EXPERIMENTAL EVOLUTION OF PHENOTYPIC PLASTICITY FOR STRESS RESISTANCE IN THE NEMATODE *CAENORHABDITIS REMANEI*

by

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DISSERTATION ABSTRACT

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Many organisms can acclimate to new environments through phenotypic plasticity, a complex trait that can be heritable, be subject to selection, and evolve. However, the rate and genetic basis of plasticity evolution remain largely unknown. Experimentally evolved populations of the nematode *Caenorhabditis remanei* were created by selecting for stress resistance under different environmental conditions. This resource was used to address key questions about how phenotypic plasticity evolves and what the genetic basis of plasticity is.

Here, I highlight ways in which a fuller understanding of the environmental context influences our interpretation of the evolution of phenotypic plasticity. In a population selected to withstand heat stress, an apparent case of genetic assimilation did not show correlated changes in global gene regulation. However, further investigation revealed that the induced plasticity was not fixed across environments, but rather the threshold for the response was shifted over evolutionary time.

Similarly, the past environment experienced by populations can play a role in directing the multivariate response to selection. Correlated responses to selection between traits and across environments were examined. The pattern of covariation in the evolutionary response among traits differed depending on the environment in which

selection occurred, indicating that there exists variation in pleiotropy across the stress response network that is highly sensitive to the external environment.

To understand how the patterns of pleiotropy are altered by environment and evolution, there is a pressing need to determine the structure of the molecular networks underlying plastic phenotypes. Using RNA-sequencing, the structure of the gene regulatory network is examined for a subset of evolved populations from one environment. Key modules within this network were identified that are strong candidates for the evolution of phenotypic plasticity in this system.

Together, the data presented in this dissertation provide a comprehensive view of the myriad ways in which the environment shapes the genetic architecture of stress response phenotypes and directs the evolution of phenotypic plasticity. Additionally, the structure of transcriptional network provides valuable insight into the genetic basis of adaptation to environmental change and the evolution of phenotypic plasticity.

This dissertation includes both previously published and co-authored material.

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For my parents, who fostered my deep love and appreciation for science from the very beginning.

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CHAPTER I

INTRODUCTION

WHAT IS PHENOTYPIC PLASTICITY?

Natural environments are constantly changing. Some changes are predictable, such as changes in light availability over the course of a day or seasonal fluctuations in temperature. Other environmental challenges—exposure to an environmental toxin or introduction of a new competing species—may happen sporadically and without warning. In order to flourish, organisms must be able to acclimate to these shifts in the environment. If the population is to persist then subsequent adaptive evolution must also occur.

It has long been recognized that the environment is critically important as a selective agent changing allele frequencies over time (Darwin 1859). However, many organisms can respond to environmental change through phenotypic plasticity. Phenotypic plasticity is defined as the ability of a genotype to consistently produce a different phenotype in response to environmental stimulus (Bradshaw 1965), and can encompass a range of phenotypic responses. Some of the most striking examples of phenotypic plasticity are morphological changes resulting from altered developmental pathways. Spadefoot toads (Gomez-Mestre and Buchholz 2006), *Daphnia* (Parejko and Dodson 1991), *Onthophagus* dung beetles (Moczek 1998), and *Pheidole* ants (Wilson 1984) are a few of the many species that can dramatically alter their appearance in response to environmental cues. For many morphological traits, the decision to produce

one phenotype or another is irreversible. However, examples also exist of reversible morphological plasticity, e.g., seasonal coat color in hares (Keogh 1967), and plumage in finches (Hill et al. 2002). Plasticity can also manifest in physiological traits, without leading to obvious changes in morphology. Cellular responses to heat stress induce the production of chaperone proteins to mitigate damage (Lindquist and Craig 1988; Wu 1995; Åkerfelt et al. 2010), while acclimation to high altitude might lead to its own set of cellular responses to hypoxia (Cheviron et al. 2013). Finally, animal behaviors are a classic example of a highly plastic phenotype (West-Eberhard 1989; Sih et al. 2004).

While phenotypic plasticity does not need to be adaptive, induced responses that have a perceived benefit to the organism are often the most interesting for evolutionary biology. Many examples exist in which it has been demonstrated that the plastic response is adaptive, spanning the tree of life from bacteria (Justice et al. 2006; Kümmerli et al. 2009) to plants (Schmitt et al. 1995; Agrawal 1998) to animals (Aubret et al. 2004; Charmantier et al. 2008; Muschick et al. 2011). However, it is still unclear what the role of phenotypic plasticity is in evolution. Does plasticity, for example, speed up or slow down genetic evolution (West-Eberhard 2003)? Can plasticity lead to innovation and novelty (Pfennig et al. 2010; Moczek et al. 2011)? What is the genetic basis of phenotypic plasticity, and how does it evolve (Snell-Rood et al. 2010)? In many natural models of phenotypic plasticity, the paucity of information about the historical evolution of plasticity makes addressing these fundamental questions prohibitively difficult.

THE GENETIC BASIS OF PLASTICITY

Most ecologically and evolutionarily important traits are complex. In traditional quantitative genetics, phenotypes are considered to be products of additive genetic effects (G), environmental effects (E) and interactions between genes and the environment (GxE). These traditional evolutionary genetics models are often simplified by assuming that organisms exist primarily in one set of environmental conditions. However, organisms can regularly or periodically experience novel and often stressful environments. In this context, the full significance of each effect—G, E, and GxE—within populations can be appreciated. These effects can be represented as reaction norms (Fig. 1.1), in which each line represents a unique genotype raised in alternative environments. Phenotypes produced by purely genetic effects in two environments will differ due to genetic variation, but the phenotype will not change for any given genotype even when raised in a novel environment (Fig. 1.1A). Phenotypic plasticity is manifest in two distinct types of effects. Additive environmental effects (E) result in parallel phenotypic changes among all genotypes (Fig. 1.1B). In this case, the genetic variation (G) and the environmental effect (E) act in an additive manner across environments. In contrast, genotype-by-environment (G-by-E) interactions can be visualized as crossing developmental reaction norms (Fig. 1C). Different genotypes exhibit differing directions and magnitudes of phenotypic change when transferred to a novel environment. The effect of the

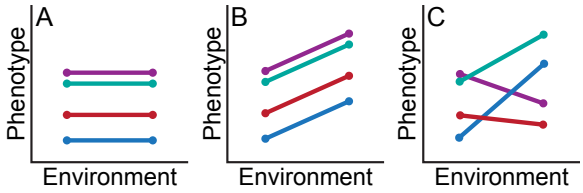


Figure 1.1. Genotype (A), environment (B), and genotype-by-environment interaction (C) effects observed in a phenotype when a given genotype is raised in two different environments. Each line represents a single genotype raised in alternative environmental conditions.

environment upon phenotype is therefore dependent upon which genotype is being considered.

An important correlate of GxE interactions is that some alleles may be differentially associated with phenotypic variation depending on environmental conditions. Therefore, underlying the non-additive effects observed in GxE interactions is so-called cryptic genetic variation (CGV), which is defined as the genetic variation that does not contribute to the phenotype under normal conditions, but which becomes expressed when the organism is exposed to environmental or mutational perturbations (Gibson and Dworkin 2004; Paaby and Rockman 2014). The accumulation of CGV is a direct consequence of canalization, a process by which organisms evolve decreased sensitivity to the genetic and environmental variability they commonly experience (Waddington 1942). Canalization buffers developmental pathways to stabilize phenotypes against common changes, and some genetic variation becomes hidden as CGV, not contributing to the observed phenotypic variance in the organism over its normal environmental range (Gibson and Dworkin 2004; Paaby and Rockman 2014). However, outside of the range of conditions in which canalization evolved, the robustness of canalized phenotypes breaks down, and CGV immediately contributes to the phenotype of the individual, resulting in increased phenotypic variance (McGuigan and Sgrò 2009; Paaby and Rockman 2014).

The exposure of CGV in both epistatic and GxE interactions is of significant importance for rapid phenotypic adaptation. Cryptic genetic variation can accumulate neutrally under a permissive environment, but when a population experiences a novel environment alleles are immediately available in significant frequencies to contribute to

evolution (Paaby and Rockman 2014). In addition, environmentally-induced CGV has particularly strong implications for defining the role of phenotypic plasticity in directing the evolutionary process. In this case, the environment becomes not just the selective agent, but also has a large role in exposing the subset of genetic variation to become expressed in the phenotype. The uncovering of similar types of CGV during the independent exposure of different populations to the same novel environments could be a mechanism for parallel evolution. Furthermore, the sudden exposure of, and subsequent selection on, cryptic mutations can lead to dramatic phenotypic change and rapid adaptation to novel environments through the process of genetic accommodation (Waddington 1953; West-Eberhard 2003; Moczek 2007).

A few studies have examined the evolution of plastic responses in natural populations of birds (Nussey et al. 2005), threespine stickleback (Wund et al. 2008; McGuigan et al. 2011), and tiger snakes (Aubret and Shine 2009). These studies, however, were limited in their ability to prove that CGV was selected on in historical populations, and none could dissect the genetic basis of CGV underlying the traits examined. In laboratory populations, where the evolutionary process can be directly observed in a controlled environment, genetic accommodation has been convincingly demonstrated in the evolution of polyphenisms in *Drosophila* (Waddington 1953; 1956; Gibson and Hogness 1996; Rutherford and Lindquist 1998), and tobacco hornworm (Suzuki and Nijhout 2006; 2008). These studies, as well as others in *Drosophila* (Gibson et al. 1999; Dworkin et al. 2003), *Arabidopsis* (Sangster et al. 2008a,b) and ribozymes (Hayden et al. 2011), have provided some insights into the molecular genetic basis of CGV and genetic accommodation. Despite being important advances, this previous work

provides an incomplete understanding of CGV in rapid evolution. First, the phenotypes uncovered and selected in these studies would probably be deleterious for natural populations. In addition, the relative importance of genetic accommodation to evolution of natural populations remains controversial, and the genetic basis of CGV in natural populations is largely unknown.

BRIEF OUTLINE OF THIS DISSERTATION

Despite the widespread occurrence of phenotypic plasticity in natural populations, much is still unknown about its role in evolution. Does plasticity contribute to evolutionary novelty? How quickly does plasticity evolve in natural populations? Still less is understood about the molecular basis of phenotypic plasticity. Many examples for which the molecular basis of plasticity has been worked out have used unnatural stresses, unlikely to be encountered by natural populations. Other studies have used mutant strains, not representative of the naturally segregating variation in the population. This dissertation addresses these limitations by harnessing a powerful experimental evolution framework to select on natural, segregating variation for resistance to ecologically relevant stresses.

Experimental evolution is a powerful system for studying evolutionary processes (Rose et al. 1990; Huey et al. 1991; Lenski et al. 1991; Matsumura 1996; Callahan 2005), because it probes the relevant components of the genetic architecture of traits. In organisms that can be cryogenically stored, such as bacteria and nematodes, this approach is particularly useful, as the ancestral and evolved populations can be compared

simultaneously, thus controlling for temporal effects in the phenotypic assay (Lenski et al. 1991).

Previous studies have used experimental evolution to investigate the evolution of phenotypic plasticity in organisms (Waddington 1953, 1956; Suzuki and Nijhout 2006). However, such studies have suffered from several drawbacks. In Waddington's classic experiments in *Drosophila melanogaster*, the phenotypes measured, differences in crossvein patterning (1953) or partial duplication of the thoracic elements (1956), have no clear adaptive benefit. In the latter case, it can be argued that the induced phenotypes would be maladaptive in a natural environment. In a more recent study (Suzuki and Nijhout 2006), the *black* mutant strain of the tobacco hornworm *Manduca sexta* was selected for the ability to change color from black to green following heat shock. While heat stress could be relevant in nature, this study did not demonstrate that plasticity in the color phenotype had any effect on fitness.

The nematode *Caenorhabditis remanei* is an ideal model for experimental evolution. Like its sister species *C. elegans*, *C. remanei* can be cryogenically stored (Brenner 1974), enabling direct comparison between ancestral and evolved populations. Unlike *C. elegans*, however, *C. remanei* is an obligately outcrossing species. Because of this, populations display two key properties—an abundance of genetic variation (Graustein et al. 2002; Jovelin et al. 2003; Cutter et al. 2006) and ample recombination—which facilitate a rapid response to selection (Morran et al. 2009). Evolved lines were created by selecting on their ability to withstand heat or oxidative shock during early larval development in three different environmental conditions. Both traits display significant heritable variation in natural populations of *C. remanei* (Reynolds and Phillips

2013). Furthermore, both heat and oxidative stress are likely to be ecologically relevant to natural populations, and by measuring and selecting on survival, the importance of the trait for fitness is much clearer.

Chapter II is in press to be published in *G3: Genes-Genomes-Genetics* (Sikkink et al. 2014b), and is the result of collaboration between Rose M. Reynolds, Catherine M. Ituarte, William A. Cresko, Patrick C. Phillips and myself. We describe the rapid loss of phenotypic plasticity for heat stress resistance in lines evolved to withstand heat stress under permissive conditions. The loss of plasticity appeared to have resulted from the genetic assimilation of the inducible response to heat in the non-inducing environment. However, analyses of transcriptional variation via RNA-sequencing from the selected populations revealed no global changes in gene regulation correlated with the observed changes in heat stress resistance. Instead, assays of the phenotypic response across a broader range of temperatures revealed that the induced plasticity was not fixed across environments, but rather the threshold for the response was shifted to higher temperatures over evolutionary time. These results demonstrate that apparent genetic assimilation can result from shifting thresholds of induction across environments and that analysis of the broader environmental context is critically important for understanding the evolution of phenotypic plasticity.

