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Functional Genomics Requires Ecology

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The problems faced by pre- and post-genomic genetics are therefore much the same—they all involve *bridging the chasm between genotype and phenotype.*
Sydney Brenner (Nobel Laureate, 2002). *The End of the Beginning*, *Science* **287,** 2173

I. THE PROBLEM: MANY GENES SEEM TO BE UNNECESSARY

Since Mendel's time, most genes have been identified by the effects mutations (including knockouts) have on the morphology, physiology, or behavior of individuals. Thus, almost by definition, there could be no ''mutant genes without phenotypes.'' In the molecular era, however, it has become possible to identify genes from DNA sequences. This alternative path to gene discovery has already led to the completion of several genome projects yielding ''complete parts lists'' for representatives of several major branches in the tree of life. This achievement is drawing widespread attention to a paradox that has troubled biologists for more than a decade: many genes lack obvious phenotypes. For example, fewer than half of the estimated 14,000 genes revealed by the recently completed genomic sequence of *Drosophila melanogaster* had been previously identified by ''forward'' (phenotype based) genetics, despite the fact that all genes had been hit multiple times during mutant screens (Rubin and Lewis, 2000). More importantly, a substantial proportion of engineered knockout and knockdown mutations of well-conserved genes in yeast (*Saccharomyces cerevisiae*) (Giaever *et al.*, 2002; Thatcher *et al.*, 1998), *Caenorhabditis elegans* (Kamath *et al.*, 2003; Maeda *et al.*, 2001), and mice (*Mus musculus*) (Shastry, 1995) produce no discernable phenotypic effects. This problem is even more troubling in light of the discovery that

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vertebrate genomes contain many fewer genes than expected (Brookfield, 1997; Lander *et al.*, 2001).

These nonessential genes must have functions or they would wither away like all pseudogenes under the continual rain of deleterious mutations. Yet the phenotypic ''invisibility'' of these genes frustrates efforts to learn what they do, because function cannot be studied effectively (or at all) without phenotypes. Whether a gene encodes an indispensable structural protein or a protein cofactor embedded in a complex biosynthetic pathway, each gene directly or indirectly influences the ultimate coordinated assembly of cells, tissues, organs, and their precise functioning. Any protein in any pathway, no matter how minor its role, is optimized to facilitate some molecular event, whether it is precise timing, expression level, or tissue specificity of other genes in the complex, the binding specificity of the complex, the function of the complex as a whole, or the ultimate regulation of downstream genes and events. The absence of a knockout phenotype, rather than providing evidence for a developmental safety net, is more likely indicating that one or more metabolic pathways are operating at reduced efficiency. The mutant, suspiciously intact in its petri dish or laboratory cage, will actually be compromised in some quantitative way, however small.

Function is fitness is function! The only function of each and every gene that ultimately matters during its evolutionary history is how it contributes to fitness (lifetime reproductive success; Darwin, 1859). Consequently, characterization of gene function will always be incomplete without fitness measurements in settings that simulate the rigorous test environment of nature. Fortunately, fitness-based assays can be exploited to reveal organismal function of mutants. These assays attempt to measure critical components of fitness in the context of important ecological conditions. Almost every biological character is potentially a component of fitness, but critical components of fitness are typically major integrative characters such as mating success, reproductive success, weaning success, survival, and social dominance. The measured components of fitness most dramatically affected will guide attempts to identify more specific effects. For example, if the effect occurs only when comparing across multiple generations then one might look for ''transgenerational characters'' such as parenting and other factors influencing the developmental environment. The important ecological conditions will differ dramatically among species and will also depend on the functions of the mutant that require testing. For many species, fitness assays will require studies conducted in nature (Endler, 1986). But for other species, critical ecological conditions can be simulated under lab settings, for example, *Dictyostelium* (Queller *et al.*, 2003), *Drosophila* (Shabalina *et al.*, 1997), plants (Hayes *et al.*, 2005), and house mice (Meagher *et al.*, 2000). Any lab simulation studies must be cautious

in their conclusions, because such studies will always miss many factors present in nature. Such assays establish the relative importance of the mutant gene for fitness, but more importantly, for the many genes with missing (cryptic) functions, fitness assays will be the most sensitive test for detecting phenotypic change, allowing molecular characterization to proceed.

A. How Big Is the Problem?

1. How Common Are No-Phenotype Gene Knockouts?

The development of molecular technologies, such as transgenes, gene targeting, gene jumping transposons, RNA-mediated interference (RNAi), and other antisense nucleic acid approaches to name a few, have granted biologists the ability to disrupt their favorite gene in a variety of prokaryotic and eukaryotic organisms. Subsequent comparative analysis of mutant and wild-type phenotypes often reveals the disrupted gene's function. Efforts are underway to provide mutants for any desired gene in *C. elegans* (http://www.celeganskoconsortium.omrf.org/), *Drosophila melanogaster* (www.openbiosystems.com; http://flyrnai.org), and every known gene in yeast (http://www.sequence.stanford.edu/group/yeast_deletion_project/deletions3.html) and mice (Austin *et al.*, 2004) (http://www.jax.org/imr/index.html). Knockout studies in each of these model organisms have consistently yielded the surprising result that many genes are nonessential. About 30% of random yeast knockouts show no phenotypic change from wild type (Thatcher *et al.*, 1998), and about 10% of mouse knockouts including a similar proportion of conserved developmental Hox gene knockouts show no phenotype (Duboule, 2000). This mouse figure will be an underestimate because the most important genes have been knocked out first and studies failing to detect a phenotype go unpublished or are slower to be published.

2. How Common Are No-Phenotype Gene Mutants?

Not surprisingly, the frequency of no-phenotype mutants generated by random mutagenesis is much higher than for knockouts of specific genes. Chemical mutagens, such as ethylmethane sulfonate (EMS) in worms and flies, or *N*-ethyl-*N*-nitrosourea (ENU) in mice can be used to induce random point mutations. These techniques yield functional knockouts, as well as hypomorphic, hypermorphic, or neomorphic alleles that differ in overall expression levels or in the spatial and temporal domains of expression. One problem with chemical mutagenesis in diploid organisms is that unless mutant alleles are dominant or semidominant, the screening process requires two generations of reproduction to generate homozygotes capable

of expressing phenotypes. Hence the absence of a phenotype among second generation offspring might merely be due to the chance lack of homozygous mutants. This will artificially boost the number of mutations scored as lacking a phenotype. As might be expected, only 4% of EMS-induced amino acid substitutions in *C. elegans* lowered lifetime fecundity in the lab, yet we know that most of these substitutions are under purifying selection based on the low ratios of nonsynonymous to synonymous mutations (Davies *et al.*, 1999). In both *C. elegans* and *Drosophila*, a low rate of phenotype discovery is further confounded by the potential rescue of defective mutants by functional RNA inherited from their mothers.

RNAi is a targeted knockdown method that works well in invertebrates (and for some vertebrate applications). The development of high-throughput RNAi is allowing functional analysis of *C. elegans* genes on a genome-wide basis (Kamath *et al.*, 2003; Sugimoto, 2004). Ectopic introduction of RNA complementary to the target gene causes degradation of the endogenous transcript. Although there are great advantages to this gene-specific, high-throughput approach, RNAi knockdowns suffer the same limitations as knockouts, no conclusions can be drawn in the absence of a phenotype.

B. Conventional Explanation: Functional Redundancy

One possible explanation to the problem of mutants without phenotypes is that other genes are serving a ''backup'' role—the genome has built-in functional redundancy. This has become the conventional default assumption, particularly for gene knockouts that are phenotypically indistinguishable from wild type. The prevalence of gene duplication events throughout evolutionary history has provided the basic framework and fuel for the functional redundancy explanation. However, we make the case in Section II.A that functional redundancy, far from being a general phenomenon, is probably limited to a small proportion of genetic mutants for which phenotypes are lacking. We expect that the majority of genes described as nonessential will reveal phenotypes when subjected to the ecological conditions under which they arose.

C. Ecological Explanation

Phenotypes too subtle to be detected in lab assays may nonetheless have large effects on the performance, health, and overall fitness of individuals living under natural conditions. Organisms living under benign laboratory conditions are free from many challenges and stresses that would confront them in the real world. For example, a 5% reduction of metabolic efficiency would have no detectable effect on the health, longevity, or reproduction of

mice in laboratory mouse cages, but it might be effectively lethal under the food-limited and socially competitive conditions in nature (Carr and Dudash, 1995; Jiménez *et al.*, 1994; Miller, 1994). What matters for a gene's evolutionary survival is that the selection coefficient is greater than the reciprocal of the effective population size. If selection exceeds this threshold, a genetic mutant with no detectable phenotype in the laboratory would be eliminated from the wild with almost as much certainty as an embryonic lethal mutation would be. In the sections below, we review theoretical and empirical studies that address our central thesis—that fitness assays are a powerful tool for elucidating gene function, especially for phenotypes that are cryptic using traditional methods. To a large extent this chapter focuses on model organisms: yeast, *Drosophila*, *C. elegans*, and mice because the tools to generate genetic mutations and gene disruptions to study specific gene function are already available. However, the principles are general and can be applied to similar enterprises in any species. In Sections II.B and II.C, we provide numerous examples where ecological approaches reveal phenotypes that were undetectable in the laboratory. We also demonstrate that even for mutations with known phenotypes, fitness testing is a useful screen for additional unknown phenotypes and to quantify the relative importance of the mutation in the evolutionary currency of fitness.

II. Genes Lacking Phenotypes: Explanations and Experimental Approaches for Their Elucidation

Various lines of reasoning have been invoked to explain the surplus of gene knockouts that yield no phenotype in animals homozygous for the mutation. These are: (1) complete functional redundancy, where redundant genes are equally efficient at carrying out a particular function and can provide complete rescue if either counterpart is rendered dysfunctional, (2) partial functional redundancy among genes that are unequal in their efficiency or have additional unique functions, (3) genetic backgrounds that mask defects, (4) genes with small effects, or (5) genes with ecological functions that are not detectable in typical laboratory tests (Cooke *et al.*, 1997). Functional redundancy has become the default assumption when a phenotype is not detected, but laboratory methods are largely ineffective for testing the alternative hypotheses listed above. Thus, the inherent limitations of laboratory analyses to replicate nature have erroneously established functional redundancy as a general explanation for minimal or absent phenotypes. In the following subsections we discuss and evaluate these alternative hypotheses.

