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PHYSIOLOGICAL ADAPTATION IN LABORATORY ENVIRONMENTS

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ENVIRONMENTAL STRESS IN THE LABORATORY

Almost any study in experimental evolution requires an altered environment in which selection is expected to occur. Sometimes the environmental variable can be biological (e.g., predators, potential mates). Often, however, it is the abiotic environment that is changed. In nature, thermodynamic variables such as temperature, pressure, and chemical activity (i.e., the concentration of salts, hydrogen ions, etc.) differ across habitats. Life itself requires input of raw materials from the environment (nutrients, water, ions, etc.) that can then be used to drive physiological processes and make more organisms.

We consider here two categories of environmental variables that have been used as selective agents in laboratory natural selection experiments. Temperature is the most important and common *physical* variable affecting the distribution and abundance of organisms in nature, as a 10°C increase in temperature causes most biochemical reactions to increase in rate two- to threefold. Typical physiological temperatures span 0°–40°C, although more extreme limits are well known (e.g., overwintering plants and insects, hot springs bacteria). Thus, selection experiments using temperature may be highly relevant to the real world. For aquatic organisms, the osmotic strength of the surrounding medium is an important environmental variable. Hypotonic or hypertonic surroundings can cause water to leak in or out, respectively, and a few selection studies have considered the effects of medium concentration.

The second category is *resource* variables, chemicals organisms need for survival and reproduction, energy sources and water being the most fundamental. Heterotrophic organisms also need chemicals such as essential amino acids, polyunsaturated fatty acids in the case of animals, oxygen in organisms using aerobic respiration, and so forth. One reason to distinguish resource from physical variables is the time course of imposed stress. The physical environment can be altered very rapidly—for example by an abrupt temperature shift or transfer into a new medium—whereas resource stress generally requires longer time scales. It takes time for an organism to consume resources, so phenotypic responses that reduce consumption rates may be part of the evolutionary response (Garland and Kelly 2006). In addition, acquisition of resources before selection is imposed (i.e., behavioral or life-history differences contributing to resource storage) further increases the time frame for evolutionary responses.

TEMPERATURE STRESS IN *DROSOPHILA*

Temperature has frequently been used as an agent of selection in laboratory experiments (see also Estes and Teotonio this volume; Huey and Rosenzweig this volume; Swallow et al. this volume). Although surprisingly little is known about the natural thermal environment of *Drosophila*, numerous studies have showed that flies respond to temperature selection (reviewed by Hoffmann et al. 2003). The experimental approaches used to study the evolution of thermal resistance have varied widely (Hoffmann et al. 2003, table 3 therein),

including acute selection for performance at extreme temperatures and laboratory natural selection of populations maintained continuously at moderately high or low temperatures. Despite the different methodologies, selection experiments highlight the ample genetic variation for thermal resistance.

Direct lab selection for increased heat survival can be difficult, as surviving flies are often sterile. Siblings of the stressed flies can be used to rear the next generation, but because siblings are only 50 percent identical genetically, this procedure provides a slower selective response. Alternative methods have therefore been developed. For example, selection for high "knockdown temperature" (T_{KD}), at which insects lose their ability to cling to vertical surfaces, results in a rapid increase in heat tolerance in *D. melanogaster* (Huey et al. 1991). In a modification of the method, knockdown times for *D. melanogaster* at 39°C increased fourfold after eighteen generations of selection (McColl et al. 1996). Similarly, flight ability following exposure to high temperature stress also responds rapidly to selection (Krebs and Thompson 2006). Perhaps the most interesting finding from thermal knockdown experiments is that T_{KD} is bimodally distributed in natural populations of *D. melanogaster* (Gilchrist and Huey 1999; Folk et al. 2006), probably due to polymorphism in a gene with major effect. Selection for high or low T_{KD} removes the low or high mode, respectively, from the population.

Measures of thermal tolerance usually exhibit consistent patterns across different assays (Hoffmann et al. 2003). For example, in natural populations of *D. buzzatii*, thirteen of nineteen assayed traits relevant to thermal adaptation were found to be significantly correlated with climatic variables (Sarup et al. 2006). However, selection for increased knockdown resistance does not always result in correlated increases in other thermoresistance traits, such as survival, recovery time, or critical thermal maximum, suggesting different genetic bases for these traits (Hoffmann et al. 1997; Bubliy et al. 1998; Folk et al. 2007). Microarray data on stress-selected populations support this conclusion. Many genes exhibit higher or lower expression in populations selected in different ways for heat tolerance (heat shock, T_{KD}, or chronic high temperatures; Sorensen et al. 2007). However, there is very little overlap between these sets of genes, suggesting there is fundamental variation in how organisms respond to different types of thermal stress.

It is not clear which of the different traits often assayed for comparing selected or natural populations is the most relevant for Darwinian fitness, and therefore the evolution of thermal resistance in nature. While T_{KD} may be an ecologically relevant indicator of adaptation to high temperature (an animal that cannot move cannot forage or evade predators), heat shock survival may not be, as extreme high temperatures are unlikely to be reached without prior gradual warming that induces the heat shock response (Sorensen et al. 2001, 2005). Indeed, a potentially important problem in selection experiments is phenotypic plasticity (Garland and Kelly 2006). Even a brief experimental treatment will cause changes in neuronal, hormonal, and intracellular signaling, gene expression, membrane properties, and so forth. Organisms respond rapidly to changes in their environment, and these responses can themselves be targets of selection.

Many thermal selection experiments using *Drosophila* have subjected populations to constant high or low temperatures (Kilias and Alahiotis 1985; Huey et al. 1991; Loeschke and Krebs 1996). Phenotypic responses to temperature change are then of less concern, but the simplicity of these experiments reduces their relevance to the real world. In nature, flies may experience the mean temperature only twice a day, as temperatures increase in the morning and decline in the evening (Feder 1997). Variable thermal regimes are clearly more ecologically relevant. Designing experiments with ecologically relevant thermal conditions is complicated by the scarcity of information regarding the temperatures *Drosophila* experience in the field. Adults can potentially avoid high temperature be seeking cooler microclimates, although these may not necessarily be available (Gibbs et al. 2003b). Immobile life stages (eggs, pupae, and to some extent larvae) are more likely to be targets for thermal adaptation (Sarup et al. 2006), but these stages have not been the subject of laboratory evolution experiments.

Survival is not the only fitness trait affected during adaptation to changing thermal environments. Life-history traits (see also Zera and Harshman this volume) also respond to temperature selection. Comparison of *D. melanogaster* populations adapted in the lab to 16.5° and 25°C showed that females had higher fertility and fecundity at their selection temperature in comparison with females adapted to the other (Partridge et al. 1995). Likewise, a comparison of *D. melanogaster* adapted to 18° and 25°C for ten years in lab culture found a higher mating success with control females for males at their selection temperature compared with males selected at a different temperature (Dolgin et al. 2006).