In Chapter III, I explore further the consequences of the broader environmental context on the evolution of stress resistance. In addition to myself, R. M. Reynolds, W. A. Cresko and P. C. Phillips contributed significantly to the work described in this chapter. Current knowledge of the molecular pathways underlying stress resistance in *C. elegans* suggest that resistance to heat and oxidative stress resistance should share a

genetic basis, and thus evolve in a coordinated fashion. Surprisingly, we found that correlated responses to selection did not generally occur. However, we find that the environmental context—in this case the environment experienced during the past selection—altered the nature of the genetic correlations within the network. Similar contingencies on the selective environment were observed for each trait individually when considering genetic correlations across environments. Such observations are difficult to explain within the canonical quantitative genetic framework. We propose that the patterns of pleiotropy underlying the stress response networks are fundamentally altered by the environmental conditions. To determine precisely how that contingency arises, however, quantitative genetic theory is not sufficient. Instead we require knowledge of the molecular interactions in the stress response networks.

Analysis of the gene regulatory networks using RNA-seq is one method that can be used to understand the structure of the stress response network. Chapter IV is the result of collaboration between R. M. Reynolds, C. M. Ituarte, P. C. Phillips, W. A. Cresko, and myself. Here, we investigate for the first time the structure of the gene regulatory network in the context of experimentally evolved populations in order to understand the evolution of plasticity. We describe the structure of the network determined from the lines evolved under one of the three environments described in Chapter II. Furthermore, we identify subgroups, or modules, within the larger network that may contribute to the evolution of plasticity in our system.

Finally, Chapter V summarizes the results from Chapters II – IV and discusses how they contribute to our understanding of the role of phenotypic plasticity in evolution.

CHAPTER II

RAPID EVOLUTION OF PHENOTYPIC PLASTICITY AND SHIFTING THRESHOLDS OF GENETIC ASSIMILATION IN THE NEMATODE *CAENORHABDITIS REMANEI*

This work is in press, to be published in the journal *G3: Genes | Genomes | Genetics* in 2014. R. M. Reynolds and I created the experimental selection lines used in this study. C. M. Ituarte and I made the transcriptional profiling libraries. I performed the statistical analyses. W. A. Cresko and P. C. Phillips were the principal investigators for this work.

INTRODUCTION

Organisms regularly experience changes in their environments to which they must adapt in order to survive. As a consequence, many organisms have evolved the capacity to respond to stressful changes in environmental conditions by coherently altering their phenotypic attributes. This phenotypic plasticity, defined as the ability of a genotype to consistently produce an alternate phenotype in response to environmental variation (Bradshaw 1965), is known to be an important contributor to fitness in many organisms, including bacteria (Justice *et al.* 2006; Kümmerli *et al.* 2009; Butler *et al.* 2010), plants (Dudley and Schmitt 1996; Huber 1996; Agrawal 1998; Harder and Johnson 2005), and animals (Parejko and Dodson 1991; Warkentin 1995; Aubret *et al.* 2004; Charmantier *et al.* 2008; Cheviron *et al.* 2013).

Like any other character of organisms, phenotypic plasticity itself has a genetic basis that can change in response to evolutionary processes. One extreme evolutionary outcome of adaptation to a novel environment is the complete loss of ancestral phenotypic plasticity after selection, which is known as genetic assimilation (Waddington 1953; 1956). More generally, adaptation in one environment can lead to changes in phenotypic plasticity across other environments due to genetic correlations generated by the pleiotropic effects of genes responding to both environments or by genetic linkage of genes with independent effects within each environment. Quantitative genetic models (Via and Lande 1985; Gomulkiewicz and Kirkpatrick 1992; Gavrilets and Scheiner 1993) predict that these genetic correlations across environments determine how plasticity across environments evolves over time.

Although there has been renewed interest in the evolution of phenotypic plasticity and its importance for affecting the rate and direction of evolution of populations experiencing novel environments (Matesanz *et al.* 2010; Pfennig *et al.* 2010; Moczek *et al.* 2011), it is still unclear how fast phenotypic plasticity can evolve or what the molecular genetic basis underlying this evolution actually is. Except for a few classes of genes, most notably the heat shock proteins (hsps), which have been well characterized for their role in regulating physiological responses to stress (Lindquist and Craig 1988) and acting as a capacitor for environmentally-sensitive variation (Rutherford and Lindquist 1998; Cowen and Lindquist 2005; Sangster *et al.* 2008a; Jarosz and Lindquist 2010; Rohner *et al.* 2013), little is known about where genetic variation for phenotypic plasticity resides in organisms' genomes. For example, from a mechanistic standpoint, it is not known to what extent the evolution of phenotypic plasticity occurs primarily via

changes in frequencies of alleles affecting protein-coding regions of genes as compared to regulatory changes affecting differential expression of genes. Dissecting the genetic basis of evolutionary change in phenotypic plasticity is particularly important because both the rate of evolution of phenotypic plasticity itself and the structure of genetic correlations across environments depend on the genetic architecture of phenotypic plasticity. Although it is likely that multiple mechanisms play a role in the evolution of plasticity, a readily testable hypothesis is that rapid evolution of phenotypic plasticity is, at least initially, more likely to involve genetic variation in transcriptional regulation.

In addition to this evolutionary context, there is increasing interest in a variety of fields as to how environmental factors such as nutrition or exposure to stress influence a wide variety of health-related outcomes such as aging (Gems and Partridge 2013). Although the direct negative effects of some environments, such as exposure to pathogens, are clear, in some cases brief exposure to stress at one point in the life cycle can lead to increased resistance to stress at a later time period—a phenomenon known as hormesis (Gems and Partridge 2008; Le Bourg 2009). In general, it appears that protection via a hormetic response is generated by the induction of stress response pathways (*e.g.*, heat shock proteins; Volovik *et al.* 2012) in advance of when they are actually needed during exposure to a more severe stressor. Hormesis is a classic example of adaptive phenotypic plasticity, although the intellectual traditions of the two fields are largely distinct.

Here we address this broad set of evolutionary and functional questions using experimental evolution in nematodes to investigate changes in phenotypic plasticity for an ecologically relevant trait: resistance to heat stress. Experimental evolution has proven

to be a powerful system for studying evolutionary processes (Rose *et al.* 1990; Huey *et al.* 1991; Lenski *et al.* 1991; Matsumura 1996; Callahan 2005), including the genetic assimilation of phenotypically plastic traits (Waddington 1953; 1956; Suzuki and Nijhout 2006). Experimental evolution is particularly useful when ancestral and evolved populations can be compared simultaneously (Lenski *et al.* 1991).

We evolved the nematode *Caenorhabditis remanei*, which, like its sister species *C. elegans*, can be frozen indefinitely and recovered later (Brenner 1974). Unlike *C. elegans*, however, *C. remanei* populations display an abundance of genetic variation (Graustein *et al.* 2002; Jovelin *et al.* 2003; Cutter *et al.* 2006) and ample recombination because of obligate outcrossing, both of which facilitate a rapid response to selection (Morran *et al.* 2009). We evolved lines by selecting on their ability to withstand heat shock during early larval development, a trait that displays significant heritable variation in natural populations of *C. remanei* (Reynolds and Phillips 2013). Plasticity for heat stress resistance was measured in populations that were raised in the selective conditions (standard lab environment at 20°) and in a high temperature environment at 30° which the populations had not experienced during selection. We further investigated the transcriptional changes occurring in the selective populations across environments. Together, these data enable a detailed investigation of adaptive physiological and transcriptional changes in phenotypic plasticity in an ecologically important trait in *C. remanei*.

MATERIALS AND METHODS

Creation of ancestral population

To obtain a heterogeneous population, we collected wild isolates of *C. remanei*. 200 woodlice (terrestrial isopods of the Family *Oniscidea*, also known as sowbugs or pillbugs) from Koffler Scientific Reserve at Jokers Hill, King City, Toronto, Ontario (+44° 1′ 46.88″, -79° 31′ 41.69″) were graciously provided to us by the Cutter laboratory (University of Toronto) and express-mailed to the Phillips laboratory (University of Oregon). All woodlice were collected within 300 meters of the main building of the field station. Of the 200 woodlice, approximately 20% contained *C. remanei*. From each of these we collected and maintained one mating pair, yielding 26 "isofemale strains." Isofemale populations were immediately expanded to a large population size following the initial mating (approximately 100-1000 offspring per line in the first generation and very large population sizes in subsequent generations). All collected strains were frozen within three generations of collection to minimize lab adaptation. To create a cohort representative of naturally segregating variation for experimental evolution, we thawed samples from each of the 26 isofemale strains and crossed them in a controlled fashion to promote equal contributions from all strains, including from mitochondrial genomes and X chromosomes. The resulting genetically heterogeneous population (PX443) was frozen after creation and served as the ancestral population for the experimental evolution. Polymorphism in this species is ~5% (Jovelin *et al.* 2003; Cutter *et al.* 2006; Jovelin *et al.* 2009), so there should have been abundant segregating variation present at the initiation of selection. All natural isolates, as well as the lines used in the experiment described

below, were grown on nematode growth media (NGM) seeded with *E. coli* strain OP50 (Brenner 1974).

Stress response phenotype

An acute stress in the context of this experiment is one that challenges the stress response of the worm within a 4-hr period. Given the short average lifespans of *Caenorhabditids* (~20 d from L4), we reasoned that any exposure in excess of 6 hr might be treated by the worm as a chronic stress and could potentially invoke a fundamentally different class of cellular stress response. To test resistance to acute heat stress, worms were stage-synchronized via a bleaching procedure ("hatch off") that kills adults and leaves only developing embryos. Embryos were rinsed, suspended in buffer without food, and given 18 to 24 hr to develop into L1 larvae. L1 larvae enter developmental arrest in the absence of food (Baugh 2013). Worms in L1 diapause suspended in liquid buffer were then exposed to an acute heat stress at 36.8° in a shaking incubator (70 rpm) for 4 hr in a sealed microcentrifuge tube. As a control, a subset of the population was kept at 20° under similar conditions. After acute stress shock, worms were transferred into a Petri dish containing Nematode Growth Medium-lite (NGM-lite, U.S. Biological) seeded with *E. coli* strain OP50. Survival was estimated 3 to 4 d later, when most worms had developed into fourth-stage L4 larvae but had yet to lay eggs. Acute heat shock resistance was quantified as the proportion of the phenotyped population that survived the heat shock and matured to adulthood, relative to the average survival of the control samples.

Experimental evolution

We propagated four laboratory-adaptation control replicates and two acute heat-selected replicates. Each replicate population comprised 1000 to 2000 mating individuals.

Exposure to acute stress occurred either every second generation or when the population produced ≥24,000 eggs, whichever occurred later. At that point, worms were stage-synchronized as described previously and subjected to the stress phenotyping protocols as described above. The control populations were randomly culled to 1000 L1 larvae during each selective generation and subjected to similar treatment as the heat shock lines, but at 20°. In the heat-selected populations, 10,000 of the L1 worms were randomly selected to undergo acute stress selection at an average temperature of 36.8°. This intensity of heat shock induces ~70% larval death in the ancestral population (s = 0.7). To maintain a similar strength of selection (s between 0.7 and 0.8) throughout the experiment as the heat-selected population adapted, the heat shock temperature was increased incrementally (up to 37.2° in the final generation of selection). The populations were maintained in standard laboratory conditions at 20° between selective events. Selection was continued until each replicate line had experienced 10 total selective events.

Each population was frozen ($N \geq 100{,}000$ individuals) after approximately every second generation of experiencing acute stress shock in order to retain a record of evolutionary change in the populations over time and to ensure that worms did not lose the ability to survive freeze and thaw. Approximately 5000 individuals from the frozen populations were thawed to continue the evolution experiment, whereas the remaining 95,000 worms remained frozen for future phenotyping and genetic and genomic analyses. Populations were thawed for selection after a minimum of 24 hr at -80°. In half of the selection lines (two control and one heat-selected population), freezing occurred a total of three times during selection, whereas this occurred five times in the remaining populations.

Phenotypic plasticity across environments

To measure phenotypic plasticity for heat shock resistance across environments, the parents of phenotyped individuals were reared in either typical lab conditions (20°) or mild heat stress (30°). Their offspring were stage-synchronized, grown, and phenotyped in the parental rearing environment, *e.g.*, worms whose parents experienced 30° were raised entirely at 30°. The heat shock assays were performed as described above, except that the control samples were also kept at the same rearing temperature during the phenotyping assay.

Additionally, we chose the ancestor and one representative population from each selective regime to measure resistance to heat shock at a range of temperatures, from 36.5° to 37.8°. The temperature during heat shock was recorded at 5-min intervals using two Thermochron iButton devices (Maxim Integrated). The average heat shock temperature was defined as the average measured temperature for both devices over 4 hr. Heat stress across the range of heat shock temperatures was measured as described for the standard (36.8°) heat resistance assays.

Statistical analysis of phenotyping data

We tested for differences in survival following heat shock using a mixed model ANCOVA using JMP10.0 (SAS Institute). Fixed effects in the model included the cultivation temperature for phenotyping (20° or 30°), the selection regime (ancestor, control-selected, or heat-selected), and the interaction between phenotyping temperature and selection regime. Independently derived replicate lines from each experimental block were nested within selection regime and treated as a random factor using Satterthwaite's approximation for degrees of freedom (Winer *et al.* 1991). As the dependent variable, we

used square-root transformed counts of survivors from each heat-shocked plate. We included the square-root transformed average count of worms from the control plates for each phenotyping assay as a covariate in the model to control for variation in the estimated number of worms in each assay. The interaction between replicates and phenotyping environment was also included in the full model as a random factor, but its effect was very small and not significant and produced a slightly negative variance component estimate. Therefore, we set the variance component equal to zero for this term in order to carry out further hypothesis testing.

Differences between reaction norms over the range of heat shock temperatures were tested by fitting a logistic regression model implemented in R (R Development Core Team 2013). We used a quasi-binomial model to allow for overdispersion in the response variable. The total number of individuals in each trial was assumed to be the average count from controls from the same treatment group that were assayed concurrently. The number of survivors from each heat shock trial was taken to be the successes in the model. In any case in which the number of survivors was greater than the assumed total, the number of survivors was assumed to be equal to the total (100% survival). Two factors, rearing environment and selection regime, as well as one continuous variable, average heat shock temperature, and all interactions were tested in the full model. We also tested for environment and environment-by-heat shock temperature interactions within each selection line.