A. Functional Redundancy: Theory and Evidence

1. Theory

It is generally thought that most cases of functional redundancy will occur among related genes. Although it is theoretically possible for some degree of functional overlap to arise in unrelated genes in integrated pathways, the majority of known genes with suspected redundant partners are themselves the direct descendents of gene duplication events (Conant and Wagner, 2004; Gu *et al.*, 2003). Gene duplication is a pervasive process that has been responsible for many evolutionary innovations and the expansion of phenotypic complexity in general. Once duplicated, newly redundant counterparts are theoretically freed from selection and become more susceptible to mutations that either destroy one paralogue entirely, or modify one or both duplicates within coding or *cis*-regulatory regions ultimately changing the extent, timing, or spatial pattern of gene expression. This leads either to subfunctionalization of the ancestral role (Force *et al.*, 1999; Gibert, 2002), with each paralogue evolving toward greater functional specificity, or more rarely, to the origin of evolutionary novelty (Carroll *et al.*, 2004b; Cheng and Chen, 1999). With the availability of genomic databases, the strength of selection following duplication events can be measured indirectly via sequence comparison. Such studies have indeed found that gene duplication events are followed by a brief period of relaxed selection. In contrast, orthologous genes (sharing an ancestral origin in related species) with divergence times comparable to within-species duplicates do not experience relaxed selection (Kondrashov *et al.*, 2002; Lynch and Conery, 2000).

So the problem is not how functional redundancy arises, it is how functional redundancy can be maintained despite the inevitable disruptive effects of mutation. In some cases, gene conversion may extend the lifetime of redundant paralogues, homogenizing sequences of duplicated genes that are in the process of drifting apart (Gao and Innan, 2004). Even without the assistance of gene conversion or natural selection, recently duplicated genes can maintain functional redundancy for millions of years assuming both genes experience similar mutation rates (Cooke *et al.*, 1997). Although recent gene duplications may explain a minority of putative functional overlap among some paralogues (Lynch and Conery, 2000), it does not explain how functional redundancy can be maintained among very old paralogues from ancient duplication events that characterize much of the genome in yeast (Wolfe and Shields, 1997), fish (Taylor *et al.*, 2001), and other vertebrates (Spring, 1997). The continued maintenance of complete functional redundancy, like we routinely find on human-engineered systems, such as airliners, is effectively impossible under evolutionary engineering. Whereas airline

mechanics have mechanisms to test redundant systems independently of each other, natural selection cannot detect a broken backup system because the crippling of one gene paralogue will have no effect on the function of its redundant counterpart. At an average mutation rate (10^{-8}–10^{-9} mutations per base pair per generation), the functional gene could remain fully operational for thousands of generations without the organism ever missing the ancestral backup system.

The best explanations proposed for the maintenance of functional backup genes rely on mechanisms that yield only partial redundancy, with the genes being maintained by selection for reasons other than exclusively backup functions. The case of recently duplicated genes described earlier is the only clear situation for which mutually redundant genes might exist (Cooke *et al.*, 1997). However, Cooke *et al.* (1997) developed models to show how partial redundancy might be maintained provided genes are asymmetrically efficient at performing their function (and experience unequal mutation rates), or as long as paralogues perform unique roles in addition to the redundant function (Cooke *et al.*, 1997). Finally, genes might appear to be functionally redundant if they are prone to functional failure in the face of frequent developmental and/or environmental perturbations which favors the maintenance of backup genes. This example is rather more closely associated with ecological contingencies, where different genes function under dissimilar ecological conditions to achieve homeostasis. Consequently, both genes are being maintained by selection due to their specialization to effectively solve different (but related) ecological problems.

2. Evidence

As we first began discussing these issues with colleagues and students, one confused student innocently asked if a no-phenotype knockout meant the animal became invisible. Fair enough. We define the term ''no-phenotype knockout'' (or mutant) to be a mutant that shows no phenotypic change from wild type. Correspondingly, a ''minimal phenotype mutant'' would have a phenotype differing mildly from wild type. It is becoming apparent that many cases of no-phenotype and minimal phenotype knockouts are due to limitations of the testing environment. Many genes are predicted to be essential for development based on their embryonic expression patterns and involvement in important developmental pathways. Yet these same genes lack clear knockout phenotypes or have unexpectedly mild effects. The homeobox family of transcription factors known as Hox genes provides many examples of this. Hox genes are responsible for patterning the metazoan body plan from worms to humans. These genes, clustered in four separate chromosomal linkage groups in tetrapods, are expressed during

embryogenesis in spatially and temporally restricted domains. Genes at 3′ ends of the Hox clusters turn on first, with anterior limits of expression in the hindbrain of the developing embryo. Additional 5′ Hox genes along the clusters are sequentially turned on, with each newly active Hox gene being expressed in progressively more caudal embryonic domains. This particular expression pattern of Hox genes, termed spatial and temporal colinearity, exhibits significant trans-species conservation. Figure 1 compares Hox genes in *Drosophila* and *Mus* and illustrates the conservation of this spatial colinearity between these two distantly related species. Also broadly conserved across taxa are the sequences of peptide residues making up the ''homeodomain.'' This region of a Hox protein, about 60 amino acids in length, recognizes and binds to DNA, permitting Hox proteins to operate as transcriptional repressors or activators of their downstream targets. These features initially distinguished the Hox genes as strong candidates for having critical functions in development. Consequently, Hox genes were among the first in mice to be systematically knocked out (Capecchi, 1997). However, the majority of the 39 mouse Hox genes are not required for embryonic or postnatal survival. Single homozygous knockouts that affect cervical, thoracic, lumbar, and sacral axial structures tend to have subtle defects, with minor transformations of vertebrae toward the identity of slightly more anterior or posterior structures. Functional redundancy among genes within paralogous groups has been and continues to be the general explanation for such results.

It was found in mice that all members of a Hox paralogous group might require disruption before a complete phenotype emerges. For example, all six alleles of the three Hox10 paralogues (HoxA10, HoxC10, and HoxD10) had to be simultaneously disrupted before the role of these genes in suppressing rib development could be identified. The ribs of tetrapods are normally attached to thoracic vertebrae only. Knocking out all six alleles of the Hox10 group produces a mouse with ribs attached to all thoracic, lumbar, and sacral vertebrae. However, even a single functional Hox10 allele from any of the three Hox10 paralogues precludes this phenotype (Wellik and Capecchi, 2003). In another study, Greer *et al.* (2000) found no phenotype after swapping the HoxA3 and HoxD3 coding sequences. It was speculated that perhaps protein identity is irrelevant and what matters is the overall amount of protein supplied by paralogues within overlapping domains. Far from being a rare exception, mild or absent phenotypes have been repeatedly documented among the thousands of mutant mouse lines produced from gene targeting and mutant screens. This general phenomenon prompted the *Journal of Molecular and Cellular Biology* in 1999 to begin dedicating a section in each issue to mammalian genetic models with minimal or complex phenotypes.

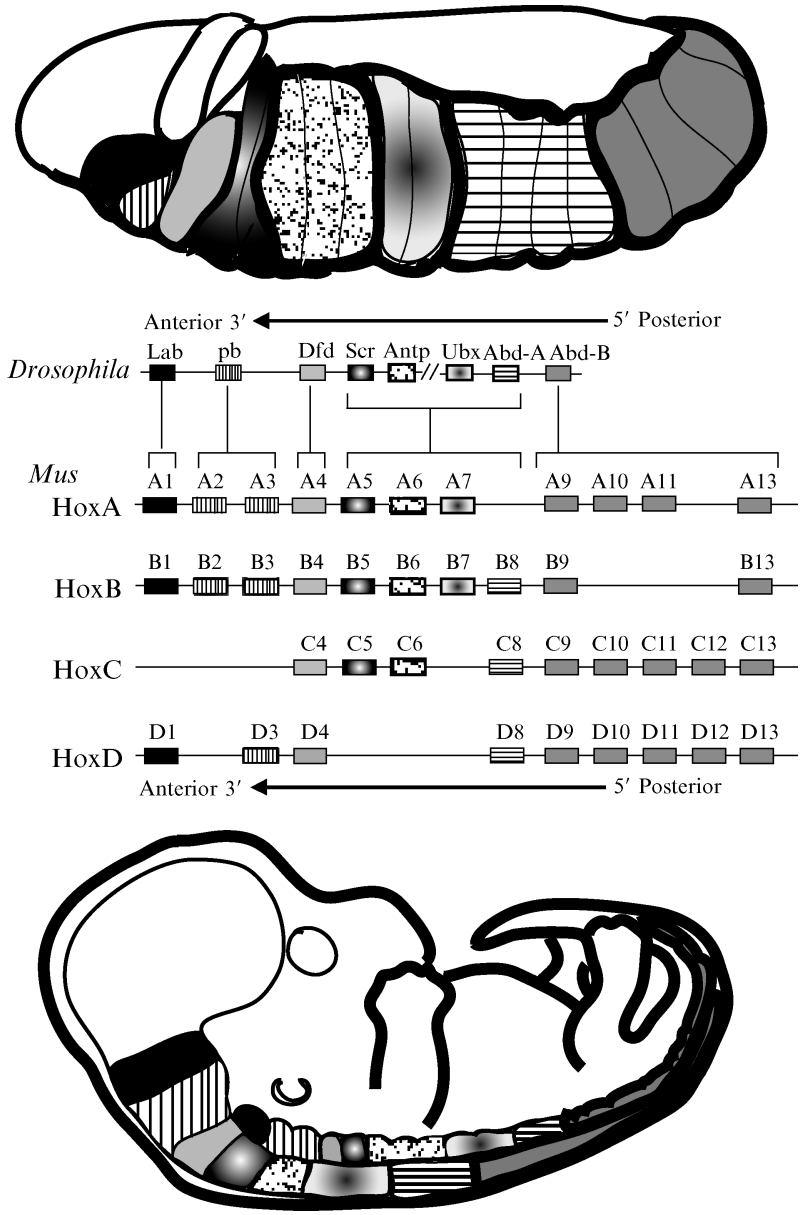

FIG. 1. A comparison of Hox (homeobox) genes in *Drosophila* and *Mus* showing the conservation of spatial colinearity and regions of the embryos controlled by each orthologous Hox gene.