TEMPERATURE SELECTION IN MICROBES

Studies of temperature selection in bacteria and other microorganisms have primarily been motivated by interest in testing theoretical predictions regarding trade-offs in adaptation (see also Futuyma and Bennett this volume). Does evolutionary adaptation at high temperatures, for example, result in reduced fitness at low temperatures? Does evolution at a single temperature result in reduced fitness at other temperatures (i.e., a narrower thermal niche) or reduced fitness in variable environments? Does adaptation to one type of stress improve bacterial strains' ability to survive other stresses, suggesting that there are general mechanisms of stress resistance? A fundamental advantage of *Escherichia coli* and most other microbes is the ability to store stocks, including the ancestral strain, in a frozen condition, then revive them. Thus, fitness changes can be directly assessed by competition experiments using ancestral and evolved strains. (More details on how these experiments are performed can be found in Estes and Teotonio this volume; Futuyma and Bennett this volume; Travisano this volume.)

Several early studies by Bennett, Lenski, and colleagues suggested that predictions of trade-offs were true. Replicated strains of *E. coli* that had evolved at 42°C for two thousand generations had higher fitness than their ancestor at this temperature, but they had

reduced relative fitness at the ancestral temperature of 37°C (Lenski and Bennett 1993). Surprisingly, the upper thermal limit for growth did not increase very much. Mutants that did achieve higher thermal tolerance lost fitness at lower temperatures (Mongold et al. 1999), also consistent with the existence of trade-offs. After twenty thousand generations at 37°C, fitness declined at extreme temperatures (Cooper et al. 2001), indicating narrowing of the thermal niche. Subsequent work reveals that trade-offs in performance at different temperatures, although common, are not universal (Bennett and Lenski 2007).

Of course, natural thermal habitats are not static. Microbes have also been selected for performance in variable habitats, either predictable (Leroi et al. 1994) or randomly changing (Ketola et al. 2004). In the former study, lines that were alternated between 32° and 42°C showed improved fitness in the variable environment, as well as at each of these temperatures when held under constant conditions. Surprisingly, they actually showed decreased fitness, relative to the ancestor, during the transition between temperatures (Leroi et al. 1994). Thus, just when phenotypic responses to changing conditions would seem to be most critical, lines selected in variable thermal regimes did not improve.

LABORATORY EVOLUTION IN AQUATIC ENVIRONMENTS: OSMOTIC STRESS

Temperature is not the only physical variable. Besides hydrostatic pressure, for which we are unaware of any experimental evolution studies, the osmotic strength of the medium is an important factor for aquatic organisms (Evans 1993). Only a handful of studies have examined laboratory selection for osmotic resistance in animals. For example, one of us (A.G.G.) once received a set of salt-selected *Drosophila* populations from J. S. F. Barker. One line developed and lived its adult life on media containing 9.5 percent NaCl (nearly three times the strength of seawater). We eventually disposed of these unreplicated lines (because they grew so vigorously that they threatened to invade other lab stocks), and Barker apparently never published anything on them, so we know nothing about their mechanisms of adaptation. Clearly, however, salt selection works in *Drosophila*.

More relevant to the real world are selection experiments using aquatic organisms. Marine teleost fishes are hypo-osmotic relative to their surroundings and therefore must ingest seawater to balance the osmotic loss of water. As the teleost fish kidney cannot produce hyperosmotic urine, mitochondria-rich cells (MRCs) in the gills, often referred to as "chloride cells," are a major site for osmoregulation through excretion of Na^+ and Cl^-. Euryhaline teleosts transferred from freshwater to seawater exhibit correlated changes in both number and structure of MRCs (Evans et al. 2005). In addition to the plasticity in this trait, levels of proliferation and hypertrophy of MRCs (and the ensuing osmoregulatory capacity) also appear to have a genetic component. Three generations of selection for high salinity tolerance in the guppy *Poecilia reticulata* resulted in an increase in both size and number of chloride cells (Shikano et al. 1998). This was correlated with increased

seawater tolerance and enhanced osmoregulatory function, in comparison with controls from the same original fish stock.

The Baltic copepod, *Eurytemora affinis*, has invaded numerous aquatic ecosystems in the United States over the past century (Lee 1999). This species is normally intolerant of fresh water, but invasive populations have colonized several river drainages. Freshwater and saline populations have diverged in their osmoregulatory capabilities and survival at low salinities. When reared at an intermediate salinity for six generations, freshwater populations survive better at low salinity, but their survival is the same at high salinity (Lee et al. 2007). Recent work suggests that the physiological differences that evolved in the laboratory mimic those observed in natural populations (C. E. Lee, personal communication).

RESOURCE LIMITATION IN INSECTS: SURVIVAL OF THE FATTEST?

Because of the Second Law of Thermodynamics (entropy always increases in an isolated system), organisms need an input of energy simply to maintain themselves. Starvation stress presents an interesting range of potential survival strategies. Organisms can acquire energy and store it in anticipation of food limitation, as do many animals in nature as winter approaches, or decrease energy consumption (i.e., metabolic rate) when food is unavailable. The first option is well documented in *Drosophila*. Starvation-selected populations accumulate large quantities of lipids, the most energy-dense storage form (Chippindale et al. 1998). Differences in energy content can explain almost all of the variation in starvation resistance in *Drosophila* populations selected for various physiological and life-history traits (Chippindale et al. 1996; Bradley and Folk 2004; Zera and Harshman this volume).

Starvation selection experiments serve as a good example of the possible effect of different experimental protocols on the results and their interpretation. Experiments in *Drosophila* usually involve exposure of selected populations to stressful conditions as young adults, typically about fourteen days after egg collection, corresponding to an adult age of about four days posteclosion (Chippindale et al. 1996, 1998; Djawdan et al. 1997). This selection procedure resulted in a twofold increase in resistance after twenty generations (Rose et al. 1992). A different experimental approach, selecting on newly eclosed flies, resulted in a less profound increase in resistance following twenty generations of selection, possibly by omitting adult feeding from the overall response to selection (Baldal et al. 2006). Both larval and adult derived energy are important sources of adult nutrition, even in unselected lines. Wild-type flies actually become less starvation resistant in the first few days of adult life, even as they are feeding (Aguila et al. 2007). In contrast, starvation-selected populations show increased starvation resistance in early adulthood (Chippindale et al. 1996).