Transcriptional profiling of pooled populations

To obtain tissue for transcriptional profiling experiments, we thawed frozen stocks of worms from the ancestral population, one representative control population, and

one heat-selected population. Worms were raised at 20° for a minimum of three generations, or until the population was at least 250,000 individuals. Each population was then allowed to lay eggs, which were age-synchronized as described above. Age-synchronized embryos were allowed to hatch and develop for 20 hr in liquid medium, at which time most individuals had entered L1 diapause. Half of the larvae developed at 20° during this period, which we define as the larval development environment, while the remainder developed at 30°. After 20 hr, larval worms were passed through a 20-μm Nitex screen to remove unhatched eggs and dead adults. Approximately 15 μl of pelleted L1 tissue (~100,000 individuals) was flash-frozen in TRIzol (Ambion) and stored at -80° until RNA isolation. For each treatment condition from each line, 6 replicates were collected from a minimum of two independently thawed populations from each line. We extracted total RNA from L1 tissue using standard TRIzol methods, and from this pool mRNA was isolated using the Dynabeads mRNA purification kit (Ambion). Purified mRNA was sheared to 200- to 600-nt fragments using a buffered zinc solution (RNA Fragmentation Reagent; Ambion). cDNA was synthesized using Superscript III reverse-transcriptase (Invitrogen), and sequencing libraries were created through ligation of adaptors with inline barcodes to enable multiplexing of samples. Samples were sequenced in five lanes on an Illumina HiSequation 2000 at the University of Oregon Genomics Core Facility.

Analysis of differential gene expression

We performed quality filtering of raw sequence reads using the *process_shortreads* component of the software Stacks (Catchen *et al.* 2011; 2013), which discards reads with ambiguous sample identity, reads with uncalled bases, and reads

failing Illumina purity filters. Reads with ambiguous barcodes were rescued if they had fewer than two mismatches from a known barcode. We obtained more than 342 million reads that passed initial quality filters. We aligned all reads that passed the quality filters to the *C. remanei* genome (C_remanei-15.0.1 assembly) available at Ensembl Metazoa (metazoa.ensembl.org/) using GSNAP (Wu and Nacu 2010). To help guide the alignment across exon boundaries, we used existing annotated gene models for protein-coding genes obtained from Ensembl Metazoa, while allowing GSNAP to identify novel splice sites as well. For this study, we chose to focus on previously annotated protein-coding genes. While this approach may miss responses in genes that are not currently annotated, this dataset does include 31,518 transcripts, including many of the genes that might be expected to respond to heat stress (*e.g.*, hsps). We then used the *htseq-count* tool from the Python package HTSeq (http://www-huber.embl.de/users/anders/HTSeq/) to count all reads aligning to protein-coding genes. Reads were counted against the gene models using the "union" mode in *htseq-count*, so that reads were only counted if they unambiguously overlapped a single gene model.

For each selection line, we tested only those genes for which we could confidently detect expression. Genes expressed at very low levels are unlikely to be detected in all libraries and are more likely to be affected by sampling variance (Bloom *et al.* 2009), thereby reducing the power for detecting differential expression among treatments. We modified the filtering procedure commonly implemented in the *edgeR* package (Robinson and Oshlack 2010; Anders *et al.* 2013) to remove these uninformative genes prior to analysis. Genes that had less than one count per million (cpm) were considered to be unexpressed in a given sample. In our smallest sequenced libraries, 1 cpm is equivalent to

about two reads aligned to a given gene. Because we were interested primarily in the effect of environmental treatment, we excluded genes for analysis unless they met the detection threshold (>1 cpm) in at least four of the six replicates for one of the temperature treatments for any line.

Differential gene expression analysis was conducted using the *DESeq* package (Anders and Huber 2010) in R, which utilizes a negative binomial distribution to test for differential expression among treatments to better accommodate the well known phenomenon of overdispersion in RNA-seq data. We tested for differences in gene expression between larval environments within each of the selection lines. Two factors, larval development temperature and replicate thaw, were included in the full model as additive effects. To assess the effect of temperature on expression, we compared the full model to a reduced model that excluded temperature. Larval development temperature was deemed to have a significant effect on the regulation of a gene if the full model fit significantly better than the reduced model at a 5% false discovery rate (FDR) after adjusting for multiple comparisons using the Benjamini-Hochberg method (Benjamini and Hochberg 1995). Similarly, we tested for the effect of evolution within the 20° larval environment by testing for significant expression differences between each pair of populations.

To understand how transcriptional plasticity evolved in the selected lines, we compared differential expression ($\log_2$ fold change between larval environments) of each evolved population to the ancestor. Because of differences in power to detect differential expression among the three lines, we used a regression approach to compare the average change across environments in each selected line and the ancestor. Three genes that were

expressed in only one environment were excluded from this regression analysis, because the $\log_2$ fold change is infinite. Furthermore, we excluded from this analysis all genes that were expressed below the detection limit in either compared line, as gene silencing potentially represents a different mechanism for genetic assimilation. Finally, we also excluded genes that did not show significant inducible expression (FDR <5%) in at least one of the two lines under comparison. We fit an ordinary least squares (OLS) linear model (using the *lm* function in R) to the $\log_2$ transformed fold change of the significantly differentially expressed genes for each pairwise comparison of the evolved lines with the ancestor.

Gene ontology enrichment analysis

We used the software program Blast2GO (Conesa *et al.* 2005) to look for over-representation of GO terms (The Gene Ontology Consortium 2000) in the sets of significantly upregulated or downregulated genes in the 30° larval environment. Blast2GO computes a Fisher's Exact Test with a FDR correction to test for significant over-representation of GO terms in a test set. Two test sets were created for each population: one with significantly upregulated genes (FDR <1%), and one with downregulated genes (FDR <1%). We tested for over-representation of generic GOSlim ontology terms using a one-tailed test against a reference set of the genes which were not differentially expressed between larval environments in the same population (FDR >5%). Ontology information was visualized using Cytoscape 3.0 (Smoot *et al.* 2011).

RESULTS

Selection increases resistance to heat stress in the selective environment

When raised in standard lab conditions at 20°, approximately 30% of individuals from the ancestral population survived a 36.8° heat shock treatment during the early larval period and were subsequently able to develop to adulthood (Figure 2.1A). Following ~30 generations of propagation under standard laboratory conditions, control populations maintained a level of heat stress resistance that was approximately comparable to that of the ancestor ($F_{1,4} = 0.99$, $P = 0.3825$). Some variation among independently evolved replicates was observed, potentially reflecting genetic drift among these populations. In contrast, selection via periodic exposure to heat shock increased resistance to high temperatures. Comparing the time points from the heat-selected lines reveals a linear increase in survival over the course of selection (linear model with time: $F_{1,4} = 10.04$, $P = 0.0397$; quadratic terms: $F_{1,5} = 0.23$, $P = 0.6485$), culminating with nearly 85% of individuals surviving heat shock in the final generation.

Plasticity for acute heat shock resistance evolves rapidly

In addition to measuring larval heat resistance following cultivation at 20°, which is the standard environment during the selection experiment, we also exposed individuals from each population to a novel environment: elevated temperature (30°) during embryogenesis. Note that *C. remanei* is much more resistant to high temperatures than *C. elegans*, which tends to have an upper thermal limit of 26° to 27° (Anderson *et al.* 2011). After cultivation at 30°, survival of the ancestral population increased to 63% after heat shock ($F_{1, 111} = 33.52$, $P < 0.0001$, Figure 2.1C), reflecting a high degree of plasticity across environments for the heat resistance phenotype (Figure 2.1B), apparently via

induction of heat resistance pathways at this sub-lethal temperature. In the populations evolved under control conditions, the novel 30° environment induced a similarly large plastic response as in the ancestral population, with no significant population-by-

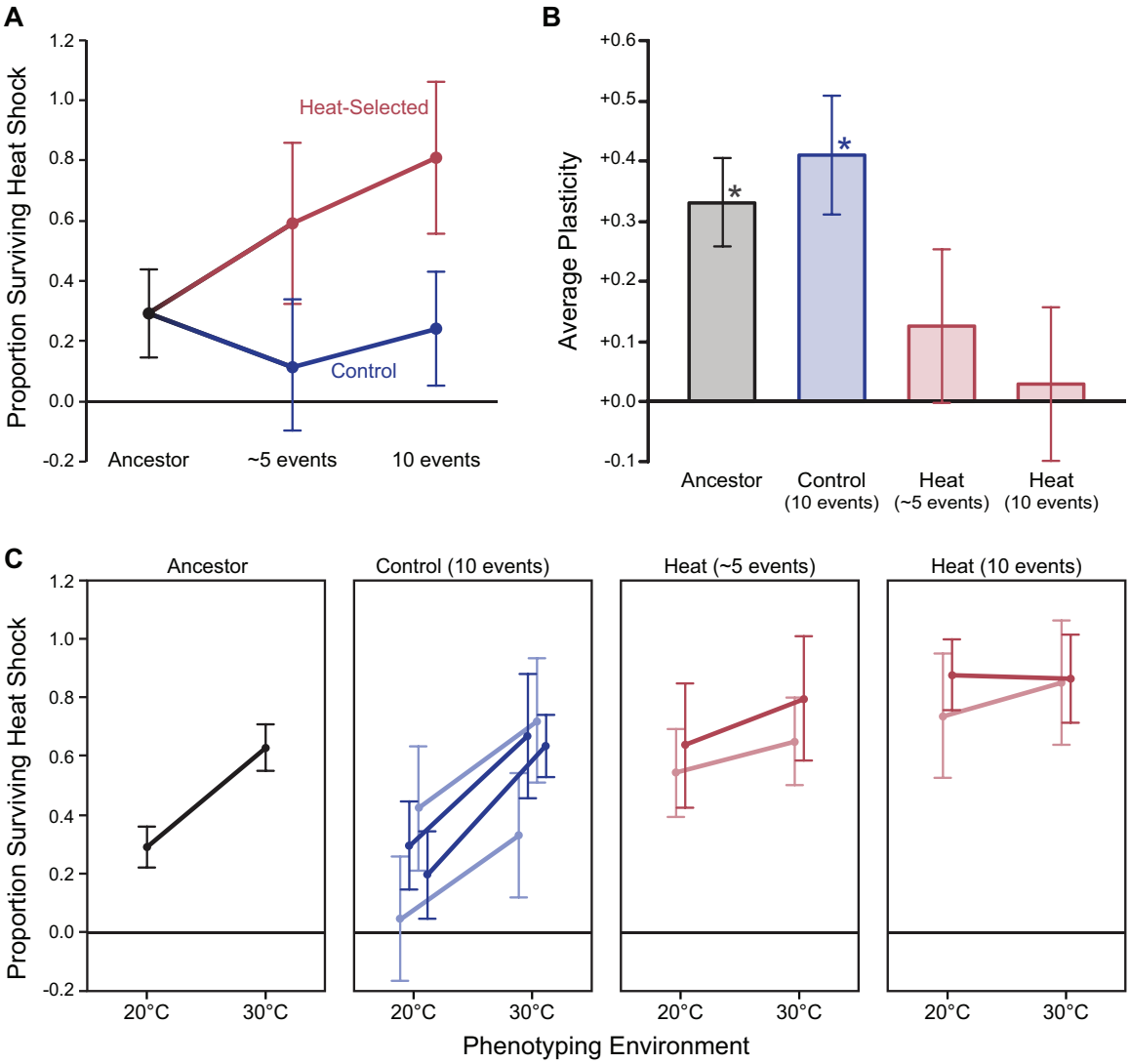


Figure 2.1 Evolved changes in heat shock resistance in selected lines of *C. remanei*. (A) Proportion of heat-shocked worms surviving to adulthood relative to control treated replicates for populations subjected to heat selection (red) and control populations (blue). (B) Plasticity for heat stress resistance, defined as the difference between survival at 30° and survival at 20° for ancestral (gray), control (blue), and heat-evolved (red) populations. Asterisks denote populations with a significant ($P < 0.05$) effect of environment (*i.e.*, plasticity) on survival. (C) Reaction norms for replicate evolved lines in the 20° and 30° environments. Least squared means from the ANOVA with 95% confidence intervals are plotted.

environment interaction ($F_{1, 113} = 2.82$, $P = 0.0959$, Figure 2.1B). Despite some variation in average survival at each cultivation temperature, plasticity in survival was highly consistent across all controls (Figure 2.1C).

In lines selected for heat-shock resistance, there was no significant increase in survival in the novel environment at 30° compared to the ancestor ($F_{1, 4} = 1.84$, $P = 0.2462$), which contrasts sharply with the response observed at 20°. Consequently, plasticity across environments declined dramatically during selection, until the complete loss of environmental sensitivity occurred after 10 generations of selection for heat resistance ($F_{1, 112} = 1.11$, $P = 0.6558$). Loss of plasticity occurred in this case because the phenotype in the 20° environment evolved to match that of the 30° environment. Note that this result is not simply a matter of scale, as these populations were still relatively far from the upper bound of 100% survival. These results support an apparent genetic assimilation of the heat-induced phenotype following selection.