How relevant are such studies as supportive evidence for functional redundancy? When the rescue of a genetic mutation is credited to a paralogous partner, an immediate question arises. Even if the paralogous partner is functionally capable of assuming the role of its crippled counterpart, what is the adaptive significance of this capacity, if any? Perhaps the redundant roles performed by paralogues are never actually required in the context of an intact genome, but remain as evolutionary baggage, performing in suboptimal capacity as useless in nature as the eyes of blind cave fish. To answer these questions, it is first necessary to know how commonly paralogues are coregulated within individual cells. Expression timing and location is a necessary requirement for predicting when genes might function redundantly and/or cooperatively to orchestrate subsequent downstream developmental processes.

A recent study supplies a plausible mechanism for apparent redundancy in yeast genes (Kafri *et al.*, 2005). Distantly related paralogues (resulting from ancient duplications) with the highest backup efficiency following mutation are dissimilarly expressed in most growth conditions and are therefore likely to perform distinct roles in the wild-type cell. Although these efficient backup paralogues show low levels of coexpression in wild-type cells, they are capable of conditionally switching their expression profiles in a coordinated manner and show an intermediate degree of similarity among their *cis*-regulatory motifs (Kafri *et al.*, 2005). The authors speculated that this partial sharing among regulatory elements enabled transcriptional reprogramming when one paralogue was disrupted. This reprogramming then led to novel expression of the intact gene, ultimately providing backup for its crippled partner (Kafri *et al.*, 2005). Dissimilar expression profiles clearly indicate that yeast paralogues are not functionally equivalent. So if yeast backup circuits are indeed evolutionarily conserved, it is overwhelmingly among genes that are not truly redundant, but serve unique functions in addition to their backup capability.

In summary, although there may be cases of selectively maintained functional redundancy, they are likely to be rare. Authors are beginning to acknowledge that the functional redundancy they observe in lab is likely to reflect the limitations of test conditions and does not indicate that paralogues are equally dispensable in long-term evolution (Gu, 2003). In cases where backup circuits are shown to buffer gene disruptions, the gene partners involved are increasingly accepted as performing unique roles in addition to buffering. It is these unique roles mediated by new regulatory motifs that we expect to be under strong selection, with the conserved functions being maintained possibly via genetic hitchiking for longer than would be expected under selective neutrality. Finally, only a few possible examples of functionally redundant mutations have been subjected to fitness assays to evaluate the alternative hypotheses. However, in cases where they were

tested, the general result is that fitness assays reveal phenotypes in formerly no-phenotype mutants/variants (Sections II.B.5 and II.C.1).

B. Ecological Functions

The most important alternative hypothesis to functional redundancy is that many genes function during specific ecological conditions, most of which are not included in standard laboratory assays. The list of such ecological conditions is long and certainly incomplete for any species, but usually includes predators, infectious agents, wounding, nutritional deficiencies, sporadic food and water availability, toxins, thermal extremes, and social ecology. Social ecology includes competition among conspecifics, which is often of special importance because it involves aggressive, physical contests that demand maximum performance from most physiological systems. Consequently, these contests represent a particularly sensitive and integrative screen for many molecular and physiological defects. Accordingly, competition among conspecifics is an ecological condition we treat in its own section (Section II.C) due to its special importance.

In Section II.C and later, we review nine examples where natural or seminatural population conditions allowed the discovery of phenotypes that were previously missed or likely to be missed in laboratory studies. Most of these nine examples involve social ecology, in part because other ecological conditions have seldom been manipulated in relevant studies. One reason that we have not manipulated many of these ecological variables in our studies on *Mus* is because the extreme competition among individuals that occurs in mouse society appears to be a sensitive screen for health, vigor, and fitness differences.

We have framed the general problem around no-phenotype knockouts/mutants. However, only two of the nine examples represent cases of specific knockouts/mutants (Sections II.B.5 and II.C.1) tested under ecologically relevant conditions. We believe that the lack of good examples in the literature underscores the general problem that no-phenotype knockouts are not typically being tested under ecological or competitive conditions. The other seven examples represent cases of natural variants (Sections II.B.1, II.B.2, and II.B.4; see also Section II.C.4) or groups of uncharacterized mutants (such as inbreeding and mutation accumulation; Sections II.B.3, II.C.2, and II.C.3) where fitness assays revealed previously unknown phenotypes.

1. MHC-Mediated Sexual Selection

Alleles of the major histocompatibility complex (MHC) have several unique features. MHC genes are characterized by tremendous polymorphisms (up to 400 alleles at some loci; Klein, 1986). They show allele sharing

among anciently diverged species, and allelic frequencies are uniform across populations. These features all point directly to some form of strong balancing selection maintaining allelic diversity at these immunologically critical loci. It has long been assumed that pathogens and parasites are the dominant form of selection acting on these loci, either by favoring MHC heterozygotes that are capable of resisting a broader array of immunological challenges than homozygotes, and/or by favoring individuals that carry rare MHC alleles. A variety of empirical and theoretical models have predicted both heterozygote and rare allele advantages to be capable of maintaining the degree of polymorphisms found in nature (Bodmer, 1972; Doherty and Zinkernagel, 1975; Hill *et al.*, 1991; Hughes and Nei, 1988). However, studies have only recently begun to yield the predicted results that in the presence of pathogens different MHC genes yield phenotypes that differ in their susceptibility to disease. In the search for pathogen-mediated selection, incorporating multiple pathogens or strains of pathogens as is encountered in nature appears to be necessary for detecting MHC effects (Carrington *et al.*, 1999; McClelland *et al.*, 2003; Penn *et al.*, 2002; Thursz *et al.*, 1997; Wegner *et al.*, 2004). In general, it is MHC heterozygotes that are better protected against infection than homozygotes.

Potts *et al.* (1991) set up seminatural populations of house mice designed to determine whether MHC heterozygotes were more fit than homozygous conspecifics. The authors reasoned that the best way to test for differences among genotypes was to allow natural selection to reveal fitness using free-living mice maintained for a year in nonsterile seminatural enclosures. The authors reasoned that mice carrying less favored MHC haplotypes would have lower survival and hence lower fitness. In a surprise twist, the authors found that mice were relatively unencumbered by the mild pathogenic conditions of the enclosures. Instead, what prevailed was massive sexual selection. Compared to random mating expectations, MHC genotypes of pups born to the original founders revealed a reduction of homozygotes in all nine populations (Fig. 2) with a mean 27% deficiency overall. In evaluating the potential causes for this deficiency, only MHC-based disassortative mating preferences could explain the pup genotype patterns. Behavioral observations of the populations suggested that females were the protagonists of the study, leaving their territories to engage in extra-pair matings when the MHC genotype of their neighboring territorial male ''smelled better'' (had greater genetic complementarity) (Potts *et al.*, 1992).

MHC-based mating preferences had been previously detected in the laboratory using inbred strains of mice (Yamazaki *et al.*, 1976; for reviews see Jordan and Bruford, 1998; Penn and Potts, 1999), although the discovery was serendipitous and could have been easily missed. Also, these studies have been difficult to interpret for many reasons, not the least of

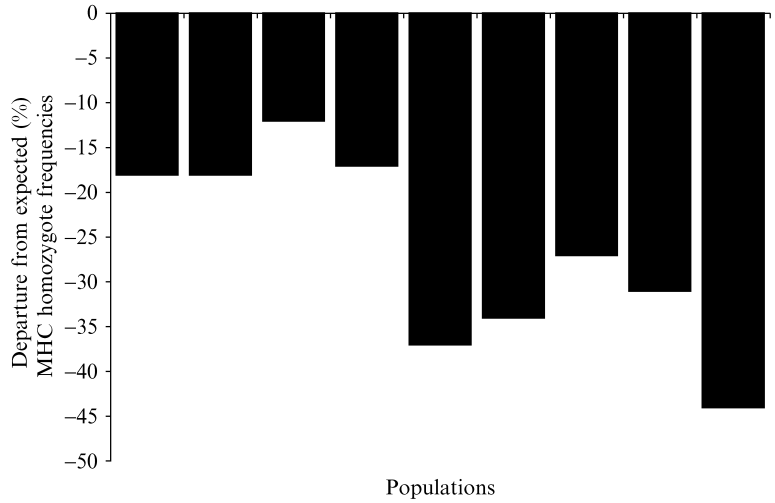

FIG. 2. The percentage deficiency of MHC homozygotes relative to Hardy–Weinberg expectations in each of nine seminatural house mouse populations. The overall mean deficiency was 27% (Potts *et al.*, 1991).

which is that reproductive behaviors of inbred animals have been affected by hundreds of generations of artificial selection (Manning *et al.*, 1992a). In the case of MHC-based mating preferences, social ecology is clearly critical to release the phenotype to selection, because the phenotype is social behavior. Testing mice in the context of *Mus* populations demonstrated that the selection coefficient arising from nonrandom mating was strong enough to maintain the allelic diversity found in surveys of wild populations, suggesting that mating preferences could indeed be the elusive source of selection maintaining MHC polymorphisms (Hedrick, 1992; Potts *et al.*, 1991).

2. MHC-Mediated Kin Recognition for Communal Nesting Partners

House mice sometimes form communal nests where two or more females share nursing duties, apparently directed without bias toward all pups (Fig. 3) (Manning *et al.*, 1992b). Evidence suggests that communal nesting functions at least in part to reduce infanticide in house mice (Manning *et al.*, 1995), presumably because one female can guard the nest while other females are foraging. Communal nursing is a rare trait for mammals and it makes females vulnerable to cheating by communal nesting partners, who would do less than their fair share of the work or bias their nursing toward their own offspring. One way to reduce this conflict is for females to

FIG. 3. Female house mouse nursing members of three litters in a communal nest.

communally nest with relatives, thereby lowering costs by directing behavior toward kin. This prediction emerges from kin selection theory (Hamilton, 1964) and was tested by evaluating communal nesting patterns in seminatural populations of house mice. When familiar sisters were present in populations, they almost always chose each other as communal nesting partners. More importantly, communally nesting females with no sisters in their population showed a significant preference for settling with MHC-similar females relative to random expectations. Due to the breeding design, MHC similarity was not correlated with relatedness, excluding the possibility that non-MHC genetic cues were being used as indicators of relatedness.