The effects of starvation selection on metabolic rate are not clear. Hoffmann and Parsons (1989) reported that mass-specific metabolic rates were lower in stress-selected populations. A potential analysis problem arises in animals that store very large quantities of

energy as lipids or glycogen. This storage may come at a low energy cost, so expressing metabolic rates per unit mass may bias estimates downward. Should glycogen and lipid sitting in a cell be counted as part of the metabolizing animal? Djawdan et al. (1997) argued that it should not and that a better measure of mass is the lipid- and carbohydrate-free mass. When they subtracted energy stores from the total mass, starvation-selected flies did not have lower mass-specific metabolic rates. Harshman and Schmid (1998) used a third approach, the per-animal metabolic rate, and also found no reduction in metabolism. In summary, it appears that lower metabolic rates can evolve, but the primary selection response is increased energy storage before starvation is imposed.

Nutritional selection need not require complete removal of food. Low food quality has been used in selection experiments in a few cases. Harshman et al. (1999) reared replicate populations on lemons (a poor larval substrate) or standard *Drosophila* medium. Many of the same physiological differences appeared as observed in the studies described here. Lemon-selected flies had greater energy stores and lower metabolic rates (quantified on a mass-specific basis). (See also Garland and Kelly 2006 for a discussion of evolved differences in plasticity in these lines.) Lemon-reared larvae developed slowly, and their slower development remained when reared on bananas. In a non-*Drosophila* example, Warbrick-Smith et al. (2006) varied the dietary protein/carbohydrate ratio of *Plutella xylostella* caterpillars and demonstrated changes in lipid storage after six generations of selection. Another example of nutritional selection concerns *Drosophila* reared for ninety generations in the presence of ethanol (Fry 2001). Ethanol is a potential energy source for all life stages in nature but potentially toxic at high levels. In fact, adult *D. mojavensis* can actually gain dry mass with ethanol vapor as its sole food source (Etges 1989). In the presence of ethanol, selected lines developed faster and survived better to adulthood than controls, without any disadvantage (i.e., trade-off) in the absence of ethanol (Fry 2001).

The rapid response to starvation selection in laboratory experiments illustrates the substantial genetic variability in natural populations, which should result in differences between natural populations exposed to different environmental conditions. Several studies report negative correlations between latitude and starvation resistance in *Drosophila* species (Karan and Parkash 1998; Karan et al. 1998; Parkash and Munjal 2000; Hoffmann et al. 2001), but these correlations are inconsistent (Harshman and Hoffmann 2000; Griffiths et al. 2005). Furthermore, Hoffmann et al. (2001) found that the latitudinal cline effect on starvation resistance in Australian populations of *D. melanogaster* was weaker than the variation within populations. Indeed, conflicting results led Karan et al. (1998) to argue that, because flies always survive starvation longer than desiccation, flies will die of starvation in nature only in humid areas.

Variation in starvation resistance to among natural populations could also result from a correlated response to selection for resistance other stresses. For example, mechanisms associated with resistance to starvation and cold stresses are antagonistic (Hoffmann et al. 2005). Latitude is generally negatively correlated with winter temperature; thus,

decreased starvation resistance with latitude may be associated with increased cold resistance. Cold tolerance is also affected by altitudinal differences (Collinge et al. 2006), and therefore clines for starvation resistance could be affected by altitudinal differences along the latitudinal gradient. Furthermore, high- and low-altitude sites are often separated by short distances thus facilitating gene flow (Blanckenhorn 1997), which may result in deviations from clines. Opposite clines and deviations from clines highlight the need to further explore the causes for evolution of stress resistance in natural populations. Furthermore, they emphasize the extent to which "laboratory natural selection experiments" oversimplify selection in nature (see also Huey and Rosenzweig this volume).

NUTRITIONAL SELECTION IN MICROBIAL POPULATIONS

In bacteria, selection using alternative energy sources has been performed on numerous occasions, using progenitor strains that have been grown on a defined diet for two thousand generations or more. When exposed to novel energy sources, replicate lines diverge markedly in their fitness (Travisano et al. 1995; Travisano and Lenski 1996). This is especially true for carbon sources that do not share the same uptake mechanism as glucose, the ancestral carbon source.

Experiments using serial dilution expose bacteria to fluctuating nutritional conditions: scramble competition for resources when cultures are diluted into fresh medium starvation, followed by resource limitation when food runs out and bacteria enter the stationary phase. The growth phase is especially important for selection. The ability to enter the exponential phase rapidly and grow quickly is critical to relative fitness (Vasi et al. 1994), but indirect evidence suggests that the ability to tolerate nutritional stress is also important. For example, thirty-six lines adapted to serial dilution versus chemostat conditions (chronic poor nutrition) exhibited little sign of trade-offs, as indicated by reduced fitness in the alternative regime (Velicer and Lenski 1999). These results would not be anticipated if rapid growth were the only target of selection in serial dilution experiments. In a different experiment, multiple lines selected for high temperature fitness had similar gene duplication events in a region containing several genes known to affect stress and starvation resistance (Riehle et al. 2001).

In most serial dilution experiments, nutritional stress is relatively brief, less than twenty-four hours. An interesting example of an alternative starvation selection regime comes from *E. coli* subjected to long-term starvation (Vasi and Lenski 1999). Replicated lines were maintained in stationary phase cultures for forty-nine days, long after energy from the media had been consumed, and the density of viable cells had decreased by 99.99 percent. In an example of artificial selection, colonies were grown from the survivors, and only those visibly different from progenitor-strain colonies were selected for experiments. Out of five mutants, three died less rapidly when grown in pure culture, exactly what one might expect. Three, but not the same three, died less rapidly in competition with the progenitor strain, in a frequency-specific manner (figure 17.1). This suggests that