Global transcriptional response to environmental change is unchanged

Given the observed pattern of rapidly evolved plasticity for heat stress resistance across environments, we hypothesized that the phenotypic evolution may be manifested in differences in gene expression profiles across environments in a large proportion of genes. Furthermore, we predicted that the phenotypic assimilation might be matched by a pattern of transcriptional assimilation as well. Specifically, genes that are differentially expressed between the 20° and 30° environments in the ancestral population would be expected to become constitutively expressed in the heat-evolved lines to match the observed phenotypic change in those populations (Figure 2.2). To test this hypothesis, we used RNA-sequencing (henceforth RNA-seq) on pooled samples from the ancestor,

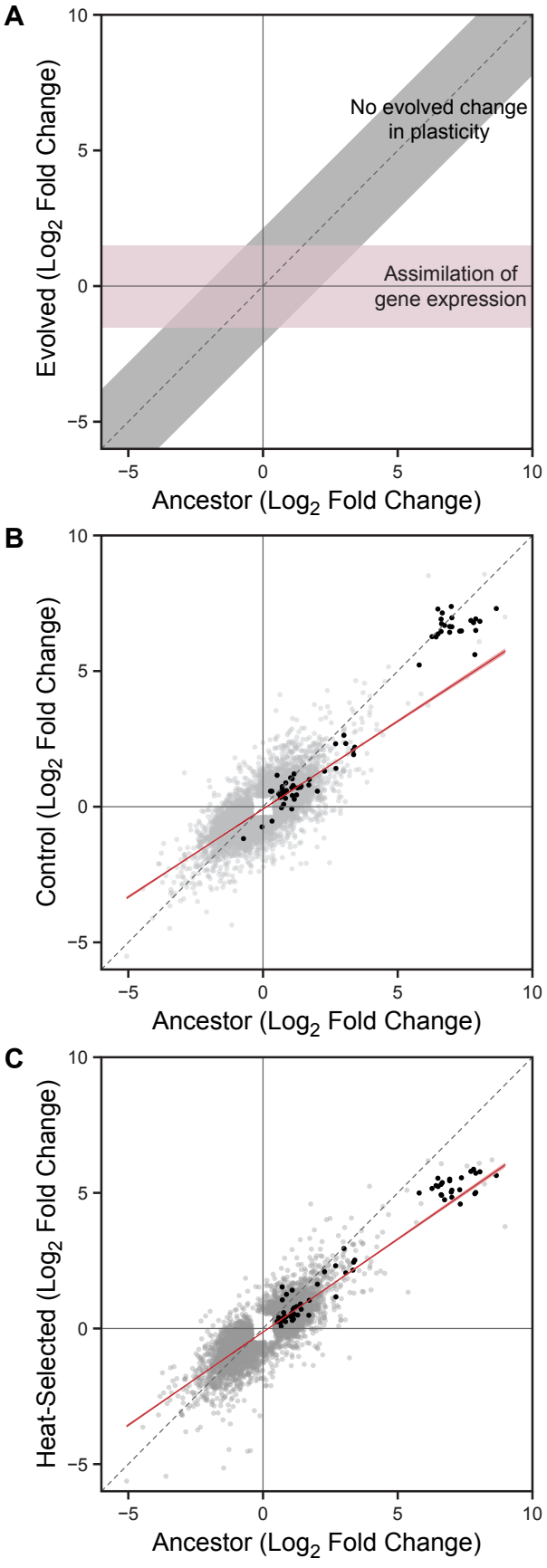


Figure 2.2 Inducible transcriptional response in evolved lines of *C. remanei*. (A) Predicted changes in transcriptional plasticity between the ancestor and heat-evolved populations under a null hypothesis in which genes have equivalent levels of plasticity in both selection lines (*i.e.*, no change in plasticity) or the alternative hypothesis in which there is genetic assimilation of gene expression in the evolved line. (B) Comparison of changes in inducibility of gene expression at 30° in ancestor *vs.* the control evolved populations or (C) ancestor *vs.* heat-selected. Gray points represent differentially expressed genes from either of the compared lines, and black points represent candidate hsp genes. Red lines in (B) and (C) indicate the linear fit from the regression model (±95% CI). Gray dashed line is slope of 1, representing the null hypothesis of equal expression between lines.

control, and heat-evolved populations, which were divided and raised at either 20° or 30° for 20 hr prior to tissue collection.

Of the genes that were expressed above our threshold for detection, we identified 8,377 genes that were differentially expressed across environments in at least one of the three populations (Appendix A, Table S2.1). Not surprisingly, exposure to the 30° environment caused upregulation of genes involved in mediating response to stress (Figure S2.1 and Table S2.2), as well as enrichment of biological processes related to metabolism, growth, and development. Processes relating to ion transport and cellular communication were downregulated at 30°. These processes were similarly enriched in all three selection lines.

To understand how expression plasticity has evolved in the selected populations, we compared the inducibility (measured as the $\log_2$ fold change in expression between environments) of the differentially expressed genes between selection lines and the ancestor (Figure 2.2). When comparing the expression change across environments between different lines, the null expectation is that these should have equal expression differences in both lines for the majority of genes (Figure 2.2A). Alternatively, under a genetic assimilation model, we would expect to observe expression differences in the control and ancestral populations but constitutive expression of these genes in the heat-evolved population.

When comparing the expression change in the ancestral population to the expression difference in either evolved population, there was a slight reduction in the slope term of the linear model, suggesting that many genes are somewhat less responsive to environmental change (Figure 2.2, B and C). However, this effect was apparent in both

the control (b = 0.648; t_{7751} = -59.49; $P < 0.0001$) and heat-evolved populations (b = 0.686; t_{6743} = -60.63; $P < 0.0001$), indicating that this observed pattern may be a signature of laboratory adaptation, rather than genetic assimilation of heat stress resistance. The reduction of slope was slightly more pronounced in the control populations than in those selected for heat resistance (t_{14494} = 4.78, $P < 0.0001$). Furthermore, a significant correlation between the responsiveness of expression in the ancestor and evolved populations remains, implying that general transcriptional assimilation is not responsible for the phenotypic assimilation.

Inducibility of candidate heat shock proteins is unchanged

Genetic assimilation may not be generated by constitutive gene expression at a global level, but rather by changes in specific pathways such as those regulating heat response. To test this hypothesis we analyzed the response of heat shock proteins, which are particularly strong candidates for regulating a heat-specific response because of their key role in mitigating damage due to cellular stress (Lindquist and Craig 1988). In addition, *hsp70* genes have been shown to respond to selection at different temperatures in *Drosophila melanogaster* (Bettencourt *et al.* 2002), and the inducibility of *hsp70* differs among related *Drosophila* species adapted to different thermal environments (Krebs 1999; Calabria *et al.* 2012), making these genes likely targets for adaptation to heat stress. We identified 89 genes in *C. remanei* belonging to four families of heat shock proteins: the HSP70 superfamily, the HSPC (HSP90) family, the DNAJ (HSP40) family, and small heat shock proteins in the HSPB family, many of which are inducible in response to heat stress in the genus *Caenorhabditis* (Heschl and Baillie 1990; Stringham *et al.* 1992; Nikolaidis and Nei 2003). As expected, most of the hsps exhibited a high

degree of plasticity across environments. However, they also retained an equivalent degree of plasticity in both evolved populations (Figure 2.2). In addition, there was no evidence that the basal level of hsp expression at 20° differed among populations. A few genes did show altered expression over evolutionary time (Table S2.3), but there was no clear pattern of constitutive upregulation across stress response pathways. Thus, despite their canonical role in mediating heat shock response, hsps do not appear to play a role here in the apparent genetic assimilation of heat shock resistance in the selected population.

Genetic assimilation of heat resistance is only apparent and is context-dependent

Given the discordance in evidence for assimilation at physiological and transcriptional levels, we sought to understand whether the transcriptional response to temperature might underlie a more complex relationship between the environment and phenotype by exploring the evolved norms of reaction for survivorship over a broader range of heat shock temperatures. As in the single temperature assays, we observed a significant interaction between heat shock temperature, population and rearing environment ($F_{2, 240} = 8.17$, $P = 0.0004$), indicating that evolved differences in plasticity due to rearing environment affect the rate of survival across the range of heat shock temperatures (Figure 2.3). In particular, as above, rearing environment had strong effects on resistance within the ancestor (rearing environment-by-heat shock temperature interaction: $F_{1, 176} = 18.95$, $P < 0.0001$), and control lines (interaction: $F_{1, 176} = 53.17$, $P < 0.0001$). However, rearing the worms at 30° also induced increased heat shock resistance in the heat selected lines when the heat shock occurred at temperatures above the selection temperature (>37°; interaction $F_{1, 35} = 20.75$, $P < 0.0001$). In fact, the heat-

selected populations appear to have evolved greater plasticity at high heat shock temperatures, largely by improving inducible heat shock resistance after being raised at 30°. Thus, while assimilation is evident at the specific temperature utilized under direct selection, plasticity is maintained—and even enhanced—at a broader spectrum of formerly lethal temperatures.

DISCUSSION

Organisms live in a constantly varying world. In response to this environmental variation, numerous lineages have evolved the ability for individuals to predictably modify their phenotypes in response to environmental heterogeneity. The importance of phenotypic plasticity in influencing ecological and evolutionary processes—such as modifying the probability of extinction or influencing the trajectory of evolutionary response—has long been known by biologists (Baldwin 1896a,b; Morgan 1896; Waddington 1942; Schmalhausen 1949; Bradshaw 1965; West-Eberhard 2003). Despite

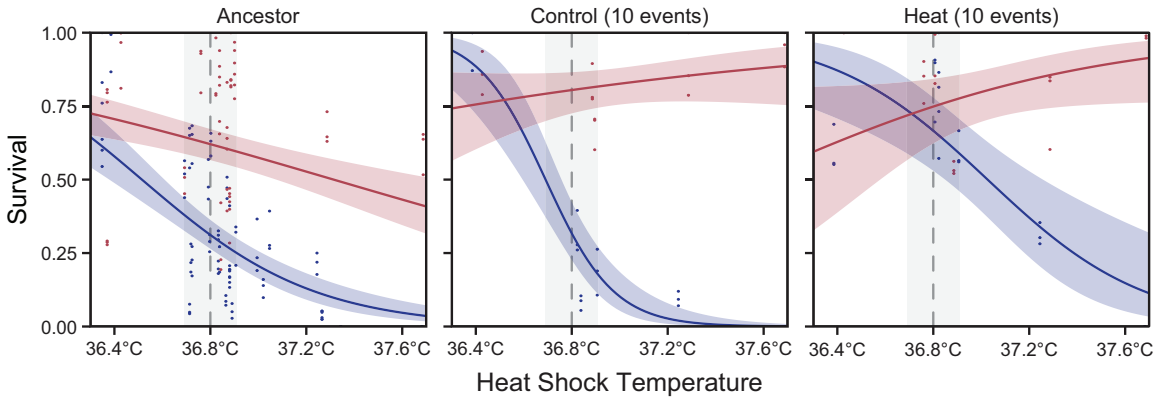


Figure 2.3 Evolved changes in heat shock reaction norm in evolved lines of *C. remanei* occur by shifting the reaction norm. Shown are predicted reaction norms across a range of heat shock temperatures for a representative of each selection line raised at either 20° (blue) or 30° (red). Points represent the proportion of worms surviving in replicate trials, and solid lines indicate the predicted probability of survival from a logistic regression with 95% confidence intervals. The gray box indicates the temperature range included in the 36.8° heat shock assays, where plasticity was initially measured.

this recognition, studies of the origin and evolution of phenotypic plasticity, in particular how quickly it can evolve and the genetic basis of plasticity, have been unsatisfactorily inconclusive. One reason is that labile phenotypes that vary in response to environmental change could naively be seen as lacking any genetic basis and therefore unable to evolve. However, evolutionary biologists now correctly understand that the ability to coherently respond to environmental variation is itself a trait that can evolve and that genetic variation for this trait can be sorted within and among populations (Via 1984). A more important reason for the lack of progress is the difficulty of using comparative studies of phenotypic plasticity in evolved populations to directly address questions of evolutionary rate and genetic mechanism. In this study, we have tackled these holes in our understanding of the evolution of phenotypic plasticity by using a powerful experimental evolution approach.

One specific form of plasticity of broad interest to molecular as well as evolutionary biologists is the increased hardiness that can often be induced by low doses of a toxin or brief exposure to a stressful environment (Calabrese and Baldwin 2003). The induced response, or hormesis, is presumably caused by the upregulation of stress-resistance factors in the initial exposure that then serve a protective function in subsequent, and potentially harsher, exposures (Gems and Partridge 2008). Within *C. elegans*, brief exposure to high temperatures has been shown to yield increased resistance to high temperatures (Lithgow *et al.* 1995), as well as increases in longevity (Gems and Partridge 2008; Le Bourg 2009). Hormesis is not usually discussed in the context of phenotypic plasticity—although it is precisely that—and in this case serves as example of adaptive phenotypic plasticity. There is evidence for genotype-by-environment

interaction for this response in *C. elegans* (Rodriguez *et al.* 2012), which is a necessary precursor to the evolution of a plastic response. Here we find an essentially similar (if not stronger) hormetic response in populations of the closely related and genetically diverse nematode *C. remanei* that have been recently collected from nature. Thus, this pattern of plasticity appears to be highly conserved across this group of nematodes.

Despite this conservation, selection for resistance to nearly lethal high temperatures rapidly produced a complete loss of plasticity for resistance to heat stress in independently evolved replicate lines. Dramatically increased fitness and a complete loss of plasticity were observed after only 10 generations of selection (Figure 2.1). This pattern of genetic assimilation was very similar to that predicted by Waddington (1953; 1956) over 60 years ago, but it occurred much more quickly than what may have been otherwise expected. The tempo of this plasticity change could only be assayed in an experimental evolution framework and led to an important subsequent question: how could such rapid evolution occur? Changes in the frequencies of alleles that affect coding sequences of genes or alleles of regulatory elements affecting the levels of expression of different genes could be responsible. We addressed the latter hypothesis and found that the global patterns of gene expression have not been altered in a way that matches the genetic assimilation of the phenotype (Figure 2.2).

In contrast to the expectation of global genetic assimilation in transcription, a more focused hypothesis is that particular candidate pathways would experience genetic assimilation. For example, given what is known about the genetics of heat shock resistance (Lindquist and Craig 1988; Morimoto 1998; Volovik *et al.* 2012), one simple means of achieving this pattern of assimilation would be the constitutive upregulation of

heat shock protein genes at permissive temperatures, thereby allowing these proteins to provide ready-made protection without the need for them to be induced before rapid exposure to lethal temperatures. Surprisingly, we also did not observe the predicted changes in gene expression levels in these key proteins. Instead, most of the hsp genes that showed differential expression in one or more selection lines showed a high degree of correlation in expression across treatments, and most of the decrease in the environmental induction of expression seemed to result from laboratory adaptation rather than specific assimilation in the heat-selected line. Thus, neither global nor hsp-focused gene expression patterns evolve in a pattern consistent with the genetic assimilation of the phenotype.