This preference for MHC-similar communal nesting partners was the first example of a genetic-based kin recognition system in vertebrates. In the context of this chapter, it is an example of the discovery of an ecologically specific function for naturally occurring genetic variants. This function would have been difficult to discover in a lab setting, but it was easily revealed under conditions allowing seminatural social ecology.

3. *Sexual Conflict in* Drosophila

The sexes are predicted to have conflict in many aspects of their biological interactions. For example, in species with sperm competition, male adaptations might reduce the fitness of females, because the chemical warfare among ejaculates might have toxic effects in females. *Drosophila*

provides us with striking examples that illustrate how mating is not necessarily a cooperative venture for the purpose of producing offspring. Indirect evidence suggests that male-specific adaptations reduce female fitness in *Drosophila* because seminal fluid from a male is toxic to females, it reduces the propensity of females to remate, and decreases the competitive ability of sperm from other males, among other effects (Ravi Ram *et al.*, 2005). Such interactions could lead to cycles of antagonistic coevolution between males and females. To test this hypothesis Rice (1996) designed an elegant set of experiments that allowed males to adapt to sperm competition occurring in females, but prevented females from making counter-adaptations. Rice predicted that such unilateral male evolution would result in reduced fitness in females when interacting with these adapted males. After 30 generations of unilateral evolution, males showed 24% increased fitness relative to control males in population assays. The unilateral evolution was allowed to continue for a total of 41 generations after which adapted males caused significantly higher female mortality and the mortality rate was correlated with the mating rate.

In this case, sperm competition within the female reproductive tract was ecologically critical for promoting the evolution of increasingly competitive sperm as well as for detecting the fitness consequences for both males and females. The genes involved in this remarkable case of experimental evolution would, when disrupted, likely look like no-phenotype knockouts without the use of the specific ecology surrounding sperm competition. These approaches have continued to reveal remarkable insights into related aspects of *Drosophila* reproduction (Gibson *et al.*, 2002; Holland and Rice, 1999; Rice and Holland, 2005; Rice *et al.*, 2005).

4. Timing of Flowering in Arabidopsis

The initiation of many behaviors in plants and animals are controlled by environmental cues such as day length. Day length can be manipulated in the laboratory to successfully initiate many seasonal behaviors, such as timing of flowering, which has been extensively studied in *Arabidopsis* under laboratory conditions. To determine if the genetic basis of timing of flowering in lab studies duplicates that in nature, quantitative trait loci (QTL) studies in these two environments were performed (Weinig *et al.*, 2002). The surprising findings were that QTL important under lab conditions were often undetectable under field conditions and vice versa. These data suggest that many ecological cues important in nature are missing under laboratory conditions and that lab conditions initiate pathways that are silent under some field conditions. In a companion study, Weinig *et al.* (2003) went on to show that different field conditions and different genetic backgrounds favored different alleles at these QTL. Taken together, these

data underscore the importance of ecology and epistatic interactions (Section II.D) for understanding gene function.

5. Dictyostelium

Under starving conditions, social *Dictyostelium* amoebas strike a coordinated venture in pursuit of a common interest—survival. *Dictyostelium*, with its simple physiology and behavior, plus the availability of molecular tools for analysis, has presented sociobiologists with an ideal model organism to study the molecular underpinnings of social evolution and cooperation. As bacterial food sources become meager, free-living *Dictyostelium* secrete and track cAMP signals to form aggregations which may often be composed of genetically distinct clones (Fortunato *et al.*, 2003). From these aggregations, a motile multicellular slug emerges and migrates to the soil surface to form a fruiting body. During this social phase of the life cycle, a ball of fertile reproductive spores differentiates from a large percentage of amoebas, while about 20% of cells assemble themselves altruistically into a slender stalk and die while elevating the spores for optimal dispersal (Bonner and Slifkin, 1949). As survival depends on directing one's genes into a spore, cheaters that manage to escape the dead-end fate of the stalk would be highly favored by natural selection.

The search for genes that mediate social conflict in *Dictyostelium* led researchers to *csA*, a gene which codes for the homophilic cell adhesion protein, gp80. Aggregation behavior in *Dictyostelium* is regulated in part by *csA* which is expressed during the preaggregation and stalk formation stages. Although no other proteins are capable of compensating for *csA*'s EDTA-resistant cell adhesion role during aggregation (Ponte *et al.*, 1998), knockouts initially had no obvious developmental phenotype. Cells lacking the *csA* gene had similar aggregation timing and development to wild-type amoebas in laboratory assays (Harloff *et al.*, 1989). Even more curious, when knockouts and wild-type amoebas were allowed to assemble into chimeric aggregates, the knockouts had a distinct advantage in becoming spores. Since the gp80 protein-binding site recognizes and binds to copies of itself on cell membranes, the advantage of knockouts is partly due to a weakened intercellular binding which loosens them to the back of the slug where they are more likely to become spores. This puzzling result suggests a strong selective advantage to mutants that lose the *csA* allele, yet its presence in wild *Dictyostelium* and its specific expression pattern suggests some critical function during aggregation.

Dictyostelium normally inhabits a complex three-dimensional environment composed of soil, decaying leaves, and other forest detritus. Yet laboratory assays are typically performed on smooth two-dimensional test surfaces of agar, nitrocellulose filters, or glass coverslips. With this in mind,

Ponte *et al.* (1998) retested knockouts in petri dishes containing either agar or moistened soil. In comparison with wild-type cells, aggregation behavior of *csA* knockouts was delayed 8–10 hr on soil, but not on agar. Moreover, fruiting body formation of knockouts was only 15% that of wild types—a reduction of approximately 99% overall when compared to performance on agar plates. Actual spore production by *csA* knockout cells was reduced overall by a similar amount, and when wild type and *csA* knockout cells were mixed on soil plates to test differential fitness, only 18% of the resulting colonies came from the knockout spores. This reduction in fitness is likely to result from the same cell adhesion deficiency that gives csA^- a spore-forming advantage on smooth surfaces. On soil or other complex substrates, the lack of *csA* protein product limits a cell's ability to get into aggregates in the first place, resulting in a strong reproductive disadvantage (Queller *et al.*, 2003).

Other genes that affect social aggregation in *Dictyostelium* have proven simpler to phenotypically characterize in standard laboratory assays. For example, the product of *dimA* responds to the signaling molecule DIF-1 which triggers differentiation into prestalk cells. By disrupting *dimA*, a cell could theoretically increase its chances of becoming a spore by avoiding the stalk entirely. However, although they outnumber wild-type cells in the prespore phase, $dimA^-$ cells have a high failure rate during spore differentiation (Foster *et al.*, 2004), preventing their reproductive domination over wild-type cells. That is not to say that stronger or additional phenotypes are not waiting to be discovered by testing *dimA* and other ''cheater'' genes that have been recovered in mutant screens (Dao *et al.*, 2000) under more natural conditions.

C. Competition Amplifies Small Performance Differences into Larger Fitness Differences

The power of competition to amplify small differences among competitors has been a major theme in the ecological literature for decades (Koella, 1988; Latter and Sved, 1994; Smith and Holt, 1996; West Eberhard, 1983). However, this same literature largely fails to appreciate the power of competition to amplify phenotypic differences among genotypes when the examined genes are not specifically ''social/sexual competition'' genes (Carroll and Potts, in press). It is perhaps then no surprise that ecological approaches as a general screen for gene function have been largely overlooked by the functional genomics community. There are hundreds of papers and dozens of meetings per year on functional genomics; few consider the role of ecological approaches for revealing gene function (Feder and Mitchell-Olds, 2003). As we illustrate in the examples that

follow, the strength of social competition to discriminate among genotypes extends far beyond genes that code for bright feathers, elaborate displays, and other sexually and socially selected traits.

Figure 4 provides a hypothetical illustration of how competition in the real world alters the fitness distribution of mutants making many more detectable under ecological versus lab conditions. Panel (A) illustrates the fitness distribution of yeast knockouts under lab conditions. When these same genes are assayed under competitive, ecological conditions the fitness distribution is shifted down, resulting in many more genes with detectable phenotypes (Panel B).

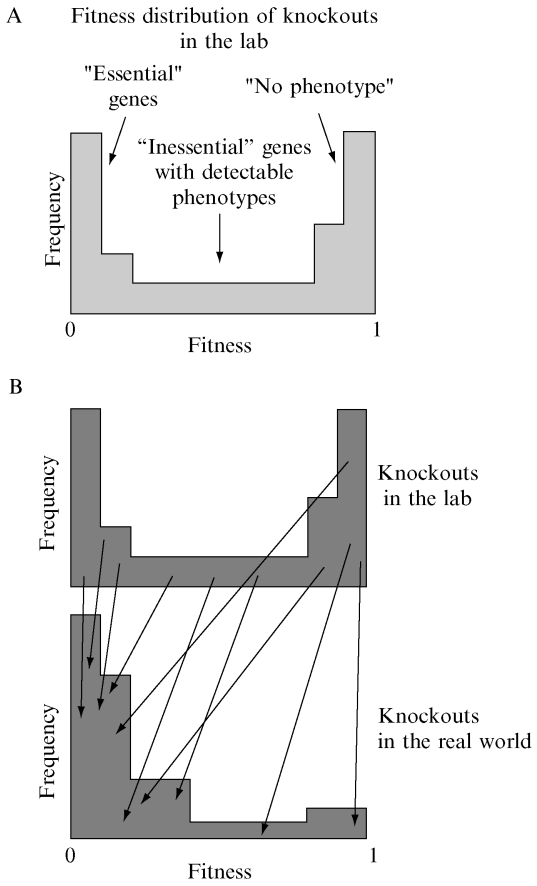

FIG. 4. Competition amplifies small performance differences into larger, detectable fitness differences (see text).