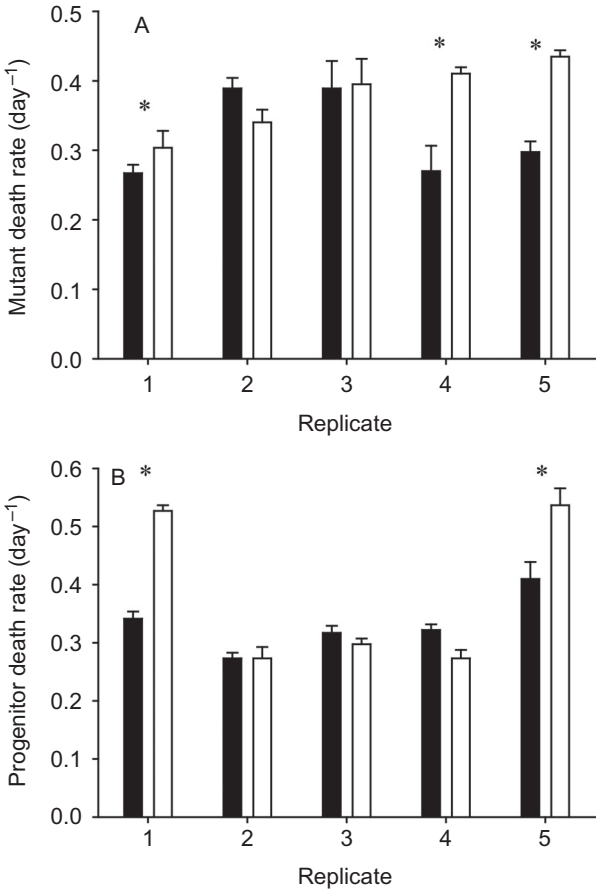

FIGURE 17.1
Death rates of stationary-phase selected and progenitor strains of *E. coli* in competition experiments. A, Death rates of selected lines at low (10 percent) and high (90 percent) relative density. Lower death rates at low density suggest that these strains are acquiring resources from the progenitor, possibly by cannibalism. B, Death rates of progenitor strains at low (10 percent) and high (90 percent) relative density. Higher death rates at low density may indicate allelopathy (bacteriocide) by the selected strain. Data redrawn from Vasi and Lenski (1999).

they were deriving nutrition from live or dead progenitor cells. In two cases, the progenitor died more rapidly when the mutant was more abundant, suggesting that the evolution of allelopathy by the mutant.

The study by Vasi and Lenski (1999) shows that bacteria can evolve to kill and eat the competition. What about in *Drosophila*? Huey et al. (2004) tested and rejected the hypothesis that adult *Drosophila* consume their conspecifics when food is scarce. Larvae are not so fastidious. We have seen active larvae with red guts, apparently from consuming the eye pigments of dead adults in their vials. Typical starvation selection experiments involve population cages containing large numbers of fly corpses, which theoretically could serve as a food source. The potential for evolution of cannibalism in starvation-selected adult flies has not been investigated. Indirect cannibalism, by feeding on microbes colonizing dead conspecifics, is another possibility.

Many bacteria can survive starvation stress by entering a dormant spore state. Hypometabolic states (hibernation, diapause, anhydrobiosis) in animals are well documented, but they have not been observed in laboratory selection studies. This may reflect

the design of the experiments. In most cases, selection continues until a certain proportion of the population appears dead. Food is restored, and those animals that can feed immediately have an advantage. In the case of bacteria, hypometabolic mutants will be flushed out of a chemostat. In experiments involving serial dilution, competition for resources will select for genotypes that can acquire resources quickly, and emergence from dormancy takes time (Vasi et al. 1994).

One selection experiment was designed with hypometabolism in mind. Maughn and Nicholson (2004) selected replicated strains of *Bacillus subtilis* for the ability to form heat-resistant spores under nutritional stress for five thousand generations. The progenitor strain sporulated 70 percent of the time under these conditions. Despite the fact that other strains exhibit varying degrees of sporulation efficiency and increasing mutation rates as selection continued, sporulation efficiency did not respond to selection. The proposed explanation for this surprising result concerns stochasticity in the decision to sporulate. *Bacillus subtilis* can take alternative developmental pathways under stress, including becoming competent for DNA uptake, growth form shifts, adaptive mutagenesis (Robleto et al. 2007), or spore formation (Maughan and Nicholson 2004). If each cell has an independent probability of taking one of these pathways, this bet-hedging strategy can allow survival of the genotype under a variety of stressful conditions. In this example, different strains may differ in which route occurs most often, but the "decision" at the individual level is stochastic. It remains to be seen whether this is a common phenomenon or whether some species or strains can evolve increased sporulation efficiency.

DROSOPHILA IN LABORATORY DESERTS

Water is perhaps the most important resource determining where organisms actually live in terrestrial ecosystems. Two-thirds of the mass of a typical animal is water, and deviations impose severe physiological and cellular stress. Several labs have subjected *Drosophila* to selection for the ability to resist desiccation stress. Desiccation selection generally results in a rapid and significant increase in desiccation resistance of adult flies (reviewed by Hoffmann and Harshman 1999; also see Bubliy and Loeschcke 2005; Gefen et al. 2006). Figure 17.2 shows the response of nine populations of *D. melanogaster* (three source populations) to desiccation selection, as indicated by the time necessary to reach 80–85 percent mortality each generation. Despite similar selection responses in different experiments, response patterns vary because of differences in genetic variation for desiccation resistance (Hoffmann and Parsons 1993; Hoffmann et al 2003). For example, three lines derived from one source population in figure 17.2 responded more rapidly to selection and evolved significantly greater desiccation resistance than the other six populations.

Because desiccation selection has been performed several times, in several labs, it provides an opportunity to examine the repeatability of evolution in different genetic backgrounds. This must be done cautiously, however, as the details of selection, measurements,

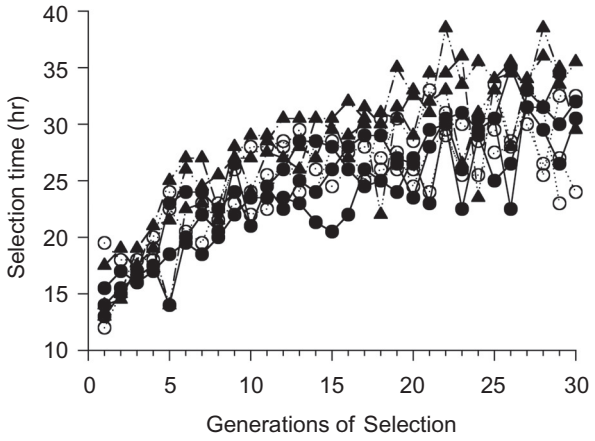

FIGURE 17.2
Desiccation resistance responds rapidly to selection. Data represent the amount of time required for about 85 percent of each cage's population to die in dry air. Three starting populations represented by different symbols were used, with three replicates of each. Note that replicate populations indicated by the triangles consistently outperformed the other two sets of replicates. Data are from A. G. Gibbs and C. H. Vanier (unpublished).

and analytical procedures can differ greatly. A few consistent patterns are evident. Desiccation resistance can evolve through one or more of the following mechanisms: an increase in body water content, reduced rate of water loss to the surrounding environment, and the ability to tolerate reduced body water content as a result of dehydration (dehydration tolerance). Desiccation-selected *D. melanogaster* generally have increased body mass, carbohydrate levels and water contents. In short-term selection experiments, the percentage of body mass composed of water does not change (Hoffmann and Harshman 1999; Folk et al. 2001; Gefen et al. 2006), whereas long-term selection (more than one hundred generations) can result in a differential increase in water content (Gibbs et al. 1997; Folk et al. 2001).