There are several explanations for the divergent observations of genetic assimilation at the level of the phenotype but concurrent lack thereof at the level of gene expression. First, the phenotypic response may be a result of changes in a few key stress response genes. However, the strongest candidates for regulating the heat shock response, the hsps, respond similarly in all lines. A second possibility is that the basal level of gene expression among lines is more important in the heat-selected line, and additional induction of expression under heat stress does not further improve survival. A few genes may be in this category and require further study (Table S2.3). Alternatively, constitutive upregulation may be important, but the target of regulation (*e.g.*, protein degradation or post-translational modification) might not be revealed from an analysis of transcript levels.

In contrast to these strictly genetic explanations, another possibility is that our initial finding of phenotypic assimilation is only apparent (Figure 2.3). In addition to the

shift of the reaction norm when raised at 20°, there appears to be a correlated shift in the 30° reaction norm as well, so that plasticity is actually increased at temperatures beyond those initially assayed. This indicates that strong genetic correlations for heat resistance exist between the stressful and permissive environments, as predicted by Via and Lande (1985), and that such correlations may strongly influence the phenotype across multiple environments. Thus, genetic assimilation of the heat resistance response was apparent only and limited to a narrow window of possible environmental perturbations.

It has been long recognized that the specifics of phenotypically plastic responses are dependent on the exact environments in which they are measured (Bradshaw 1965). For example, Waddington saw genetic assimilation as a specific form of canalization, or reduction in phenotypic variation, and hypothesized that canalization could be broken outside the range of environmental variation under which assimilation occurred (Waddington 1942). Our results clearly support this point of view. Even in the context of a significantly reduced set of environmental stimuli, as examined here, it is apparent that the phenotypic and environmental space is complex and multidimensional. Although the evolution of genetic assimilation might be seen as potentially limiting subsequent evolutionary change, traits that presumably exhibit canalization in one range of environmental variation are likely to be periodically exposed to ranges of environmental conditions under which canalization is broken. Therefore, rather than limiting the evolutionary response to selection via the induction of genetic canalization, changing environments instead likely provide a continually shifting substrate for the evolution of plasticity. The dynamic balance between canalization and plasticity is therefore one of the major drivers—and outcomes—of evolution in a complex environmental milieu.

BRIDGE

In Chapter II, we examined a case in which genetic assimilation appeared to have evolved rapidly in selected lines of *C. remanei*. However, this observation was shown to be an illusion resulting from a myopic perspective of the environmental context in which the organisms exist. Therefore, consideration of the broader environmental context is vital in any study of phenotypic plasticity. In Chapter III, we investigate this problem further by asking whether evolution under different environmental conditions affects responses to selection by altering genetic correlations among traits and across environments.

CHAPTER III

VARIABLE PLEIOTROPY AND ENVIRONMENTALLY INDUCED CHANGES IN CORRELATED RESPONSES TO SELECTION

This work is in preparation for submission to the journal *Evolution* in 2014. The experimental selection lines were created and phenotyped by R. M. Reynolds and myself. I performed all statistical analyses. W. A. Cresko and P. C. Phillips were the principal investigators for this work.

INTRODUCTION

All phenotypic evolution is dependent on environmental context for at least two reasons. First, the fundamental idea of evolution by natural selection is that the specific circumstance of the environment causes individuals with particular phenotypes to have higher probabilities of surviving or reproducing in greater numbers than individuals with other phenotypes (Darwin 1859). Second, an individual's phenotype is itself the result of the complex interplay between the genetic information encoded in that individual's DNA and a potentially wide variety of attributes of a given environment that influence the manifestation of the genetic information in the phenotype. The distinction and interplay between these two roles of the environment is exemplified in the case of the snowshoe hare (*Lepus americanus*). A brown snowshoe hare starkly stands out to predators when found on the snow, while a white snowshoe hare is highly visible against the burnt grass of the late summer. Yet whether a given hare is brown or white depends on season-

specific signals that alter pigment-controlling pathways within that individual's hair follicles (Keogh 1967). Mismatches between the phenotype and environment are not always so readily apparent, however, because the environment includes not only external factors, but also the microenvironment of the individual itself. For example, cytoplasmic factors contributed by an individual's maternal parent can interact with paternal zygotically expressed proteins to negatively affect the functioning of the cell and thus the fitness of the organism (Reed et al. 2008).

These truisms of the environment being the filter of genetic change into the phenotype, and a key arbiter of the distribution of genetic variation via the fitness effects on the associated phenotypes, have rightly served as the basis for evolutionary ecology for the last century and a half (Falconer and Mackay 1996; Roff 1997; Lynch and Walsh 1998). However, individuals do not exist as single phenotypes in a well-defined set of environments. Each individual is composed of an effectively infinite number of phenotypic dimensions that are influenced by a wide array of systematic and stochastic environmental exposures. The unique life trajectory of each individual is the result of the interaction of the specific set of environmental exposures and the combination of alleles represented in their genome. Which elements of the environment generate fitness differences and which have direct influences on phenotype? Which of the thousands of phenotypic attributes can be said to be the targets of natural selection and which simply covary as a result of that selection or related environmental perturbations? These are the essential questions of modern evolutionary quantitative genetics.

One consequence of the complexity of interactions within an organism is that changes in one feature or in a subset of traits should have ramifications that spread

throughout the organism. This can be due to the direct functional interactions of the traits involved (Arnold 1983), or, more subtly, because the traits are coupled together because of a shared genetic basis (Lande 1979). This genetic coupling can be generated by either pleiotropy, when a single allele influences more than one trait, or by linkage, when alleles at two or more loci tend to be inherited with one another, usually via physical linkage on a chromosome (Falconer and Mackay 1996). More than a half-century of work in molecular biology has revealed that most organismal traits are underlain by genetic networks of dozens to many hundreds of genes. The existence of such networks supports the view of universal pleiotropy first espoused by Sewall Wright during the formation of modern evolutionary genetics (Wright 1968), suggesting that genetic coupling among traits should be the rule rather than the exception.

Evolutionary quantitative genetics provides a strong conceptual framework for untangling the patterns of natural selection and genetic inheritance for suites of interacting complex traits. For the most part, these approaches have relied on statistical associations—among traits, between traits and fitness, and among relatives—for making inferences. Such associations are necessarily averages over genes, genetic networks, traits, and individuals within a population. The existence of complex genetic networks begs the question of whether variation in the nature and structure of pleiotropy should have an important influence on evolutionary outcomes or whether a perspective of pleiotropy developed nearly 100 years ago remains sufficient for understanding the evolution of complex traits.

When multiple traits are affected by natural or artificial selection, the multivariate formulation of the breeder's equation (Lande 1979; Lande and Arnold 1983) describes the responses to selection among the set of phenotypes as:

$$\Delta\bar{\mathbf{z}} = \mathbf{G}\boldsymbol{\beta}, \quad (1)$$

where $\Delta\bar{\mathbf{z}}$ is the vector of average phenotypic responses to selection, **G** is the additive genetic variance-covariance matrix, and $\boldsymbol{\beta}$ is the vector of selection gradients for each trait. Equation (1) can be used to describe the evolution of multiple traits within a single environment (e.g., brain size and body size; Lande 1979), as well as related traits across multiple environments (e.g., body size at high and low temperatures; Via and Lande 1985; Via 1987). In either case, genetic correlations can lead to correlated responses to selection (Lande 1979; Via and Lande 1985). Evolutionary trajectories will be biased by the genetic covariation to adapt along the "genetic lines of least resistance" (Arnold 1992; Schluter 1996; McGuigan and Blows 2007). If these genetic lines correspond with selective gradients on a fitness landscape then evolution will occur unimpeded. However, in extreme cases when genetic covariances and fitness landscapes are in conflict, populations can be slowed or prevented from achieving certain phenotypic combinations, even if such combinations are strongly favored by selection (Steppan et al. 2002).

Despite the fundamental role that this framework has played in our understanding of the evolution of quantitative characters, recent studies have demonstrated that genetic correlations between traits (Grant and Grant 1995; Fischer et al. 2007) and across-environments (Czesak et al. 2006; Stinchcombe et al. 2010) do not necessarily predict the realized evolutionary response. First, because the G-matrix is symmetric, the naïve expectation is that genetic correlations will result in symmetry in the correlated responses

to selection as well (Lande 1979). However, this is rarely the case when selection occurs over multiple generations (e.g., Falconer 1960; Shiotsugu et al. 1997; Cortese et al. 2002). Proposed reasons for the asymmetry include changes in allelic effects due to allele frequency changes (Bohren et al. 1966) and changes in linkage disequilibrium (Villanueva and Kennedy 1992). Furthermore, it is apparent that the genetic architecture of complex traits can be strongly influenced by the environment (Hoffmann and Merilä 1999; Charmantier and Garant 2005; Paaby and Rockman 2014), which also contributes to the unpredictability of the evolutionary response. However, the effect of the environment on correlated responses to selection has very rarely been tested (Baker and Cockrem 1970; Fry 2001). Do we need a better understanding of the genetic architecture underlying quantitative trait variation in order to more fully understand the evolution of complex phenotypes?

The G-matrix itself is a composite of wide variety of possible influences on genetic variation and covariation, averaged over all of the loci in the genome. Using the two-trait case for simplicity, and ignoring possible contributions of between-gamete disequilibria, the G-matrix can be decomposed as

$$\mathbf{G} = 2\mathrm{E}\left[\sum_{i}^{n}\sum_{j}^{n}\begin{pmatrix} (x_{i1}-\overline{x}_{i1})(x_{j1}-\overline{x}_{j1}) & (x_{i1}-\overline{x}_{i1})(x_{j2}-\overline{x}_{j2}) \\ (x_{i2}-\overline{x}_{i2})(x_{j1}-\overline{x}_{j1}) & (x_{i2}-\overline{x}_{i2})(x_{j2}-\overline{x}_{j2}) \end{pmatrix}\right] \quad (2)$$

where x_{i1} is a random variable describing the average effect of a particular allele at locus *i* on trait 1, x_{j2} is the average effect of a particular allele at locus *j* on trait 2, etc., and the expectation (E) is taken over all alleles at all possible pairwise combinations of *n* genes (Lande 1980; Phillips and McGuigan 2006). Terms on the diagonal describe the contributions of single and pairs of linked loci on the additive genetic variation for a

given trait. Terms involving x_{i1} and x_{i2} in the off-diagonal describe the influence of pleiotropy on the additive genetic covariance between traits, whereas terms involving x_{i1} and x_{j2} (and vice versa) describe the effects of linkage on these covariances. The critical point here is that genetic covariances (and therefore predicted correlated responses to selection) are averages over alleles at a given locus and over many loci within the genome. Variability in these allelic effects yield the overall pattern of genetic variation and covariance, but strong heterogeneity in these effects has the potential to generate evolutionary responses beyond those predicted by **G** alone (Barton and Turelli 1987). We were only beginning to glimpse the molecular underpinnings of pleiotropy when this theory was just being formulated. In the light of core understanding of genetic networks from the field of molecular biology, however, it now seems clear that these patterns must be deeply complex for most biological systems (Phillips 2008; Costanzo et al. 2011).

One important example of a suite of complex traits that are regulated by linked genetic networks is the response to environmental stress. For example, in *C. elegans*, many of the proteins that respond to stressors such as heat, oxidative damage, or starvation are known, and their interactions within the stress response network have been characterized in detail. In most instances, they display strong pleiotropies with one another (Fig. 3.1). One particularly well-studied pathway is the insulin/insulin-like growth factor signaling (IIS) pathway. Notably, IIS regulates nuclear localization of the FoxO transcription factor, DAF-16 (Lee et al. 2001; Lin et al. 2001). Genes directly regulated by DAF-16 contribute to resistance to heat stress (Hsu et al. 2003; Morley and Morimoto 2004), oxidative stress (Honda and Honda 1999; 2002; Oh et al. 2006), osmotic stress (Lamitina and Strange 2005), heavy metals (Barsyte et al. 2001), and

pathogens (Evans et al. 2008). In addition, IIS likely interacts with HSF-1, a transcription factor known to regulate a number of heat shock proteins to mediate heat stress response (Wu 1995), and SKN-1, whose regulatory targets are important for resistance to oxidative stress (An and Blackwell 2003). The IIS pathway clearly plays a central role in mediating response to a variety of stresses, mediated through a core set of regulatory hubs, such as DAF-16, which simultaneously affect resistance to a diverse array of cellular stressors. Therefore, the molecular biology of this system would predict that pleiotropy should influence evolution of the different stress responses, leading to correlated responses to selection on any stress phenotype.