Intraspecific competition can take two major forms. Direct (interference) competition results in direct encounters between competitors, for example fights for territory ownership. During indirect (exploitation) competition, a resource is used by one individual thereby removing it from the resource pool for other competitors who might never be encountered directly. Traits that influence the efficiency of resource exploitation are favored for indirect competiton, whereas traits for fighting ability (or other forms of direct competition) are paramount for direct competition.

Direct and indirect forms of competition often have dramatically different dynamics in their quantitative influence on fitness. During indirect competition, the difference in competitive ability is often proportional to fitness outcomes. For example, individuals that feed 10% more efficiently have 10% more offspring. In contrast, during direct competition, difference in competitive ability is often amplified into much larger fitness effects. As in the inbreeding example later, outbred males may only be 10% better duelists, but since they win most fights over territories (and nonterritorial males do not breed), the fitness consequences are dramatically amplified. Consequently, it may be easier to detect fitness consequences of a similar genetic defect (mutation) in species with direct competition compared to species with indirect competition.

Later we provide examples in yeast, *Drosophila*, and mice where competition amplified fitness differences dramatically, turning no-phenotype mutants into major phenotype mutants.

1. *Gene Knockouts in* Saccharomyces

Many gene knockouts in yeast (*S. cerevisiae*) reveal no phenotypic change from wild type when grown under normal laboratory conditions. To determine if competition might reveal phenotypes, Thatcher *et al.* (1998) measured the fitnesses of a random collection of these disruption mutants in direct competition with their wild-type progenitor. Figure 5 shows the fitness distribution of 34 no-phenotype yeast knockout mutants (under no competition) when subjected to direct competition with wild type. Approximately 1/3 maintained their no-phenotype status, but 2/3 expressed significant fitness declines ranging from 0.3 to 22%; two knockouts showed a significant fitness increase compared to wild type. Competition became a microscope that made the formerly invisible phenotypes visible and subsequent studies have now incorporated this approach (Giaever *et al.*, 2002).

2. *Mutation Accumulation in* Drosophila

Similar results have been demonstrated in *Drosophila* by Kondrashov and coworkers (Shabalina *et al.*, 1997). They allowed mutations to accumulate

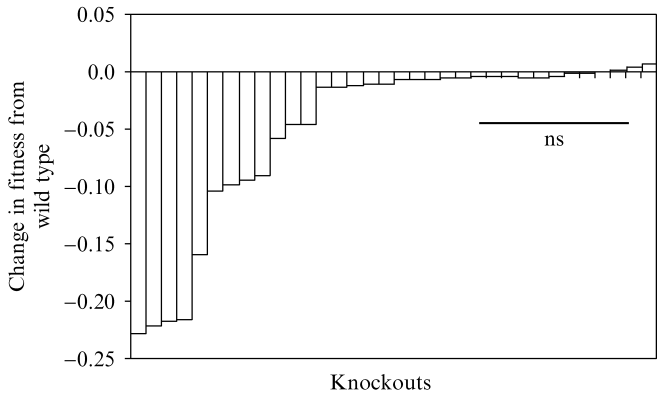

FIG. 5. Fitness distribution of 34 no-phenotype yeast knockout mutants during direct competition with wild type (Thatcher *et al*., 1998). These 34 mutants showed no phenotypic change from wild type when grown separately under standard laboratory conditions (ns = not significant).

in populations of *Drosophila* for 30 generations. These mutation accumulation lines were allowed to compete with wild type to test for fitness declines either under benign or harsh competitive conditions. In benign conditions, food was not limited, eliminating most competition among adults and among their larvae. Consequently larval survival was high. In harsh conditions food was limiting, promoting competition among adults and among larvae, which resulted in larval survival of approximately 10%. The fitness declines of mutants under harsh population conditions were approximately 70% (2% per generation). However, the final fitness decline of mutants under benign population conditions was only 5%, an order of magnitude lower than under harsh conditions (Fig. 6). This represents a case where ecological stressors other than social stressors were also manipulated. However, since social competition and harsh ecological conditions were not manipulated independently, it is unclear what proportion of the large fitness declines were due to each variable or their interactions. Similar competition-amplified fitness effects have been demonstrated for inbreeding in *Drosophila* (Charlesworth and Charlesworth, 1987).

3. *Inbreeding in* Mus

The primary cause of inbreeding depression is the expression of deleterious recessive alleles that are expressed at a higher rate in inbred individuals (Latter, 1998). These negative consequences have been well established for centuries. Two major studies have been conducted on mice and the reproductive consequences of one generation of full-sib matings

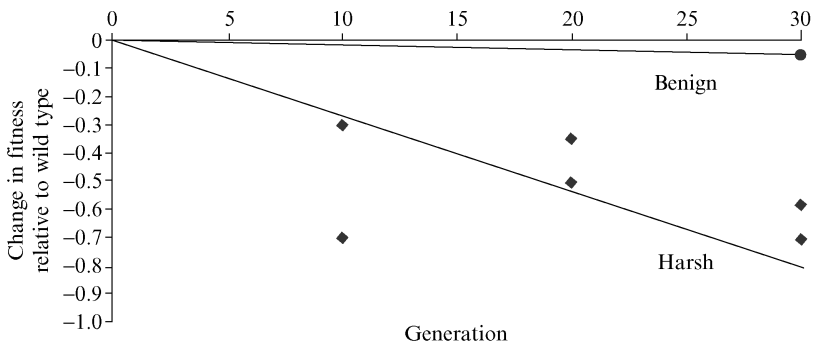

FIG. 6. Competitive performance (fitness) under harsh versus benign conditions of *Drosophila* lines allowed to accumulate mutations for 30 generations. Competition is against wild type. Means and regression lines are shown (adapted from Shabalina *et al*., 1997, with permission: © 1997, National Academy of Sciences, USA).

were estimated at about a 10% decline (Connor and Belucci, 1979; Lynch, 1977); almost all of the effect was due to reduced litter size. No attempt was made to measure fitness in any type of competitive social conditions.

Meagher *et al.* (2000) repeated these experiments with the goal of adding fitness measures in competitive social conditions. Wild-caught mice were bred so that the F_2 generation came from either outbred or full-sib matings. These progeny became the founders for six experimental populations. It was found that outbred males had five times more offspring than inbred males (Fig. 7A). This represented a tenfold amplification over the reproductive declines observed for males in breeding cages. Significant fitness declines were found for inbred females, but they were an order of magnitude smaller than the observed male declines (Fig. 7B). There was no significant difference between laboratory and enclosures results for females. These gender differences were attributed to the fact that males compete aggressively over territories and nonterritorial (subordinate) males have little reproductive success. In contrast, females had no limiting resources. It remains an open question whether the fitness consequences of inbreeding in females would approach males if they had to compete over critical resources such as food or nest sites.

The dramatic fitness declines in inbred males were due both to a 41% reduced ability to gain territories and decreased survival. This was particularly true for territorial inbred males where 90% had died by the end of the experiment as compared to only 24% of outbred territorial males (Fig. 8). These results suggest that inbred males had difficulty maintaining territories, as well as gaining them.

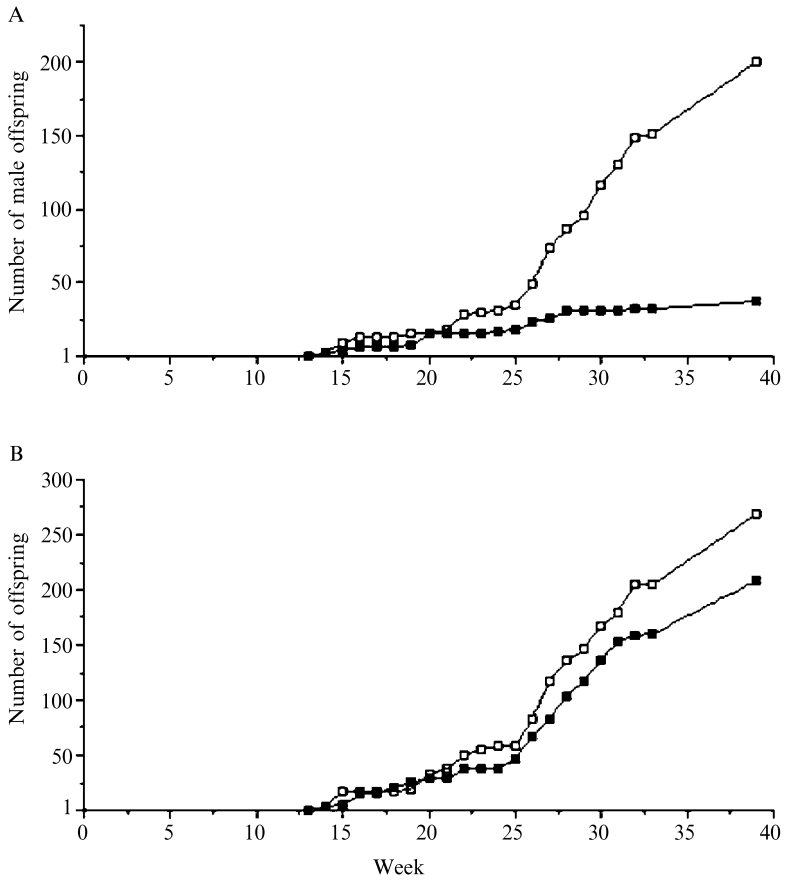

FIG. 7. Relative reproductive success of inbred (solid) and outbred (open) males (A) and females (B) (Meagher *et al*., 2000). Male reproductive success is measured using a genetic marker on the Y-chromosome, which explains why only sons are counted in (A).

Figure 7 shows the relative reproductive success of inbred and outbred males and females over time. This analysis demonstrates that the relative differences were increasing at the end of the experiment, suggesting that all the inbreeding depression estimates were conservative. If the populations had been allowed to continue to obtain lifetime reproductive success measures, the fitness differences between inbred and outbred animals would have been much larger.