Greater water storage may be related to the high-carbohydrate contents in desiccation-selected flies (Hoffmann and Parsons 1993; Gibbs et al. 1997; Chippindale et al. 1998; Gefen et al. 2006). Glycogen binds three to five times its own mass in water (Schmidt-Nielsen 1997), and therefore accumulation of glycogen could increase body water storage capabilities. Gibbs et al. (1997) estimated that 47 percent of the additional water in selected flies could be bound to intracellular glycogen stores. This water cannot be released unless glycogen is metabolized. Djawdan et al (1997) reported that *D. melanogaster* shift to carbohydrate catabolism under desiccation stress, thereby making free water available to replace that which has been lost. Similarly, carbohydrate catabolism during desiccation stress was significantly higher than during starvation in four out of five *Drosophila* species (Marron et al. 2003). The second location for water storage is the hemolymph. Folk et al. (2001) estimated that selected flies had a

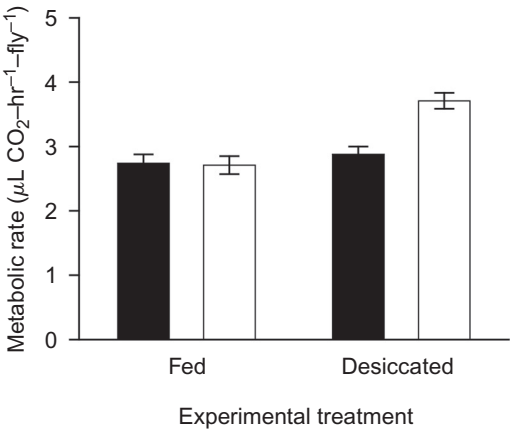

FIGURE 17.3
Metabolic rates of desiccation-selected and control flies. Selected flies (filled bars) do not exhibit an increase in metabolic rate when exposed to dry air, whereas control flies (open bars) do. Carbon dioxide production was measured using flow-through respirometry, in the presence or absence of a food and water source. Data are from A. G. Gibbs and C. H. Vanier (unpublished).

hemolymph volume four times greater than controls, equivalent to 68 percent of their extra water content.

Water loss rates are significantly lower in desiccation-selected flies than in controls (Hoffmann and Parsons 1993; Gibbs et al. 1997; Williams et al. 1997). This may be the result of reduced respiratory water loss, as selected lines are less active (Williams et al. 2004) and exert better spiracular control than control populations (Williams et al. 1997, 1998). This may, in turn, be caused by a reduced behavioral response to desiccation stress (Williams et al. 2004). For convenience, metabolic rates are typically measured in dry air; when flies have access to food and water, the differences in metabolic rate can disappear (figure 17.3). In this example, reduced plasticity in activity and metabolic rate are part of the overall selection response.

Behavioral responses to desiccation are very apparent in natural populations of *Drosophila* and differ according to habitat. Desiccation-sensitive species become very active shortly after exposure to dry air, whereas desert-dwelling species remain inactive for fifteen hours or more (Gibbs et al. 2003a). We note that experimenters have generally measured water loss and metabolic rates early in desiccation stress, when behavioral differences between species or selected populations and their controls are most evident. Thus, some of the reported differences may be artifacts of when measurements were made. Both desert-adapted and desiccation-selected flies may exhibit the same behavioral and physiological responses as mesic or control populations, but these may be delayed until the flies actually "feel" stressed.

Despite consistent changes in water loss rates, carbohydrate storage, and behavior, different studies can yield what appear to be conflicting results. Both age and sex of experimental flies are potential sources of discrepancy. Desiccation resistance of *Drosophila* species decreases with age after the first week of adult life (Lamb 1984; Nghiem et al. 2000; Gibbs and Markow 2001). In contrast, Chippindale et al. (1998) reported that resistance of desiccation-selected males decreased during the first four days posteclosion, but that of females remained unchanged. Female flies generally tend

TABLE 17.1 Water Contents of Control and Desiccation-Selected *Drosophila*

Line	Initial Water Content (mg)	% Water Lost	Net Water Lost (mg)	Net Water Remaining at Death (mg)
C1	1.087	47.36	0.515	0.572
C2	1.058	50.43	0.534	0.524
D1	1.290	60.39	0.779	0.511
D2	1.178	58.22	0.698	0.480

NOTE: Flies were weighed before the experiment and immediately after dying from desiccation stress. Masses are milligrams per individual. Data calculated from Telonis-Scott et al. 2006.

to be more desiccation resistant than males, but Gefen et al. (2006) reported only minor differences between newly eclosed desiccation-selected males and females. Large sex-related differences in older flies would then reflect different patterns of water accumulation in adult males and females.

Apparently conflicting results between lab selection experiments may also stem from different terminology. In contrast to Gibbs et al. (1997), Hoffmann and Parsons (1993) reported that D flies had increased dehydration tolerance compared to their controls. However, this discrepancy may result from different definitions of dehydration tolerance. Hoffmann and Parsons (1993) calculated dehydration tolerance as the total water loss prior to death. Other authors have used similar definitions (Telonis-Scott et al. 2006; Archer et al. 2007), whereas Gibbs et al. (1997) described dehydration tolerance as the body water content at time of death. Table 17.1 uses data from Telonis-Scott et al. (2006) to illustrate how these different definitions can result in different interpretations. Desiccation-selected flies (D1, D2) have higher body water contents than controls (C1, C2) and therefore have more water available to lose. Selected flies lose a greater fraction of their initial water content before they die, but the actual amount remaining in their bodies when death occurs does not differ (table 17.1; Gibbs et al. 1997; Gefen et al. 2006), so it appears these seemingly conflicting reports may actually be in agreement. The higher initial bulk water stores of desiccation-selected flies allow higher total water loss prior to death, which may occur at a similar body water content regardless of selection treatment.

The major routes for water loss from *Drosophila* are cuticular transpiration and respiratory water loss (Gibbs et al. 2003a). Cuticular waxes (the main barrier to water loss) do not differ between selected and control populations (Gibbs et al. 1997), suggesting that desiccation selection had not resulted in a less permeable cuticle. Another possibility that has not been tested in experimental evolution studies is an increase in melanization. In interspecific comparisons, population comparisons, and studies of body color mutants, darker adult *Drosophila* lose water less rapidly than lighter ones and are more resistant to desiccation (Rajpurohit et al. 2008).