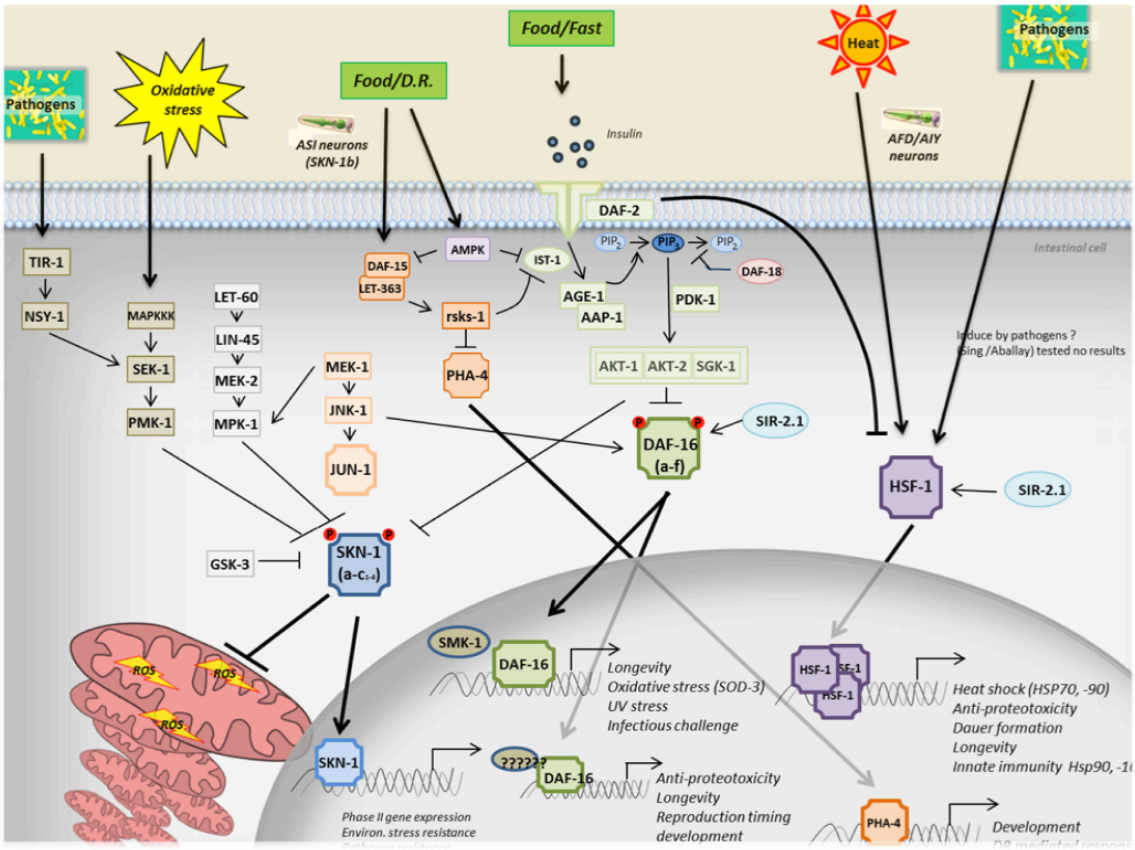


Figure 3.1. Stress response network in *C. elegans*. Many stressors activate IIS, which regulates several key transcription factors, such as DAF-16, SKN-1, and HSF-1. As a group, the target genes of these transcription factors are responsible for resistance to many different types of cellular stress, leading to an expectation of strong pleiotropy within the molecular network. Image courtesy of John Willis.

Traditionally, the G-matrix has been estimated using controlled breeding experiments, often including hundreds of families (Lynch and Walsh 1998; Steppan et al. 2002). However, different combinations of pleiotropic effects can lead to the same estimate of genetic correlation between traits, but will place different constraints on the evolutionary response (Gromko 1995). An alternative approach is to use experimental evolution in the laboratory to study patterns of changes in the covariances among traits and across environmental conditions (Rose et al. 1990). Experimental evolution guided by laboratory selection enables the impact of genetic correlations among traits to be more accurately estimated. Here, we use this approach to investigate variation in patterns of pleiotropy both between traits and across environments. We imposed selection on two traits, heat stress resistance and oxidative stress resistance, for which the shared molecular pathways lead to a prediction of pleiotropy. We measured both direct and correlated responses to selection in three different selective environments. In particular, we asked whether correlated responses to selection were symmetrical and constant across environments as predicted by theory, or if instead the selective environment alters the patterns of pleiotropy within the stress response network.

METHODS

Experimental evolution

The ancestral population used for selection was created as previously described (Sikkink et al. 2014b). In brief, natural isolates collected from Ontario, Canada, were used to create 26 isofemale strains. These strains were crossed in a controlled manner to create a population that was representative of the naturally segregating genetic variation.

The genetically heterogeneous population obtained from the crosses (PX443) was frozen after its creation, prior to use for experimental evolution. All natural isolates and the selection lines described below were raised on Nematode Growth Medium-lite (NGM-lite, U.S. Biological) seeded with *Escherichia coli* strain OP50 (Brenner 1974).

We evolved populations of *C. remanei* in three different chronic environments within which they spent their entire lives (Fig. 3.2). Worms evolved in the chronic control environment were raised at 20°C on plates containing NGM-lite seeded with *E. coli* strain OP50—standard lab conditions for worm husbandry (Brenner 1974). The chronic heat environment differed from the control environment in that the temperature was increased to 30°C. To apply a chronic oxidative stress, 160μM paraquat (methyl viologen) was added to the NGM-lite before the plates were poured. After thawing the ancestral population in standard lab conditions at 20°C, we allowed two generations in those conditions for recovery from the freeze. Worms were then divided among lines in each of the chronic environments (Generation 0), and lines were maintained within that environment for the entirety of the experimental evolution.

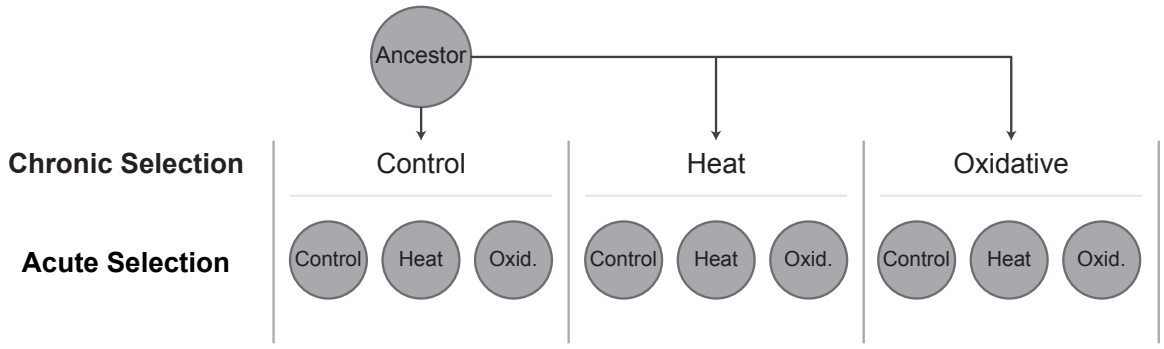


Figure 3.2. Schematic of the experimental evolution design. Lines were raised throughout selection (~30-40 generations total) in one of three chronic selective environments. In each environment, selection lines were generated by selecting individuals at random (Control), or by selecting survivors of an acute heat shock or oxidative shock.

Within each of the chronic environments, evolved lines experienced one of three different acute treatments: a control, acute heat stress, or acute oxidative stress (Fig. 3.2, Table 3.1). An acute stress in the context of this experiment was one that challenged the stress response of the worm within a four-hour period. In contrast to the chronic environmental treatments, acute stressors were a very high-intensity stress applied during a single developmental stage of the lifecycle over a relatively short period of time.

Acute selection occurred either every second generation or when the population produced ≥24,000 eggs, whichever occurred later. At that point, worms were treated with a bleach solution (Stiernagle 2006), and allowed to develop into L1 larvae in buffer. Without food, *C. remanei* enter diapause at the L1 stage (Baugh 2013), resulting in a population that is fully stage-synchronized. During the development period, the conditions of the buffer matched the chronic selection conditions. That is, lines that were typically raised at 30°C, experienced the same thermal environment during stage synchronization. Similarly, 160μM paraquat was added to the buffer for lines selected

Table 3.1. Experimental evolution lines and selective conditions.

Chronic Selection		Acute Selection[a]		No. of Lines
Control	20°C, NGM	Control	—	4
Control	20°C, NGM	Heat	36.8-37.1°C	2
Control	20°C, NGM	Oxid.	1-1.5 mM H_2O_2	2
Heat	30°C, NGM	Control	—	2
Heat	30°C, NGM	Heat	36.8-37.8°C	2
Heat	30°C, NGM	Oxid.	1-2.25 mM H_2O_2	2
Oxid.	20°C, NGM + 160μM paraquat	Control	—	2
Oxid.	20°C, NGM + 160μM paraquat	Heat	36.4-36.8°C	2
Oxid.	20°C, NGM + 160μM paraquat	Oxid.	0.75-2 mM H_2O_2	2

[a]Acute selection increased during experimental evolution in order to maintain a strength of selection (*s*) of ~0.7-0.8 throughout.

under chronic oxidative stress (but note that this treatment was applied to only one replicate of each chronic oxidative line during selection; the other replicate was treated the same as those from the control environment). Synchronized L1s were then selected via one of the acute selection regimes described below.

Acute heat-stress populations. After age-synchronization, approximately 10,000 individuals were haphazardly selected to undergo acute heat selection. L1 larvae were placed in a shaking incubator (70 rpm) in a sealed microcentrifuge tube for four hours. Initially, heat shock occurred at an average temperature of 36.8°C. This intensity of heat shock induces ~70% mortality in the ancestor (s = 0.7). The heat shock temperature for each line was increased incrementally to maintain a similar strength of selection in all environments throughout the experiment. Heat-selected lines from the control environment were the same as those analyzed in Sikkink *et al.* (2014b).

Acute oxidative-stress populations. To select for resistance to oxidative stress, approximately 10,000 worms were haphazardly selected to undergo acute oxidative selection. L1 larvae were placed in a sealed microcentrifuge tube containing a solution of 1mM hydrogen peroxide (H_2O_2) and rotated at 70 rpm for four hours. Because hydrogen peroxide decomposition is temperature-dependent, oxidative selection occurred at 20°C, regardless of the chronic environment for the line. In the ancestral population, 1mM H_2O_2 induces ~80% larval death (s = 0.8). The concentration of H_2O_2 was increased as necessary to maintain a similar strength of selection for all environments.

Control populations. In the acute control populations, populations were haphazardly culled to 1000 L1 larvae during each selective generation. To maintain consistency with the other selective regimes, the selected larvae were rotated in sealed

microcentrifuge tubes for four hours. Control lines from the chronic heat environment were kept at 30°C during this period, while those from the chronic control and chronic oxidative environments were maintained at 20°C. Paraquat was not added to the buffer in the chronic oxidative lines during acute selection. Control lines from the chronic control environment are the same as those analyzed in Sikkink et al. (2014b).

For each selection line, we propagated two independently evolved replicates (Table 3.1), each derived from independently thawed ancestral stocks. One exception was the lab-adapted lines from the chronic control environment. Four replicate populations (two from each ancestral thaw) were propagated under these conditions, because we expected selection to have the weakest effect. Selection was continued until each replicate line had experienced 10 total selective events in the acute stress environment.

We froze each population ($N \geq 100{,}000$ individuals) after approximately every second generation of acute stress selection. This was done to ensure that worms did not lose the ability to survive freeze and thaw, and also to provide a record of evolutionary change over time in each of the populations. Approximately 5000 individuals from each population were thawed to continue the evolution experiment after a minimum of 24 hours at -80°C. The remaining worms remained frozen for future analyses. In one replicate set of evolved lines, freezing occurred a total of 3 times during selection, while this occurred 5 times in the second set of populations.

Stress response phenotypes and measures of phenotypic plasticity

To test resistance to acute stress, frozen stocks of worms that had undergone 10 generations of acute selection were thawed in the chronic maintenance environment they had experienced during the course of their evolution. We allowed populations to recover

in their maintenance environment for two generations prior to phenotyping to minimize effects attributable to freezing. In the third generation, populations from each of the maintenance conditions were divided into three different environmental treatments, matching the three chronic environments used during selection: the standard lab environment (20°C), chronic heat stress (30°C), or chronic oxidative stress (160μM paraquat). Note that for one third of the individuals from a given selection regime, these environmental treatments would be identical to the maintenance environment they had experienced during the previous 30-40 generations, while the remaining two-thirds would be experiencing a novel growth environment. When a population had produced eggs, worms were stage-synchronized as described previously, in conditions matching the environment during the third generation.

Worms in L1 diapause suspended in liquid buffer were then exposed to either an acute heat stress or an acute oxidative stress. These stresses were administered in a manner similar to the acute selection described above. The acute heat stress occurred at an average temperature of 36.8°C (recorded in 5-minute intervals using two Thermochron iButton devices (Maxim Integrated)) in a shaking incubator for four hours in a sealed microcentrifuge tube. As a control, a subset of the population was kept under similar conditions in the respective environmental treatment for the population. Acute oxidative stress was assayed in a microcentrifuge tube in liquid buffer containing 1mM H_2O_2 for four hours on a rotator kept at 20°C. A subset of the population was maintained in liquid buffer under similar conditions without H_2O_2 as a control for the oxidative stress assays. After acute heat or oxidative shock, worms were transferred into a Petri dish containing NGM-lite seeded with *E. coli* strain OP50 and maintained at their respective chronic

environmental treatment during the remainder of development. Survival was estimated 2-4 days later, when most worms were L4 larvae and had yet to lay eggs. Acute stress resistance was quantified as the proportion of the phenotyped population that survived the acute stress and matured to adulthood, relative to the average survival of the control samples from the same treatment.

Statistical analysis

Reflecting the nature of the selection imposed, resistance to acute stress is best interpreted as the proportion of individuals surviving following the acute shock challenge. The total number of individuals in each trial was assumed to be the average count from the three control plates from the same line that were concurrently subjected to a mock treatment. In any case in which the number of surviving worms from the shock treatment was greater than this total, the number of survivors was assumed to be equal to the total (100% survival).

We tested for evolved differences in acute heat or oxidative resistance using a generalized linear mixed model (GLMM) with a logit link and binomial error distribution, using a maximum likelihood estimation based on the Laplace approximation implemented in the *lme4* package (Bates et al. 2014) in R (R Development Core Team 2013). The evolutionary replicate was included as a random effect in the model. We also included observation level random effects to correct for overdispersion. The acute selection regime was modeled as a fixed effect, and we performed contrasts between each evolved line and the ancestral population. If this contrast for a selection line was significant, then we classified that population as having a significant response to selection.

To test for correlated responses to selection between traits, separate analyses were performed for each chronic environment and each acute stress resistance phenotype. We define a direct response to selection as one that occurs on the same phenotype as was under selection, while a correlated response occurs in a phenotype that was not under selection in that evolved line. The ancestral population was included in all models. Only data collected in the 20°C phenotyping environment were included to enable comparison between chronic environments while accounting for phenotypic plasticity.

In addition, we tested for across-environment responses by analyzing the set of acute heat or acute oxidative selection lines from all the chronic selection regimes. In this second set of models, responses in each phenotyping environment were tested in separate models, with the ancestor included each time. In this case, we define the direct response to selection as the response to selection when the phenotyping environment matches the chronic environment experienced during evolution. A correlated response to selection occurs in either of the two other phenotyping environments.