The same 10% reduction in litter size was observed under colony housing as was found in the two major previous studies on *Mus* inbreeding (Connor

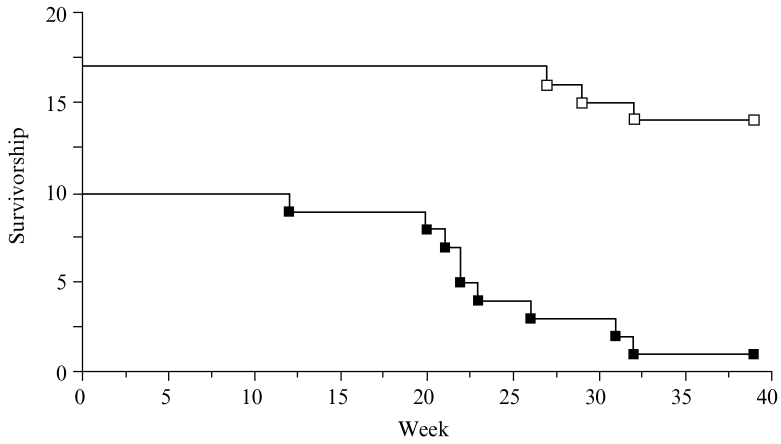

FIG. 8. Survivorship analysis of inbred (solid) and outbred (open) territorial males (Meagher *et al*., 2000).

and Belucci, 1979; Lynch, 1977), suggesting the inbreeding load in all three wild-caught populations were similar. However, the analysis of adult male fitness added an additional 500% effect; outbred males had five times more offspring than inbred males.

A recent survey of inbreeding studies demonstrates that in most cases stress amplifies the deleterious effects of inbreeding (Armbruster and Reed, 2005). Since inbreeding depression is primarily the expression of defective mutant genes (Latter, 1998), these results are particularly instructive as to the power and sensitivity of fitness assays for other gene function studies involving mutants or knockouts. Competition and other forms of stress increase the deleterious effects of mutants, making such tools useful for revealing phenotypes of mutants.

4. Resolving the Paradox of the Selfish t *Complex*

The mouse *t* complex on chromosome 17 is a classic example of a selfish gene which increases its own genetic representation at the expense of its bearer. Across the globe, all subspecies of house mice (*M. musculus* and *M. domesticus*) carry versions of this segregation distorter complex, held genetically intact by four nonoverlapping inversions that effectively prevent crossing over and recombination within its 400 megabase span. Although females transmit the *t* complex in Mendelian frequencies, a heterozygote male will transmit the *t* complex to up to 100% of his offspring. This nearly perfect meiotic drive in males is accompanied by a well-characterized cost—homozygosity at the *t* complex causes lethality

or sterility in males, depending on which combination of *t* haplotypes is inherited. However, this costly phenotype is only sufficient to keep the *t* complex from achieving complete fixation in populations. It is not sufficient to prevent the *t* complex from spreading to high frequencies. Early studies estimated that the dual effects of segregation distortion and homozygote lethality should yield population frequencies around 70% (Bruck, 1957). Yet the *t* complex staggers along at puzzlingly low levels around 6–25% (Ardlie and Silver, 1998; Dunn and Levene, 1961; Figueroa *et al.*, 1988; Lenington *et al.*, 1988; Myers, 1973), less than half of its expected frequency. In the 50 years since its discovery, the *t* complex has been studied empirically to determine the effects of fertility, fecundity, juvenile survival, and female choice (Dunn and Suckling, 1955; Dunn *et al.*, 1958; Johnston and Brown, 1969; Lenington *et al.*, 1994; Levine *et al.*, 1980). Models have been constructed and computer simulations have been run to sort out the effects of drift, migration, and selection (Baker, 1981; Berry *et al.*, 1991; Durand *et al.*, 1997; Levin *et al.*, 1969; Lewontin, 1968; Petras and Topping, 1983). Since then, many phenotypes of the *t* complex have been discovered and much theory has been published regarding the population dynamics of genetic elements possessing the peculiar characteristics of the *t* complex. Yet, perhaps not surprisingly, many of these results are in conflict with one another and no single study accounts for a significantly large proportion of the discrepancy between observed and expected frequencies. What these studies do show is that there are clearly many different relevant factors which limit the spread of the selfish *t* complex, making it nearly impossible to integrate all available data into a cohesive model for predicting the fitness of *t* haplotypes in nature. In an attempt to measure *t* haplotype fitness directly, Carroll *et al.* (2004a) analyzed pup genotypes to estimate lifetime reproductive success in 10 seminatural populations of wild house mice over the approximate span of a generation. This study of competing *t*-bearing and non-*t*-bearing mice revealed a strong heterozygote disadvantage in both males and females. Heterozygote disadvantage had been predicted by previous models, but had not been convincingly demonstrated by laboratory assays.

The novel phenotype emerging from long-term competitive populations was a significant impairment of heterozygous *t*-bearing males in their ability to gain territories—only 32% of heterozygous males gained territories, whereas 67% of non-*t*-bearing males gained territories (Carroll *et al.,* 2004a). Female mice overwhelmingly prefer to breed with dominant males, which helps explain why in a single generation the *t* complex was at frequencies nearly 50% lower than expected (when both segregation distortion and male homozygote sterility were considered). An additional novel phenotype was the increased mortality of both *t*-bearing male and

female population founders under competition. These data collectively suggest that selection against *t*-bearing heterozygotes in natural populations can easily resolve the paradox of why *t* frequencies in nature are so low.

Although the populations were not run long enough to determine an equilibrium between heterozygote disadvantage and meiotic drive, the dramatic loss of *t* haplotypes from the enclosures in a single generation suggests this trend would lead to the ultimate exclusion of *t*-bearing animals from the reproductive pool. Yet the *t* complex has survived over millions of years, and it is tempting to speculate that heterozygote disadvantage of *t*-bearing mice is a phenotypically plastic phenomenon affected by social and ecological context. Without competition, *t*-bearing animals are quite prolific. *t*-Bearing males that successfully emigrate to found new populations could easily produce rapid increases in *t* frequencies by virtue of meiotic drive, serving as primary reservoirs of *t* haplotypes. In larger populations, individuals carrying *t* haplotypes will face competition and suffer lowered fitness, driving down *t* complex frequencies. Ardlie and Silver (1998) obtained *t* frequency data from a variety of natural populations. Their results suggest that small- and medium-sized populations (<60 individuals) experience the largest fluctuations in *t* frequency and carry more *t* haplotypes than large populations. Large populations (>60 individuals) tend to carry low numbers of *t* haplotypes (average 3%) or none at all. This prediction of density-dependent selection not only explains why *t* complex phenotypes have been so difficult to pin down in the laboratory, it also adds another dimension to the detection of subtle phenotypes, underscoring the argument that the appropriate context for studying a gene is the ecological circumstance in which its function evolved.

D. Genetic Background Problem

It has become clear that many phenotypic effects of mutants depend on epistatic interactions with background genes (Leiter, 2002; Nadeau, 2001, 2003). Ten years ago, Threadgill *et al.* (1995) radically raised the awareness on this issue by illustrating the tremendous influence genomes can have on specific gene disruptions. Knocking out the gene for epidermal growth factor receptor caused early embryonic lethality in the CF-1 mouse stain. However, CD-1 mice carrying the mutation survived past birth for up to 3 weeks (Strunk *et al.*, 2004; Threadgill *et al.*, 1995). Such epistatic interactions could explain some cases of no-phenotype mutants. The common solution to this problem is to breed a mutant onto many different inbred strains, but this is a slow and expensive process (Bucan and Abel, 2002). No approach is perfect, however, one feasible alternative is to test phenotypes in the context of wild-derived, segregating backgrounds. This approach has the advantage

of reducing some of the inescapable effects of drift and artificial selection that afflict inbred strains. However, the reluctance of wild rodent females to breed in the laboratory potentially introduces extreme selection for animals predisposed to breed under artificial conditions.

Inbred strains come with a tremendous load of accumulated genetic baggage from the unavoidable side effect of spontaneous deleterious mutations becoming genetically fixed through inbreeding and low effective population size. Data documenting these effects in inbred strains primarily come from the mouse literature. The mouse, with human homologues to 99% of its genes, has held distinction as the principal animal model for human disease, making it vitally important to characterize phenotypic variation among the established strains. However, even sublines of strains separated in different breeding colonies have been shown to carry fixed mutational differences (Simpson *et al.*, 1997; Weiss *et al.*, 1989). Phenotypic divergence of sublines has been documented for such phenotypes as aggressive behavior (Sluyter *et al.*, 1999), response to cocaine (Henricks *et al.*, 1997), susceptibility to Theiler's virus-induced demyelinating disease (Nicholson *et al.*, 1994), and susceptibility to experimental *Salmonella* infections (McClelland *et al.*, 2004). The Jackson Laboratory currently manages a comprehensive database supplying information on phenotypic strain differences in mice (www.jax.org/phenome). Mutation accumulation lines in *C. elegans* have shown similar effects, including degradation in behavior (Ajie *et al.*, 2005) and other specific components of fitness occurring over a short period of time (Estes *et al.*, 2005).

A related problem that arises with inbred mice during characterization of knockout phenotypes is the potential misinterpretation of phenotypes that arise from linked lethal mutations to the gene of interest. In mice, gene targeting is typically performed in embryonic stem (ES) cells from the 129 inbred stains. Subtypes of this strain carry a number of known defects that can greatly confound interpretation of the targeted gene when they occur within its flanking regions. This problem garnered enough concern to a prompt a Banbury Conference on Genetic Background in Mice, which generated numerous recommendations for its remedy (Silva, 1997; Wolfer *et al.*, 2002). However, although such recommendations may be relevant when a phenotype is detected, they are not expected to improve the detection of null phenotypes.

As a whole, inbreeding in laboratory animals creates strong selection to adapt to the peculiar conditions of laboratory housing and breeding (Miller, 1994) so that inbred animals often display aberrant behaviors and physiological traits (Manning *et al.*, 1992a) compared to their wild counterparts. For detecting behavioral and physiological phenotypes that are expressed in only a subset of genetic backgrounds, breeding mutations

onto a wild, segregating background might be a straightforward compromise. Of course, the disadvantage of breeding onto outbred genomes is that this approach produces higher variance in data sets due to uncontrolled segregating genes. This was the major impetus for producing inbred laboratory strains in the first place. However, when testing performance or fitness differences, a wild outbred background will greatly facilitate expression of the full range of physiology and behaviors that a mutant animal would normally experience in nature. Despite the inherent selection favoring wild mice willing to breed in the laboratory, we argue that using outbred genomes may often be a more effective approach than the current approach of relying on many inbred strains that can have aberrant physiology, behavior, and accompanying epistatic effects.