If desiccation selection does not affect cuticular permeability, by default respiratory water loss must be reduced. This hypothesis is very difficult to test in such small insects,

but reduced metabolic rates in these lines are consistent with a reduced need to open the spiracles to allow gas exchange (Hoffmann and Harshman 1999; Gibbs 2002; but see Williams and Bradley 1998). Comparative studies of water balance yield similar conclusions; desert species have lower metabolic rates for their size than mesic ones (Gibbs et al. 2003a).

Desiccation selection experiments are generally designed to press the physiological limits of organisms, but that does not preclude behavioral responses. Desiccation-selected *Drosophila* are less active than control populations when water is available and do not become more active when desiccated (Hoffmann and Parsons 1993; Williams et al. 2004). These differences are supported by measurements of metabolic rate in the presence and absence of water (figure 17.3). Control populations have higher metabolic rates when desiccated, in contrast to selected populations. In this case, selected lines have lost the plastic response of increasing metabolic rate, at least during the early stages of desiccation. We have also recently noticed an interesting phenomenon in desiccation-selected flies in our lab. Flies are reared in thirty-five-milliliter vials during preadult

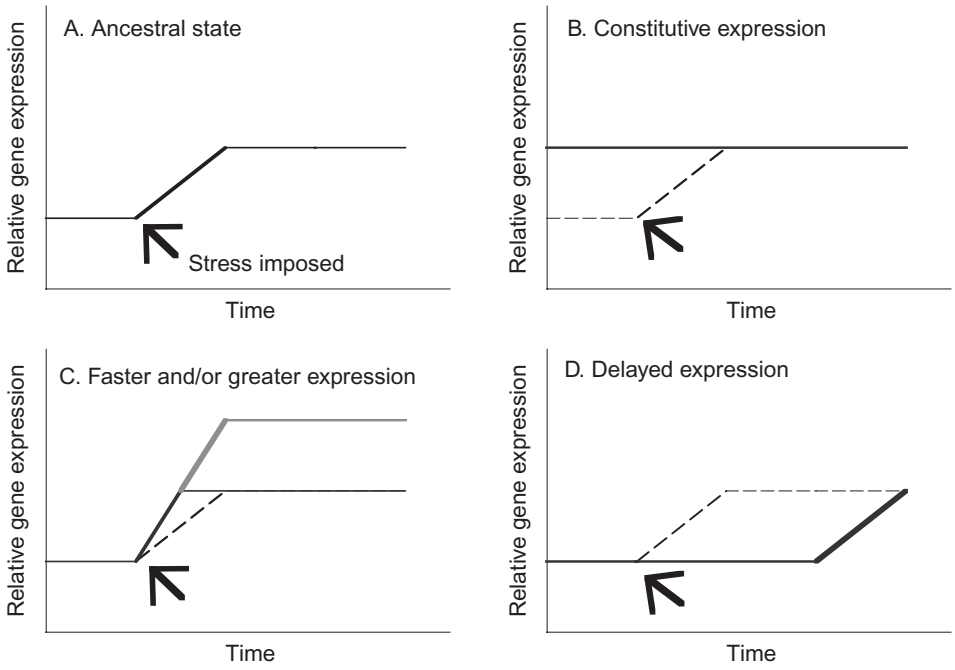

FIGURE 17.4

Potential effects of selection on gene expression. A, In the ancestor, expression of a given gene increases when stress is imposed (arrow). B, Stress selection results in high constitutive expression of the gene. In this and following panels, the dashed indicates the ancestral condition; the solid line, the evolved pattern. C, Selection results in faster induction and/or higher expression under stress (see also Garland and Kelly 2006). D, Induction is delayed, relative to the ancestor, when stress is imposed. This pattern may occur in resource selection, in which increased storage of resources delays stress responses.

stages and the first few days of adult life, then are transferred into population cages (Gefen et al. 2006). Because not all populations can be transferred simultaneously, flies that are transferred first are provided with media until all populations have been dumped. Desiccation-selected flies congregate and feed on the medium, while control populations remain dispersed around their cages. It appears that we have selected for flies that respond to the physical trauma of being dumped into a population cage, by using the last-minute opportunity to acquire additional water and nutrition that they will need to survive the selection bout. Alternatively, they may simply spend more time feeding, both before and after transfer in cages.

Because some *Drosophila* species inhabit desert environments, desiccation selection provides an opportunity to compare evolutionary responses in the lab to those in nature (Gibbs 2002). Desert flies are subjected to highly variable temperatures as well as low humidity (Gibbs et al. 2003b), so comparisons need to be made with caution. Some of the same differences appear in lab-selected and desert *Drosophila*, particularly reduced water loss rates. Desert flies are not more tolerant of dehydration (after correction for phylogeny; Gibbs and Matzkin 2001), nor do desert-adapted flies store more water and carbohydrate than mesic ones. One possible reason for the lack of water and carbohydrate storage in natural populations is that food and water sources are the same in nature. Carrying extra mass in the form of water or energy might compromise flight and the ability to avoid predators. Preliminary work from our lab suggests that, despite their larger mass, desiccation-selected flies do not have larger wings, which results in greater wing loading and potentially diminished flight performance. This is of little consequence in a predator-free, confined ecosystem like a population cage, but potentially important in nature.

WHAT'S THE GENE?

An ultimate goal of evolutionary studies is to understand the nuts and bolts of the evolutionary process. What are the "genes that matter," in nature or in the laboratory? Experimental evolutionists have taken two general approaches to this issue (see also Dykhuizen and Dean this volume; Rosenzweig and Sherlock this volume). The first is to investigate candidate genes that have been independently identified by researchers in other disciplines. A prime example is heat shock genes. HSP70 is a heat-inducible chaperone that helps to minimize cellular damage by binding and enabling refolding of heat-damaged proteins (Feder and Hoffman 1999). These genes are expressed in response to numerous stresses, and most of them are expressed constitutively at a basal level (Hoffmann et al. 2003).

In nature, HSP expression and the threshold for expression often correlate with the levels of stress to which species are exposed naturally and their stress resistance (Feder and Hofmann 1999). In the laboratory, long-term maintenance of *Drosophila* at constant temperatures results in lower basal HSP70 expression (Krebs and Loeschcke 1994;

Cavicchi et al. 1995; Bettencourt et al. 1999), perhaps because it has deleterious effects below the threshold temperature, while HSP expression is higher in lines selected in variable thermal environments (Sorenson 1999). A similar pattern is seen in the protozoan, *Tetrahymena thermophila*, with higher induction of *Hsp90* in lines selected in the most variable conditions (Ketola et al. 2004). Changes in basal HSP level can evolve independently of an increase in HSP induction (Bettencourt et al. 1999).