RESULTS

Direct response to selection for stress resistance phenotypes

To minimize the confounding effects of phenotypic plasticity, we first measured acute heat and oxidative stress resistance in the most permissive conditions—the 20°C control environment—regardless of the chronic selective environment previously experienced by the evolved lines. Heat stress resistance increased significantly in all lines that had experienced acute heat selection, regardless of the chronic selective environment in which selection occurred (Fig. 3.3, Table 3.2). We also observed slight, but significant,

increases in heat resistance in the lines selected in the 30°C chronic heat treatment, even in the absence of acute heat selection (Fig. 3.3D). Therefore, the mild, chronic heat stress imposed by the 30°C selective environment leads to adaptation to a more severe heat shock, even when the more stressful environment had never been experienced by that population (see also Sikkink *et al.* 2014b).

Similarly, acute oxidative selection increased resistance to hydrogen peroxide stress, a related but distinct oxidative stress (Fig. 3.3, Table 3.2). This direct response to selection occurred in under each of the three chronic selection environments. Unlike heat stress, however, exposure to chronic mild oxidative stress throughout selection did not increase oxidative stress resistance in the control lines.

Correlated responses among phenotypic traits are contingent on chronic selective environment

In worms, heat and oxidative stress are expected to share aspects of their respective stress response pathways (Fig. 3.1). Furthermore, hyperthermia has been reported to increase reactive oxygen species in cells (Flanagan et al. 1998), potentially requiring heat-stressed populations to adapt simultaneously to heat and oxidative challenges even in the absence of pleiotropy within the stress response network. We therefore hypothesized that such pleiotropic or physiological links between different stress types would lead to significant correlated responses in traits that were not under direct selection in our evolved lines of *C. remanei*. Surprisingly, heat and oxidative stress resistance were not generally correlated in our selected lines. In lines evolved under permissive conditions (the "chronic control" environment), we saw no evidence for correlated responses to selection in either of the selected populations (Fig. 3.3, Table 3.2).

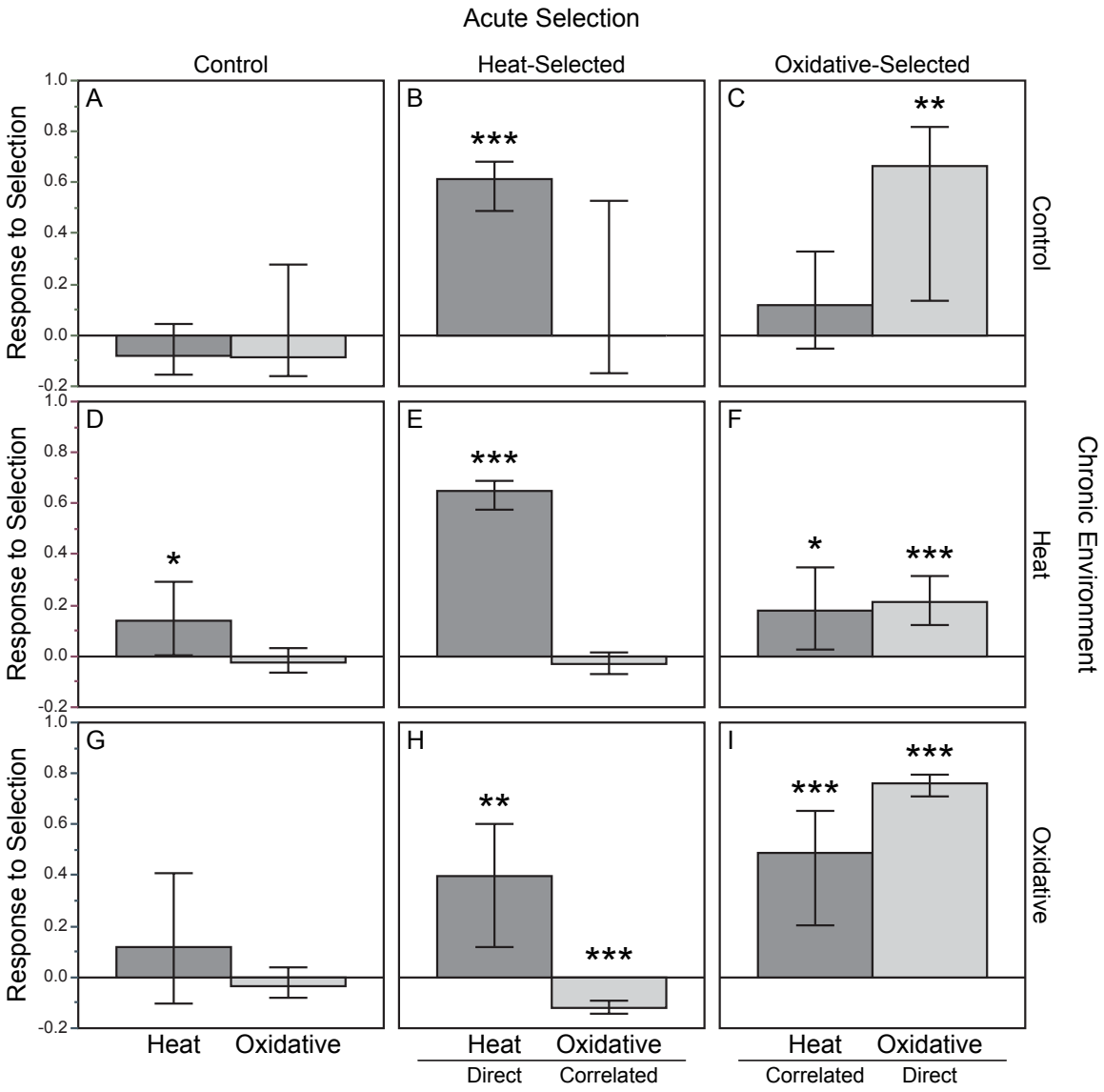


Figure 3.3. Direct and correlated responses to selection between traits in experimentally evolved lines. Response to selection is defined as the mean difference in survival between the selected line and the ancestral population. Responses in both heat shock resistance (dark grey) and oxidative shock resistance (light grey) are shown for populations evolved under chronic control (A-C), heat (D-F), or oxidative (G-I) environmental conditions. Data are conditional means from the GLMM for all independently evolved replicates for each treatment combination, measured at 20°C in all lines (±95% CI). Significant deviation from the ancestral population is indicated by * $P<0.05$, ** $P<0.01$, *** $P<0.001$

Table 3.2. GLMM results indicating effect of selection on heat and oxidative resistance phenotypes in each chronic environment.

		Heat resistance			Oxidative resistance		
Chronic Selection	Acute Selection	β (SE)	*z* value	*P*-value	β (SE)	*z* value	*P*-value
Control	Intercept[a]	-1.09 (0.22)	-4.88	<0.001*	-1.61 (1.01)	-1.60	0.111
	Control	-0.47 (0.35)	-1.34	0.181	-0.85 (1.14)	-0.74	0.457
	Heat	2.96 (0.41)	7.25	<0.001*	-0.02 (1.26)	-0.01	0.990
	Oxid.	0.56 (0.44)	1.26	0.209	3.24 (1.26)	2.58	0.010*
Heat	Intercept[a]	-1.08 (0.19)	-5.82	<0.001*	-1.60 (0.10)	-16.05	<0.001*
	Control	0.65 (0.33)	1.96	0.050	-0.17 (0.21)	-0.83	0.405
	Heat	3.41 (0.34)	9.92	<0.001*	-0.24 (0.18)	-1.32	0.187
	Oxid.	0.82 (0.37)	2.23	0.026*	1.12 (0.22)	5.19	<0.001*
Oxid.	Intercept[a]	-1.09 (0.40)	-2.74	0.006*	-1.61 (0.14)	-11.45	<0.001*
	Control	0.55 (0.62)	0.89	0.374	-0.25 (0.26)	-0.98	0.325
	Heat	1.71 (0.58)	2.94	0.003*	-1.45 (0.29)	-5.03	<0.001*
	Oxid.	2.15 (0.62)	3.46	<0.001*	4.22 (0.33)	12.91	<0.001*

[a]Model intercept indicates the mean phenotype in the ancestral population.
*Response to selection is significant at $P<0.05$.

A similar lack of correlated responses was observed when selection occurred in the chronic heat environment. As noted above, the significant increase in heat resistance observed in the oxidative-selected line in this environment is more parsimoniously attributed to the direct effect of adaptation to the chronic heat selective environment, rather than a correlated response to acute oxidative selection, as the response is essentially identical to that observed in the control treatment.

A very different pattern was observed when selection occurred in the chronic oxidative selection environment. In the acute heat-selected line evolved under these conditions, there was a significant negative correlated response in oxidative stress resistance (Fig. 3.3H). In these lines, resistance to acute oxidative stress actually decreased in comparison to the ancestor despite long-term maintenance in an oxidative environment. In the acute oxidative-selected line from the same environment, the

correlation between the two stress resistance phenotypes flipped sign, leading to increases in both heat and oxidative stress resistance under acute oxidative selection (Fig. 3.3I). This change in direction of the correlated response was observed in both sets of independently evolved lines, suggesting that the pattern is unlikely to be explained by random drift (Appendix B, Fig. S3.1). Overall, then, the realized genetic covariance between these phenotypes is highly contingent on the environment in which selection occurs, and can be rapidly altered by selection for a given trait. Furthermore, the asymmetry in the response indicates that the correlated response is not attributable solely to linkage disequilibrium between heat-adaptive and oxidative-adaptive loci, but rather requires variation in pleiotropy in the underlying genetic network.

Across-environment correlations are contingent on selective environment

In our ancestral population, exposure to mild heat stress (30°C) prior to acute stress induced a plastic response that protected against both heat and oxidative stress, which made preconditioned individuals up to twice as likely to survive subsequent acute stress (Fig. 3.4; Sikkink et al. 2014b). Raising worms in the oxidative environment did not improve resistance to either acute stress. In fact, prior exposure to paraquat decreased resistance to heat stress in the ancestral population.

Although selection occurred entirely within a single chronic stress environment, genetic correlations across environments could lead to evolved changes in phenotypic plasticity for a single trait. A direct response to selection occurred if the phenotyping environment matched the chronic selective environment for that particular line. If genetic correlations exist, then they should lead to correlated increases or decreases in survival in the other (novel) phenotyping environments as well.

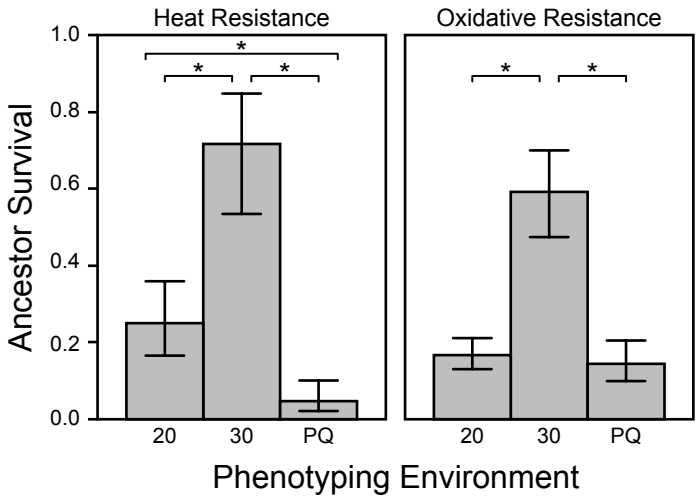


Figure 3.4. Phenotypic plasticity for stress resistance in the ancestral population. Mean survival (±95% CI) is shown for individuals raised in each of the three phenotyping environments. Pairwise comparisons across environments which show significant plastic effects on survival are indicated by * (Tukey HSD; $P<0.05$).

Across-environment correlations were observed more frequently than between-trait correlations in our selected lines (Fig. 3.5). In acute heat-selected lines, we always observed a significant direct response to selection (Table 3.3). In addition, a correlated increase in heat resistance was detected in lines selected under each of the three environmental conditions. However, as above, the precise pattern of the correlated response depended on the chronic environment in which selection occurred. For example, when populations were selected to withstand heat stress in the control environment, resistance to the acute stress showed a significant correlated response in the paraquat environment, but not in the 30°C environment (Fig. 3.5A). In contrast, if selection occurred in the chronic heat selective environment, correlated improvements in heat resistance are apparent in the 20°C environment, but are absent from the paraquat phenotyping environment. If selection occurred in the chronic oxidative selective environment, there was a correlated response at 20°C, but no corresponding change at 30°C (Fig. 3.5A).