E. Fitness Differences too Small to Measure

Genes with small effects may have functions that are ultimately too subtle for even the most exhaustive analyses to detect. Although these phenotypes might defy our keenest efforts to identify them, they are hardly invisible to natural selection, because what matters for a gene's survival in nature is that the selection coefficient is roughly greater than the reciprocal of the effective population size (Kimura, 1985; Tautz, 2000). As effective population size increases, even a vanishingly small selective advantage would be enough to maintain a seemingly functionless gene against the effects of mutation and drift. Just as population size exposes genes to the discriminating sweep of natural selection, sample size might be a crucial factor for obtaining the statistical power to detect small genetic effects. For this reason, studies in bacteria, yeast, and other organisms that can be tested within the context of large populations might be amenable for testing the generality of small effects as an alternative explanation for genetic redundancy. For example, Thatcher *et al.* (1998) used competitive yeast cultures to monitor fitness declines in mutant versus wild-type *Saccharomyces cervisiae* (Section II.C.1). This strategy permitted the detection of fitness declines as small as 0.3%, reducing the number of yeast genes with no known phenotypes from 100% to only 20%. Similarly, Smith *et al.* (1995) used a novel assay to measure the ''genetic footprints'' of random gene mutations competed in batch culture. By this method, fitness declines were detected in over 60% of 255 randomly derived mutant strains (Smith *et al.*, 1995; Thatcher *et al.*, 1998). In mice, competitive population studies are capable of detecting fitness differences on the order of 10–15%. This means that many mutations with strong selective effects ($s > 0.01$) will still be undectable in fitness assays. For such genes, sequence analysis will remain the leading method for inferring function by detecting evidence of selection.

Genetic sequence comparisons among related species with divergent population sizes could help determine whether a gene is maintained due to a small fitness effect or whether its maintenance is not a direct effect of population size but is likely due to an unidentified yet significant function.

F. Why Is Behavior so Critical When Measuring Fitness?

In the postgenomics era, we may hope to find few if any genes chiefly dedicated to specific behaviors. Rather, genes that affect behavior are pleiotropic so that a behavioral phenotype will result from mutations in genes that affect many physiological processes whether these are fundamentally metabolic or neurobiological. Stated otherwise, behavior is the whole organismal response to various combinations of specific cellular, molecular, and physiological processes. Therefore, the collective outcome of these processes can be studied by measuring behavioral performance.

In most metazoans, fitness is achieved primarily through successful behavior such as predator avoidance and intra- and interspecific competition for resources. The remaining organismal biology largely becomes infrastructure for these activities because behavior puts physiology to its greatest tests. Thus, defects in this behavioral infrastructure below the detectable threshold (e.g., cryptic-phenotype mutants) might still manifest noticeably during the performance of behaviors that demand energy, endurance, neuromuscular coordination, and so on. This is particularly true in light of the numerous examples where relatively small differences in physiological performance are amplified into large fitness differences by intraspecific competition (Section II.C). There are few physiological systems in house mice (and other behavior-rich metazoans) whose deficiencies will not result in fitness-reducing behavioral impairment. Under this view, almost all genes become behavioral genes and consequently, when phenotypes are cryptic, behaviorists may be the best biologists at detecting the resulting phenotypes, as well as the components of fitness most affected.

Studying behavior under natural conditions sufficient to measure fitness is one major way to reveal phenotypes of mutants. Unfortunately, there is almost no mention of this approach from either the phenomic or functional genomic communities. This failure to appreciate the power of behavior-related fitness measures is a major rationale for writing this chapter.

G. Why Seminatural May Often Be More Efficient Than Natural: Should You Test Your Mouse Against a Cat or Another Mouse?

Whether your favorite organism is predator or prey, the ultimate measure of fitness is lifetime reproductive success. When resources are limiting,

there are generally fewer breeding opportunities than there are fertile individuals, and since the most physically robust, pathogen-free, predator savvy individuals are those that win reproductive opportunities, this means that reproduction falls to those who win the competition for food, basking spots, predator-free hiding sites, and other limited resources. For this reason, competition in experimental populations might serve as a useful proxy for natural selection, even when experimental populations lack many of the important components of natural selection. In nature, the losers of intrasexual competition are killed by starvation, predators, disease, and other difficult to measure effects. By eliminating these natural selective factors while simultaneously creating competition for the resources that would serve to restrict them, potential breeders are excluded from territories not by predators and starvation, but by competitors. Reproductive winners are those that successfully gain access to mates and to sites appropriate for the rearing of offspring.

Staged seminatural conditions are impossible for many species. For these species nature becomes the only place to obtain realistic fitness measures. Many long-term field studies have shown that an amazing level of detail can be revealed by studying animal populations in nature. Just a few examples include lions (Packer *et al.*, 2005), Darwin's finches (Grant, 1986), Florida scrub jays (Wolfenden and Fitzpatrick, 1996), and acorn woodpeckers (Koenig and Mumme, 1987).

For species that are amenable to a seminatural approach, measuring selection in competitive experimental populations offers a practical compromise between nature and the laboratory. For vertebrates in particular, selection is difficult to measure in the laboratory. Forcing reproduction in caged breedings can only give a narrow range of results regarding the mechanisms underlying reproductive differences among genotypes. However, studies performed in the wild have problems of their own. Stochastic environmental conditions (weather, food, shelter, and so on) add noise to already statistically complex data sets, and lifetime measures of fitness which could be easily measured in artificial populations, are confounded in nature by the loss of subjects to dispersal and various sources of mortality. That is to say, testing your mouse against another mouse might be a less stochastic, more tractable solution for determining exactly which one is more adept at evading the cat.

H. Gene Function Studies Will Seldom Be Complete Without Fitness Assays

Even if a phenotype is detected in the laboratory for a gene knockout or mutant, there remain at least two important aspects of gene function

that require fitness studies in order to comprehensively understand the function(s) of that gene. First, we need to find the true fitness consequence of lab phenotypes because their relative importance in the real world may be difficult to predict from lab-assayed phenotypes. Second, there may be additional, important phenotypes that were missed in the laboratory screens.

1. Relative Importance of a Particular Gene Must Ultimately Be Measured in the Currency of Evolution: Fitness

Fitness measurements are important for determining how essential or nonessential a gene is—the strength of selection acting against its knockout. Such measurements provide a quantitative measure of the relative importance (essentialness) of a gene. It will often be difficult to estimate the actual fitness declines of a given lab-assayed phenotype that is not lethal or near lethal. This is because estimations require extrapolation from minor phenotypes in the lab to their fitness consequences in the context of complex epistatic and ecological interactions as well as the harsh competitive conditions of nature. This is demonstrated by all four of our examples in Sections II.C.1 , II.C.2, II.C.3, and II.C.4 where phenotypes were initially invisible or minor, but had major fitness consequences under harsh competitive conditions. The relative fitness decline is the accurate measure of how important that mutation would be to its bearer in nature.

Are phenotypes trivial if detectable only in fitness assays? The answer is obviously no if you consider the inbreeding results in Section II.C.3. Being an inbred male is equivalent to having a lethal gene with 80% penetrance. The reduced health and vigor of inbred males prevent them from effectively competing against conspecifics. This should be of foremost interest to conservation biologists concerned with the genetic health of species communities and of no less interest to the biomedical community concerning human welfare. It is not that inbreeding-associated declines in health and vigor are trivial, but rather, that our previous phenotyping methods were insensitive. For example, quantitative defects in most metabolic pathways and organ function would go undetected until they became debilitating. Many neurological disorders in animals, such as migraine headaches, would go undetected under most lab assays. However, these conditions in humans would be considered disease and they would be detectable during competition in mouse and other vertebrate populations.

The danger of misinterpreting laboratory artifacts or detecting nonsense phenotypes is yet another important reason for characterizing gene function using an ecological approach. Genes have evolved to function in the context of the natural environment, so artificial environments can cause the expression of inappropriate phenotypes. For example, the genetic basis of flowering time in *Arabidopsis* is one of this model organism's most studied

traits and many QTL have been identified in laboratory studies (Section II.B.4). It was a great surprise to find out that when similar QTL studies were conducted in natural field experiments, many new loci were found that had not and could not have been detected in laboratory experiments (Weinig *et al.*, 2002). Furthermore, many QTL important in the lab had no detectable effects in nature.

2. *Discovery of Additional Phenotypes*

A single gene can influence many phenotypic traits (pleiotropy) and this is probably the general rule rather than the exception (Fraser and Marcotte, 2004). Consequently, if a phenotype is already known for a mutant or for a natural genetic variant, additional unknown phenotypes may await discovery. Most of the examples previously described in Sections II.B and II.C are cases where fitness assays revealed major new roles for genes that already had well-characterized phenotypes. For example, our early MHC experiments used seminatural populations in house mice to test for pathogen-associated selection (Sections II.B.1 and II.B.2). Consistent with the idea that homozygotes would be more susceptible to pathogens, we found a deficiency of MHC homozygous offspring. However, analysis of the components of fitness revealed not one but two novel phenotypes for MHC genes: first, the observed deficiency of homozygotes was not because they were dying from pathogens, but rather because females were preferring to mate with MHC dissimilar males (Potts *et al.*, 1991) (Section II.B.1). Later we were able to show that these same MHC genes also allowed the recognition of unfamiliar kin during the choice of communal nesting/nursing partners (Manning *et al.*, 1992b) (Section II.B.2). Most genetic mutants will probably have multiple phenotypes, many of which may be invisible in laboratory tests, but may be revealed during ecological competition.

III. Gene Function Studies Demand Integrative Approaches

The era of functional genomics affords a great opportunity for organismal biologists to collaborate with molecular biologists to truly evaluate how genes function through all levels of biological organization (Feder and Mitchell-Olds, 2003). One might say that the ultimate reductionist act has been committed—sequencing of genomes. Genome projects will largely be failures until the functions of these genes are clarified, a task that will often require organismal and ecological approaches. This endeavor promises to be a major application of integrative biology that could begin to heal the divisive wounds that tore apart our great biology departments in the last decades of the twentieth century.