Naturally occurring protein polymorphisms have been associated with performance differences in stress resistance or clinal variation in natural populations (Dahlhoff and Rank 2000, 2007). These provide candidate genes to examine in laboratory-selected populations. One can also use candidate physiological processes to identify candidate genes. For example, oxidative damage induced by free radicals has been implicated in aging-related loss of function, responses to hyperoxia, reoxygenation damage following hypoxia, salinity stress, desiccation stress, and so forth. It stands to reason that selection to survive such stresses might involve increased expression of proteins that reduce oxidative damage—for example, superoxide dismutase. Even if these efforts are successful (e.g., Deckert-Cruz et al. 1997), however, observations of allele frequency shifts in the lab need to be followed up with more experiments, such as measuring survival of different genotypes or manipulating gene expression in model organisms (Sun et al. 2002).

Despite occasional successes, the use of information from other fields has not been very successful in finding genes that actually are under selection in laboratory experiments. In experimental evolution, many genes will respond to selection, most with relatively minor effect. Even obvious candidates are components of pathways; existing genetic variation in ancestral populations may favor selection on other pathway members. One exception may be T_{KD} in *Drosophila*, where the bimodal distribution of knockdown temperatures is consistent with polymorphism for a gene of major effect existing in natural populations (Gilchrist and Huey 1999; Folk et al. 2006).

Genomic methods provide a second avenue to identify genes under selection (see also Rosenzweig and Sherlock this volume). Whole-genome microarrays allow rapid screening of differences in gene expression, as well as identification of sequence variation between selected and control lines. The first such study used yeast (Ferea et al. 1998), but bacteria provide the most detailed information to date. Microarray experiments have revealed parallel changes in gene expression in replicate lines (Riehle et al. 2001; Cooper et al. 2001, 2003; Crozat et al. 2005). Genes that show up in microarray analyses include heat shock genes, PIMT (a protein repair gene), topoisomerase, and spo1, which is involved in metabolism of the important signaling molecule, ppGpp. Gene duplication and loss also affect thermal tolerance (Riehle et al. 2003). It is encouraging to see that molecular evolution is repeatable to some extent, although the specific mutations that occur usually differ among replicates (Woods et al. 2006). One could just as easily imagine that, for example, different subunits of multiunit proteins, or different enzymes in metabolic pathways, would mutate and have similar effects on fitness. Protein expression experiments also indicate parallel evolution (Pelosi et al. 2006).

As of this writing, one *Drosophila* microarray study using laboratory-selected populations has been published (Sorensen et al. 2007). The study includes lines selected for resistance to high and low temperatures, desiccation and starvation stress, and aging. The source populations used to found the selection lines are problematic, including an eclectic mix of outbred populations, isofemale lines, and even populations that had previously been selected for stress resistance. Clustering analysis revealed that selection replicates had similar expression patterns, again indicating the repeatability of molecular evolution. There was relatively high overlap in the functional categories of genes responding to selection (e.g., metabolic enzymes), but relatively little overlap in specific genes. Thus, the situation in *Drosophila* appears more complicated than in bacteria, with selection apparently acting on the same stress response pathways, but not necessarily the same genes. Of course, the differences between microbial and insect responses could also reflect differences in population size (thousands vs. millions) and number of generations under selection (twenty vs. twenty thousand).

The study by Sorensen et al. (2007) used flies that had been selected for stress resistance but had not been directly exposed to stress. It is likely that selection will affect induction of gene expression as well (i.e., expression plasticity). For example, genes involved in thermal responses may be turned on more quickly or to a higher level in heat-selected lines. Alternatively, if populations have been subjected to resource selection, they may have slower responses to stress. Desiccation-selected *Drosophila* may not mount a response until they have been desiccated for several hours, when controls may already be dead. It is interesting, then, to compare the results of Sorensen et al. (2007; selected lines not exposed to stress) with those in which flies have been directly exposed to stress. Harbison et al. (2005) exposed flies to severe starvation stress (estimated to cause 50 percent mortality) and quantified gene expression across the genome. Over 3,400 genes were differentially expressed during starvation, with nearly half having higher expression. In contrast, Sorensen et al. (2007) found that 230 genes were down-regulated in starvation-selected lines, and none were up-regulated. Only one gene was common to these data sets. Thus, the selection response and the phenotypic response to starvation stress are dramatically different.

The lack of concordance between these studies extends to the functional categories of genes. Starvation selection affects energy acquisition and storage before starvation actually occurs, and it is not surprising that a few carbohydrate metabolism genes are down-regulated in selected lines. However, genes involved in transcriptional regulation are represented much more heavily (more than seventy-five genes out of approximately two hundred annotated genes; Sorensen et al. 2007). In contrast, most of the genes that are differentially regulated during starvation stress are involved in biosynthesis and protein metabolism (Harbison et al. 2005). It will be very interesting to see if selection affects the regulation of these same genes, and certainly more genomic comparisons of selection responses and plastic responses to stress are needed. Figure 17.4 illustrates the types of changes in gene expression one might expect. Genes that are normally turned on (or off) by

stress might evolve differences in constitutive expression, or the speed, degree or timing of the stress response might change (see also Garland and Kelly 2006). To understand which changes are most common, what is needed is a time series of gene expression measurements, in replicate selected and control populations. This would be a large undertaking, but certainly within the scope of current technology and bioinformatics.

Identification of candidate genes is only the first step to demonstrating that these genes actually have an effect on fitness in laboratory populations. A few such experiments have been performed. Sun et al. (2002) overexpressed superoxide dismutase in *D. melanogaster* to confirm its hypothesized role in aging. In *E. coli*, Crozat et al. (2005) transferred candidate genes into the ancestral genetic background and demonstrated that these could account for one-third of the gain in fitness. These studies are clearly just the start of efforts to investigate the function of target genes identified by experimental evolution studies.

UNINTENDED SELECTION IN LABORATORY ENVIRONMENTS

In nature, environmental variables are often highly correlated. For example, high-pressure deep-sea habitats are generally cold, hydrothermal vents being an extremely rare (but extremely interesting) exception. The solubility of oxygen in water is negatively related to temperature; thus, even oxygen-saturated aquatic environments can have less available oxygen than colder, subsaturated regions. In terrestrial environments, the saturating vapor pressure of water increases dramatically with temperature, so that a parcel of air containing the same absolute quantity of water vapor will have a lower relative humidity as it warms. The combination of high temperatures and low humidity in deserts, therefore, provides a double stress. A potential interaction between cold and desiccation stress also exists. Long-tem exposure to subfreezing temperatures can lead to organismal and cellular dehydration (Ring and Danks 1994). Thus, selection for resistance to either cold or desiccation stress might cause a correlated response of resistance to the other.

