal of Entomological Science* 38:93–103.

Savolainen, P., Y. P. Zhang, J. Luo, J. Lundeberg, and T. Leitner. 2002. Genetic evidence for an East Asian origin of domestic dogs. *Science* 298:1610–1613.

Schadt, E. E., J. Lamb, X. Yang, J. Zhu, S. Edwards, D. Guhathakurta, S. K. Sieberts, et al. 2005. An integrative genomics approach to infer causal associations between gene expression and disease. *Nature Genetics* 37:710–717.

Scheiner, R., M. B. Sokolowski, and J. Erber. 2004. Activity of cGMP-dependent protein kinase (PKG) affects sucrose responsiveness and habituation in *Drosophila melanogaster*. *Learning & Memory* 11:303–311.

Schliekelman, P. D. 2008. Statistical power of expression QTLs for mapping of complex trait loci in natural populations. *Genetics*. Available: www.genetics.org/cgi/rapidpdf/genetics.107.076687v1.

Shirley, R. L., N. A. Walter, M. T. Reilly, C. Fehr, and K. J. Buck. 2004. Mpdz is a quantitative trait gene for drug withdrawal seizures. *Nature Neuroscience* 7:699–700.

Silver, L. M. 1995. *Mouse Genetics: Concepts and Applications*. New York: Oxford University Press. Available: www.informatics.jax.org/silverbook/.

Singer, M. C. 2000. Reducing ambiguity in describing plant-insect interactions: "Preference," acceptability" and "electivity." *Ecology Letters* 3:159–162.

Singh, B. N., and M. B. Pandey. 1993. Selection for high and low pupation height in *Drosophila ananassae*. *Behavior Genetics* 23:239–243.

Spuhler, K. P., D. W. Crumpacker, J. S. Williams, and B. P. Bradley. 1978. Response to selection for mating speed and changes in gene arrangement frequencies in descendants from a single population of *Drosophila pseudoobscura*. *Genetics* 89:729–749.

Steinberg, S., M. Dicke, L. E. M. Vet, and R. Wanningen. 1992. Response of the braconid parasitoid *Cotesia* (=*Apanteles*) *glomerata* to volatile infochemicals: Effects of bioassay set-up, parasitoid age and experience and barometric flux. *Entomologia Experimentalis et Applicata* 63:163–175.

Swallow, J. G., and T. Garland, Jr. 2005. Selection experiments as a tool in evolutionary and comparative physiology: Insights into complex traits: An introduction to the symposium. *Integrative and Comparative Biology* 45:387–390.

Swallow, J. G., P. A. Carter, and T. Garland, Jr. 1998. Artificial selection for increased wheel-running behavior in house mice. *Behavior Genetics* 28:227–237.

Taper, M. L. 1990. Experimental character displacement in the adzuki bean weevil, *Callosobruchus chinensis*. Pages 289–301 *in* K. Fujii, ed. *Bruchids and Legumes: Economics, Ecology and Coevolution*. Dordrecht: Kluwer Academic.

Toma, D. P., K. P. White, J. Hirsch, and R. J. Greenspan. 2002. Identification of genes involved in *Drosophila melanogaster* geotaxis, a complex behavioral trait. *Nature Genetics* 31:349–353.

Trut, L. N. 1999. Early canid domestication: The farm-fox experiment. *American Scientist* 87:160–169.

Tryon, R. C. 1940. Genetic differences in maze learning in rats. *Yearbook of the National Society for the Study of Education* 39:111–119.

Weber, K. E. 1996. Large genetic change at small fitness cost in large populations of *Drosophila melanogaster* selected for wind tunnel flight: Rethinking fitness surfaces. *Genetics* 144:205–213.

Wehner, J. M., R. A. Radcliffe, and B. J. Bowers. 2001. Quantitative genetics and mouse behavior. *Annual Review of Neuroscience* 24:845–867.

Welbergen, P., and F. R. Vandijken. 1992. Asymmetric response to directional selection for licking behavior of *Drosophila melanogaster* males. *Behavior Genetics* 22:113–124.

Wells, T., and D. A. Carter. 2001. Genetic engineering of neural function in transgenic rodents: Towards a comprehensive strategy? *Journal of Neuroscience Methods* 108:111–130.

Whitehead, A., and D. L. Crawford. 2006. Variation within and among species in gene expression: Raw material for evolution. *Molecular Ecology* 15:1197–1211.

Winslow, J. T., N. Hastings, C. S. Carter, C. R. Harbaugh, and T. R. Insel. 1993. A role for central vasopressin in pair bonding in monogamous prairie voles. *Nature* 365:545–548.

Yalcin, B., S. A. Willis-Owen, J. Fullerton, A. Meesaq, R. M. Deacon, J. N. Rawlins, R. R. Copley, A. P. Morris, J. Flint, and R. Mott. 2004. Genetic dissection of a behavioral quantitative trait locus shows that Rgs2 modulates anxiety in mice. *Nature Genetics* 36:1197–1202.

Young, L. J., R. Nilsen, K. G. Waymire, G. R. MacGregor, and T. R. Insel. 1999. Increased affiliative response to vasopressin in mice expressing the V1a receptor from a monogamous vole. *Nature* 400:766–768.

Young, L. J., and Z. Wang. 2004. The neurobiology of pair bonding. *Nature Neuroscience* 7:1048–1054.