A. Integrating the Fitness Component of Phenomics

Our central thesis is that testing fitness will often be integral to understanding gene function. Competitive population studies are capable of providing the most direct fitness measures while simultaneously providing a comprehensive comparison of genotypes with respect to important variables such as male and female activity patterns, dominance, reproduction, longevity, and offspring-rearing capacity. However, setting up population studies are by no means trivial, especially for larger metazoans and nonsocial species. Researchers working on vertebrate species might be wise to start with simpler approaches to learn as much as possible about the gene or trait of interest using tools that are readily available in a laboratory setting. Despite the surfeit of mutants with no obvious phenotypes, there are nevertheless many cases where a little or a lot of concentrated effort in the laboratory will be rewarded. The basic problem is how to best proceed with phenotype analysis. As behavior represents the combined organismal response to all molecular, cellular, and physiological processes, it is certainly the most complex, but also perhaps the most fruitful area to begin the search. Most researchers find it prudent to begin with a battery of behavioral tests. A variety of guidelines and recommended protocols exist for this purpose, which are intended to help improve across-laboratory standardization and rigor (Bolivar *et al.*, 2000; Crawley, 2000; Crawley and Paylor, 1997; Hatcher *et al.*, 2001). Accordingly, the relatively new field of behavioral phenomics is an especially ripe area for the elucidation of gene function. Organisms with complex behavioral repertoires present the greatest challenge for efficient phenotyping. At the forefront of testing technology, sophisticated equipment is becoming available for automated behavioral monitoring and testing of mice and rats (Gerlai, 2002; Tecott and Nestler, 2004). The vast datasets these instruments are capable of producing are once again raising the bar for bioinformatics to facilitate the handling, processing, organization, and retrieving of tremendous information flow. The hope is that improved across-laboratory consistency, reliability, and comparative analysis will not only help reveal hidden phenotypes, but will simultaneously avoid the opposite pitfall—detecting a phenotype when none exists or misinterpreting a phenotype.

Phenotypes represent not only the effect of a disrupted gene, but depend also on genetic background (Strunk *et al.*, 2004; Threadgill *et al.*, 1997), age (Crabbe *et al.*, 1999; Heiman-Patterson *et al.*, 2005; Hultcrantz and Li, 1993; McIlwain *et al.*, 2001), experience (McIlwain *et al.*, 2001), and environment (Crabbe *et al.*, 1999). Therefore, although the entire behavioral phenome is likely to occupy an enormous space, a large segment of the phenome will

undoubtedly reside within ecological space, involving the extended interplay of genes and environment. Phenomics technologies are still largely based on measuring the physiology and behavior of individual animals, and therefore have a long way to go before replicating the complex social milieu of experimental population studies. Nevertheless, automated technologies have many uses, from tracking motion, to measuring the duration of such complex behaviors as eating and grooming. Some of the more clever technologies are even beginning to integrate a more naturalistic social environment into the testing design. One such example is IntelliCage, manufactured by NewBehavior Inc. (Zurich, Switzerland; http://www.newbehavior.com). This instrument enables the simultaneous tracking and testing of multiple interacting animals. Although laboratory-based phenomics testing does not yet offer a substitute for long-term fitness studies, these technologies have proven to be extremely informative and continue to make rapid technological advances as researchers demand more from their assays.

B. How Do Fitness Measures Contribute to Understanding the Molecular Basis of Phenotypes?

One criticism of the ecological approach espoused here is that ''fitness differences in population cages will not easily lead to understanding the function of these genes in a more mechanistic sense.'' However, we are presenting the ecological approach for understanding gene function not as a substitute for mechanistic studies, but as a vital first step in the process, because determining the function of a gene and the mechanistic basis of its associated phenotype is greatly aided by a full characterization of the phenotype. Most diseases are first discovered as an organismal defect, usually with symptoms that do not reveal the molecular and physiological basis of the malady. Once the disease phenotype is characterized, we then go on to characterize its molecular, cellular, and physiological bases. This has often taken decades. Diseases characterized in seminatural conditions are no different than diseases characterized any other way. The struggle to elucidate biochemical and biological details will proceed in identical ways as diseases identified by any other means. The advantage of an ecological approach is that forward and reverse genetic studies are both possible once fitness defects of knockouts or known mutations are revealed. We are therefore much farther ahead at characterizing the mechanistic basis of a mutant than when we are fooled into thinking there is no defect, which is the case any time functional redundancy is falsely invoked.

Our proposed approach simply identifies disease states that are difficult to detect in other ways. It gives voice to mice who can now tell us, ''Bearing a *t* allele causes me discomfort; I am only half the mouse I used to be.'' As a consequence, we can combine an advantage of human medicine (where the patient tells you it hurts) with the advantages of experimental animal studies. Our ecological approach revealed defects in *t*-bearing mice having massive evolutionary consequences, equivalent to a lethal gene with 29% penetrance. We can now proceed to identify and characterize the molecular basis of these defects which were invisible under four decades of traditional approaches.

In this age of evo-devo, developmental and evolutionary biologists are increasingly eager to share ideas and insights across fields, using the principles of natural selection and evolution along with biological and molecular tools to attack problems of mutual interest. Despite these melding of interests, there is a general lack of appreciation for the idea that genes may be developmentally critical if they are regulated during embryogenesis but only manifest phenotypes at later stages of development or adulthood, and furthermore, that genes which are only expressed during later stages of development and adulthood are nevertheless essential if they mediate successful reproduction. This includes, but is not limited to genes which enable procurement of resources critical to obtaining mates. For this reason, phenotypic changes that show up under competitive circumstances are utterly relevant to the study of development. The ultimate and only meaningful test of all development is how it influences adult performance (fitness). Developmental genes that fail this test will be discarded by natural selection. Successful embryogenesis is the intermediate process on the way to high-performance adults. Thus, testing adult performance is requisite for evaluating successful embryogenesis.

If we are going to take seriously the challenge of determining the function of genes in the postgenomic era, we must have sensitive methods for detecting less obvious phenotypes. The ultimate function of many genes will be to increase competitiveness by enhancing what might be called ''general health and vigor.'' Enhanced vigor can be achieved in innumerable ways such as increasing metabolic efficiency, neuromuscular coordination, and so on. Each of these mutants will have a molecular and physiological basis and when we discover it we will not call it general vigor anymore, we will call it by its specific name, such as a metabolic defect. But without sensitive methods to identify organismal defects, these molecular defects will largely remain undetected. The ecological approaches proposed here do not replace current functional genomic tools; they add a sensitive screen allowing detection of important but cryptic functions.

C. Nonmodel Organisms and Functional Genomics

Female zebra finches, with an acoustic call structure far simpler than that of their musical mates, were long assumed to lack the vocal skills capable of allowing males to distinguish them individually. That is, until Christopher Sturdy (2004) discovered that males can and do respond to their mate's call—it just takes the right social environment. Male zebra finches respond to their mate's call twice as often as to that of an unfamiliar female when he finds himself in the presence of a mated pair of zebra finches. But a male's brain simply does not activate the same way when he is alone (Vignal *et al.*, 2004). Clearly, his ability to judge the importance of social context is more sophisticated than our own naïve attempts. The field of Sociogenomics (Robinson, 1999) takes such experiments a step further, by asking not just ''why,'' but ''how.'' The goal of Sociogenomics is to dissect the molecular underpinnings of social life, and as such, focuses well beyond the familiar model organisms examined in this chapter, to all creatures displaying potentially complex social behaviors, from *Dictyostelium* to hymenoptera to birds and other beasts. To understand social behavior and how it evolves, sociogenomic researchers track down genes and regulatory pathways that underlie development, physiology, and behavior using the same genomics tools as do conventional molecular and developmental geneticists. What distinguishes this field from that of connected molecular and genetic research is its special focus on species that live in societies and its emphasis on naturalistic conditions as a prerequisite for study (Robinson *et al.*, 2005). The related fledgling field of evolutionary and ecological functional genomics (Feder and Mitchell-Olds, 2003) similarly seeks to understand which genes effect ecological success and influence fitness in nature and how they do it. Integration of these two approaches with conventional genomics offers the opportunity to broaden genetic studies to include phenotypes that are not found in model organisms and moreover, to allow inferences into the evolution of traits through comparative studies with outgroups of species carrying genes of interest.

IV. Summary

The enterprise of determining the function of genes is by far the most difficult portion of genome projects. This reflects the sheer complexity of the genome, with genes interacting to influence function (epistasis), genes influencing more than one function (pleiotropy), the involvement of many genes to effect one function (polygenic traits), and countless gene-associated

phenotypes yet to be discovered. A particular problem emerging from targeted gene-disruption technologies is that many of these gene knockouts seem to have no phenotypic effect on the organism. The conventional explanation of such observations is to invoke functional redundancy in genomes. Although this may explain some cases, our review of the literature here suggests that many, if not the majority of such observations represent situations where if the mutant gene was tested under the ecological stresses and contingencies in which they evolved, functional defects could be measured as substantial declines in specific components of fitness. Here we review and develop this ecological approach for evaluating the functional effects of gene mutants, knockouts, or variants. Such ecological approaches are already in use in nonmodel organisms, largely for evaluating functional consequences of genetic variants. Thus the research program does not represent anything particularly new other than pointing out what should be obvious—to succeed over long-term evolution, alleles must outperform the fitness contribution of genetic variants (and mutants) within the ecological conditions where they function. Yet, when one looks at what is published in functional genomic journals or topics at functional genomics meetings, one seldom observes attempts to test gene function under the ecologies in which they evolved. In the same journals functional redundancy emerges as the default explanation in cases where genes are knocked out but with little to no phenotypic effect. When functional redundancy is accepted as the explanation for no phenotypic change, research on that mutant largely comes to a halt. Here we review many cases where fitness-based assays under seminatural ecological conditions revealed phenotypes (often major phenotypes) that were missed in laboratory studies. Developing such a research program provides a great opportunity for the development of a truly integrative biology, where we begin to understand how genetic change influences molecular, cellular, and physiological changes that ultimately control the fitness-influencing performance of whole organisms. We conclude that functional genomics will often require an understanding of ecology and behavior to gain a useful understanding of gene function.

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