Physiological ecologists have been well aware of and particularly interested in interactions between environmental variables for decades, but studies in experimental evolution have generally not considered these. We discuss here one hypothetical example. Consider a laboratory natural selection experiment using *Drosophila* populations exposed to high temperatures throughout the life cycle. The larval stages are semiaquatic, usually feeding in the upper one to two centimeters of the medium. At high temperatures, the solubility of oxygen in the media declines. Although larvae are generally considered to respire through the spiracles, they spend much of their time foraging below the surface. Their cuticle is very permeable to water, and oxygen fluxes across the surface are certainly possible.

Oxygen availability affects larval growth rate, developmental time, survival, and adult body size (Loudon 1989; Greenberg and Ar 1996; Frazier et al. 2001; Harrison

et al. 2006). In a selection experiment, Henry and Harrison (2004) reared populations of *D. melanogaster* in atmospheric oxygen levels ranging from 10 to 40 percent. After six generations, larvae from hypoxic populations had wider tracheae than larvae from normoxic and hyperoxic populations, when all were reared in the same conditions (Henry and Harrison 2004). Wider tracheae allow more rapid diffusion of oxygen to the tissues. Interestingly, neither hypoxia nor hyperoxia significantly affected the body mass of third-instar larvae. Instead, adult mass was correlated with rearing oxygen levels, suggesting an important effect of oxygen during pupal development, perhaps related to the reorganization of the tracheal system during metamorphosis.

Drosophila larvae respond to hypoxia by moving to the upper regions of the medium or even out of the medium entirely. This can affect growth rates and body size in two ways. First, larvae that exit the medium cannot feed, so their development will be delayed. Eventually, they must return to the medium, but surface crowding may occur. Food quality under these conditions will be compromised by the accumulation of ammonia (Borash et al. 1998) and other waste products, also contributing to slow development and smaller adult body size. Thus, a seemingly simple temperature selection experiment might inadvertently reduce larval oxygen availability and thereby indirectly select for resistance to hypoxia, larval starvation, and resistance to toxins.

Interactions between the environmental variables of temperature and food quality were examined in one experiment using *Drosophila* (Bochdanovits and de Jong 2003). Larvae were reared at high and low temperatures and on high- and poor-quality food, with adults being maintained at an intermediate temperature. Larvae from the cold selection lines produced consistently larger adults, especially when reared at high temperature. They also had greater survivorship, though development times did not differ. Larvae selected on poor-quality food were not smaller, even though they had lower feeding rates. Significant interactions between selection temperature and food quality appeared for all traits assayed. These complicated results suggest that low temperatures select for efficient nutrient processing. As the authors note, the findings highlight the potential importance of food quality in nature for this widespread species.

Unintentional selection has been well documented in the case of life-history evolution (Chippindale 2006). It may also be common in environmental selection experiments (see also Garland 2003). The example we have discussed is purely hypothetical (so far), but perhaps one example is already known from desiccation selection. In these experiments, selection is imposed by removing flies' access to food, which is their source of water. Thus, the control treatment is usually to have water accessible but not food—in other words, starvation is imposed (Gibbs et al. 1997). Control lines, therefore, undergo starvation stress, and they respond by storing more lipid than selected populations (Chippindale et al. 1996; Djawdan et al. 1997). This makes sense, as starved flies normally metabolize lipids (Marron et al. 2003). Carbohydrate accumulation in desiccation-selected flies (Djawdan et al. 1997) also makes sense, as it is metabolized when flies are desiccated (Marron et al. 2003). Thus, the control treatment undergoes starvation

selection that is relatively mild, but enough to generate a significant physiological response. Is it then a proper control, or would fed flies (Telonis-Scott et al. 2006) be better? One way to address this issue would be to have two control treatments, both fed and starved.

In summary, even seemingly simple selection regimes may contain hidden complexity that can result in unintended selection. Laboratory experimenters need to consider the entire ecology of their model environments, including potential interactions between environmental variables, and even control environments can be problematic. This does not mean selection experiments are not useful, only that they are not as easy to interpret as one would hope.

CONCLUSION

Selection experiments have demonstrated that responses to physical and resource variables are different. Physical variables generally induce direct responses to a change in environmental conditions. This is evident in the improved fitness of bacterial lines selected under variable temperature regimes. Selection in constant physical conditions tends to foster specialization (niche narrowing). In contrast, in insects, selection for survival of resource limitation results in prestress resource accumulation, as well as conservation of existing resources. Thus, preparation for stress is an important component of resource adaptation. Resource selection experiments using microbes have not examined resource acquisition, but other mechanisms have been shown to evolve, such as shorter lag phase and faster growth rates when food is available. In extreme circumstances, allelopathy and cannibalism can evolve.

To what extent does evolution in "simple" laboratory environments mimic nature? The jury is still out (see also Huey and Rosenzweig this volume). Similar physiological differences are often found in stress-selected laboratory populations, natural populations along environmental clines, and species living in different habitats. However, natural environments are far more variable in time and space, making it difficult to identify which environmental variables are most important in shaping populations. That is the great advantage of laboratory selection, but lab environments can have their own complexity. This is well known in the context of life-history evolution (Chippindale 2006; Zera and Harshman this volume), but environmental physiologists also need to keep this in mind.

Finally, high-throughput molecular techniques are beginning to change our understanding of the details of evolution (see also Rosenzweig and Sherlock this volume). These studies are certain to change our understanding of basic biology. Candidate genes identified in genomic analyses of stress-selected populations provide testable hypotheses for their function in stress resistance and under non-stressful conditions. Thus, selection experiments have the potential to inform mechanistic biology (and for the reverse perspective, see Dykhuizen and Dean this volume).

SUMMARY

Environments change on time scales ranging from seconds and minutes to millions of years. Natural environments are complex, sometimes making it difficult to identify general principles of adaptation. In principle, laboratory models simplify the environment to the point where environmental variables can be manipulated independently, thereby providing a complementary approach to interspecific comparative analyses. The types of environmental variables can be broadly separated into physical ones, such as temperature, and resources, such as food and water. One potential advantage of laboratory selection experiments using model organisms is that genomic approaches can provide unparalleled insight into the mechanisms of adaptation. These experiments are in their infancy, but they will eventually allow deeper understanding of both evolutionary and mechanistic biology. Laboratory environments can also contain unintended complexity. This has been recognized in the context of life-history evolution as, for example, resources necessary to survive stress may be acquired well before they are actually needed. Interactions among environmental factors have not generally been considered in experimental evolution, despite their recognition by ecological physiologists outside the laboratory. The relevance of laboratory studies to nature may therefore be problematic, and indeed the complex interactions found in nature may also arise in laboratory settings.

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