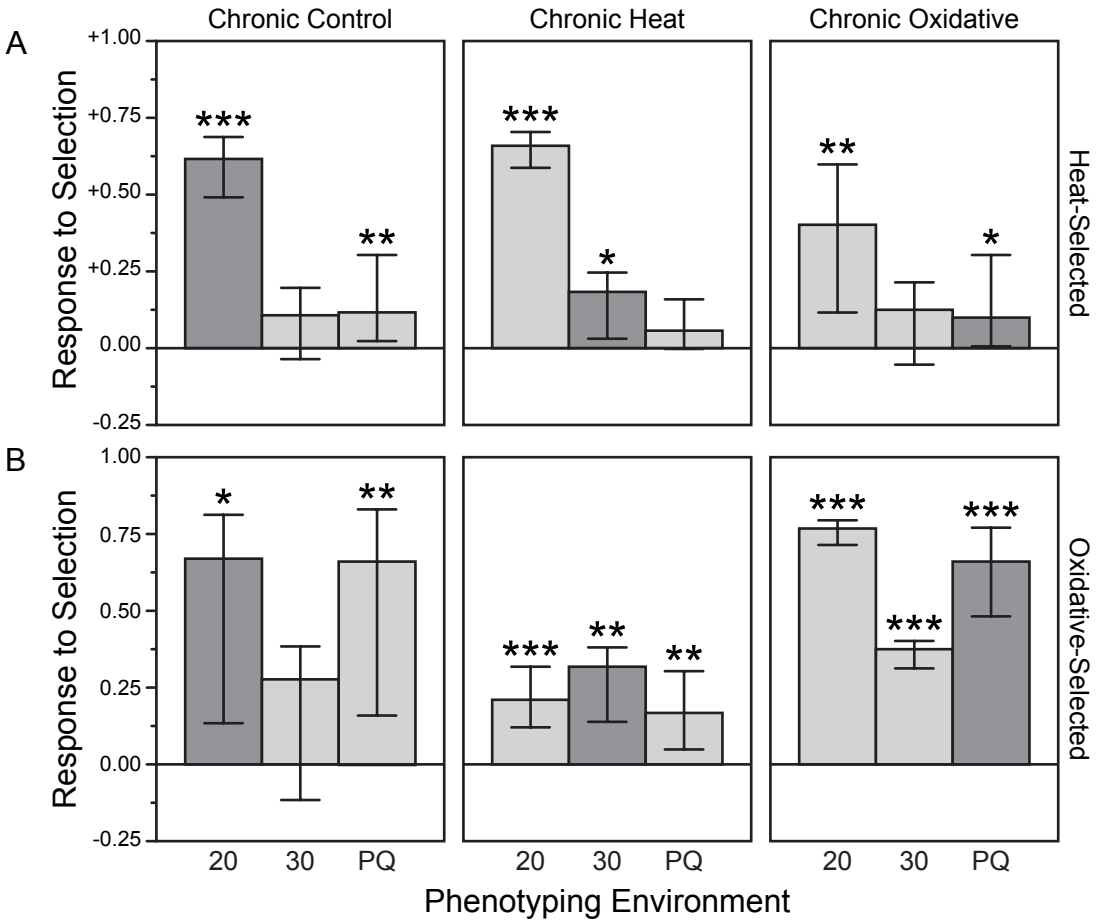


Figure 3.5. Direct and across-environment correlated responses to selection for acute stress-selected lines. Differences in survival from the ancestral population for (A) the acute heat-selected lines or (B) oxidative-selected lines are plotted (±95% CI). Dark grey bars indicate the effects of direct selection, i.e. the phenotyping environment matches the chronic selective environment. Light grey bars indicate across-environment correlated responses for the selected trait. Significant deviation from the ancestral population is indicated by * $P<0.05$, ** $P<0.01$, and *** $P<0.001$

The pattern of across-environment correlations also varied depending on the trait under selection. In the oxidative-selected lines, we observed significant direct responses to selection in every selective environment (Table 3.3). Unlike the heat-selected lines, however, there were also significant correlated responses in nearly every phenotyping condition, regardless of the selection environment. The only correlated response which was not statistically significant occurred at 30°C when selection happened in the chronic

Table 3.3. GLMM results for across-environment correlated responses to selection within the acute stress-selected lines.

		Heat resistance (Heat-Selected Lines)			Oxidative resistance (Oxidative Selected Lines)		
Chronic Selection	Phenotyping Environment	β (SE)	*z* value	*P*-value	β (SE)	*z* value	*P*-value
Control	20°C	2.96 (0.41)	7.25	<0.001*	3.24 (1.26)	2.58	0.010*
	30°C	0.59 (0.38)	1.56	0.118	1.52 (1.01)	1.50	0.133
	PQ	1.39 (0.51)	2.72	0.006*	3.18 (1.14)	2.79	0.005*
Heat	20°C	3.41 (0.34)	9.92	<0.001*	1.12 (0.22)	5.19	<0.001*
	30°C	1.35 (0.60)	2.26	0.024*	1.99 (0.70)	2.85	0.004*
	PQ	0.77 (0.40)	1.91	0.056	0.97 (0.30)	3.20	0.001*
Oxidative	20°C	1.71 (0.58)	2.94	0.003*	4.22 (0.33)	12.91	<0.001*
	30°C	0.72 (0.48)	1.47	0.140	2.69 (0.49)	5.48	<0.001*
	PQ	1.21 (0.59)	2.06	0.039*	3.23 (0.46)	7.02	<0.001*

*Response to selection is significant at $P<0.05$.

control environment (Fig. 3.5B). Even in this case, the mean increase in oxidative stress resistance was generally positive and large, although the variability in the response prevented it from reaching statistical significance. Overall, then, the patterns of covariation across environments for oxidative stress resistance differ markedly from those observed for heat stress resistance. Thus, even for single traits compared across environments, we see evidence for changes in genetic architecture that are dependent on the specifics of the prior selective history.

DISCUSSION

Organisms live in a world that is constantly changing, and they must be able to cope with fluctuations in the external environment in order to persist. Phenotypic plasticity can provide immediate, short-term acclimation to shifting conditions, while natural selection enables adaptation to more persistent changes in the environment.

Understanding the functional and genetic integration of complex organisms in the face of this environmental variation has been one of the central themes of evolutionary biology for the last century (Berg 1960; Cheverud 1984; Klingenberg et al. 2001). The perspective that has emerged from evolutionary quantitative genetics over that time period has focused on patterns of multivariate selection (e.g., correlational selection, Lande and Arnold 1983; Phillips and Arnold 1989) and genetic covariation among traits (Lande 1979; 1980; 1984) as central drivers of this integration, and these must almost certainly be the central agents that structure these systems. However, is the current formulation of this theory sufficient to capture the potential complications that may arise from the heterogeneous structure of complex genetic networks and the shifting patterns of selection imposed variable environments? Consistent with theory, we find that the multivariate response to selection depends strongly on the environmental context in which that selection occurs. However, the nature of the correlated response to selection can itself vary with the environment in non-canonical ways, both in responses within and between environments (i.e., the evolution of phenotypic plasticity). In particular, even when strong pleiotropy is predicted on the basis of the molecular structure of the phenotypic response network (Fig. 3.1), we tend to not observe correlated responses to selection. When we do observe correlated responses to selection within specific environments, they can be asymmetrical and of opposite sign (Fig. 3.3 and Fig. 3.5). We walk through each of these issues in turn.

For our two-trait system, the standard result for multivariate selection displayed in Equation (1) can be broken into parts as:

$$\begin{aligned} \Delta z_H &= G_{HH}\beta_H + G_{HO}\beta_O \\ \Delta z_O &= G_{OO}\beta_O + G_{HO}\beta_H \end{aligned} \tag{3}$$

where *H* and *O* subscripts denote the responses to heat and oxidative shock, respectively. We seek to understand two fundamental aspects of our results: (1) that different environments display different patterns of correlated responses to selection and (2) that the correlated responses to selection can sometimes be asymmetrical. Note that the conditions encapsulated in the β terms in (3) were held constant across all environments. Thus, one possible explanation for the changing pattern of correlated responses is that the elements of **G** change across environments, in other words, that there is "cryptic genetic *covariation*" for the relationship between heat and oxidative stress. In terms of the underlying components of **G** (Equation (2)), this would mean that the allele-specific effects (x) vary across environments; i.e., genomic components contributing to both trait variances and covariances display environment-specific norms of reaction.

Estimating norms of reaction and genotype-by-environment influences on genetic variances for the same trait across multiple environments is a fairly regular part of studies of phenotypic plasticity (e.g., Schmalhausen 1949; Scheiner and Lyman 1991; Gutteling et al. 2006; Beckerman et al. 2010; Diamond and Kingsolver 2010). Norms of reaction for genetic correlations across traits are less frequently studied (e.g., Donohue and Schmitt 1999; Bégin and Roff 2001; Pollott and Greeff 2004). Note that in this instance we are talking about shifting patterns of pleiotropic effects for three different classes of genetic correlation: across multiple traits within the same environment, across the same trait across multiple environments, and across multiple traits across multiple environments. Our analysis of correlated responses both within and between environments shows that each of these can shape the correlated responses to selection and that they can vary by environmental context.

In contrast to the "cryptic covariance" hypothesis, there is also a "cryptic *selection*" hypothesis. Under this scenario, shifting to a different environment does not change the structure of the G-matrix but instead reveals selection operating on components of the multivariate phenotype. For instance, moving from the relatively benign lab environment to a condition of chronic oxidative stress may induce selection on different aspects of the stress response pathway, which are in turn also correlated with the acute heat and oxidative stress phenotypes directly measured here. The multivariate response to selection under this scenario would look something like:

$$\begin{aligned} \Delta z_H &= G_{HH}\beta_H + G_{HO}\beta_O + G_{HU}\beta_U \\ \Delta z_O &= G_{OO}\beta_O + G_{HO}\beta_H + G_{OU}\beta_U \end{aligned} \quad (4)$$

where the subscript U represents a new, unmeasured trait under selection in the new environment that is also genetically correlated to the measured traits. The possible action of selection on unmeasured traits is one of the weaknesses of the canonical representation of multivariate selection, which fundamentally assumes that one is measuring all of the relevant traits (Mitchell-Olds and Shaw 1987; Wade and Kalisz 1990). However, unlike natural populations, our experimental evolution design allows us to measure the system under the case of no direct selection on the measured traits (i.e., when $\beta_H = \beta_O = 0$ in (4)). This should reveal any cryptic selection generated by shifts in the environment. We did indeed observe some increases in stress resistance in some of chronic environments in the absence of acute selection (Fig. 3.3), indicating that β_U is not zero in many cases. However, in no cases did this source of selection change the interpretation of the presence or absence of the correlated response to selection of one acute selection treatment on the other. Thus, the G_{HU} and G_{OU} terms are either not very large or operate in

the same direction as selection on the acute responses. We therefore conclude that "cryptic selection" is not the cause of the results that we observe.

The final possibility, then, is that the conditions of Equation (3) hold, but that **G** is changing not because of environmentally induced changes in allelic effects, but because a rapid response to selection in the different environments generates different components of the pleiotropic gene network to change in frequency in different conditions. Under this scenario, the effects of any particular allele in Equation (2) do not change with the environment, but the structure of **G** itself changes because different subsets of alleles are responsible for the response to selection in different environments, and these alleles in turn display different patterns of pleiotropy across the genetic network. This can generate a feedback loop that generates a correlated response to selection in some environments and not others. This hypothesis is especially appealing as an explanation for the asymmetry in correlated responses that we observed in the chronic oxidative stress environment (Fig. 3.3). Shifting frequencies of alleles with different patterns of pleiotropy is one of the predominant explanations for asymmetrical responses to selection (Bohren et al. 1966). A related possibility is that the response to selection is not solely determined by the additive pleiotropic effects. Instead, alleles that display strongly asymmetrical effects cause higher order moments of the genetic distribution beyond the variance/covariance (e.g., multivariate skewness) to contribute to the response to selection (Barton and Turelli 1987).

We cannot currently distinguish between the "cryptic genetic covariance" and "rapid evolutionary response" hypotheses. Indeed, they are not mutually exclusive. Nevertheless, both of these scenarios suggest that variance in pleiotropy across the

genetic network and/or variance in pleiotropy across environments is the primary determinant of the results we observe here. Determining whether one or both of these possibilities is correct will require actually determining the alleles responsible for the evolutionary change we observe and then measuring their pleiotropic effects across multiple environments. Such an effort is at least conceivable using this model system.

Conclusions

We have measured all possible responses in a 3x3x3 response hypercube (Fig. 3.6). The edges of this cube are determined on two sides by the various combinations of selection imposed on the population (acute vs. chronic). The remaining side is the current environment experienced by the population when its phenotypic response is assayed. One way of viewing these responses as a metaphor for various kinds of evolutionary change is that the acute selection treatments represent periodic bouts of strong selection ("past transient" events), whereas the chronic selection treatments represent more stable changes in the environment ("past consistent" events). The phenotyping environment then represents the present environmental circumstances (Fig. 3.6). Here we observe a multitude of possible responses that fill the hypercube in unexpected ways. In most systems we can only observe the "present" and wonder how the population came to achieve this specific state. Using an experimental evolution framework allows us to capture the entire suite of complexity induced by historical, as well as

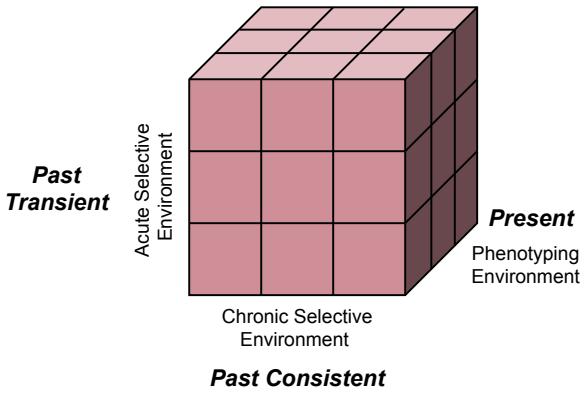


Figure 3.6. Selection history of a population represented as a hypercube. Various aspects of the past and present environmental influences on evolution are depicted on each face.

ephemeral, shifts in the environment. It is sobering to consider that this hypercube represents a very small subset of the entire multidimensional evolutionary and phenotypic space filled by this species.

Modern evolutionary quantitative genetics, as codified primarily by the Chicago School (Lande 1979; 1980; Lande and Arnold 1983; Lande 1984; Arnold and Wade 1984), has tended to emphasize either the separation of genetics from the selective context of the environment (in order to describe the multivariate response to natural selection; Lande 1979), or the separation of the complexities of environmentally contingent natural selection in favor of better describing the environment-specific expression of phenotypes via phenotypic plasticity (Via and Lande 1985). Both approaches rely on an assumption that we know and can measure all of the attributes of both the environment and of the individual that *matter* (Barton and Turelli 1989). Here we observe that the multivariate response to selection, as well as the evolution of phenotypic plasticity, is highly contingent on environmental context. This is perhaps not surprising in and of itself. However, the manner in which it is contingent—changes in genetic covariance structure via direct environmental perturbations or via the response to multivariate selection—are unknown. Indeed, we believe that they are fundamentally unknowable from the analysis of phenotypes alone. The structure of the molecular genetic network that underlies the G-matrix, particularly varying patterns of pleiotropy across the network, needs to be determined.

BRIDGE

In Chapter III, we showed that genetic correlations both between traits and across environments are strongly influenced by the environmental context, which can lead to very different responses to selection. Changes in pleiotropy in the underlying stress response networks likely explain the observed variation in correlated responses. However, we suggested that it is impossible to disentangle the effects of cryptic covariation and multivariate selection without knowledge of the underlying molecular networks. Chapter IV is a critical first step toward addressing these fundamental questions. In this chapter, we elucidate the structure of the gene coexpression network in the subset of our lines that was evolved in permissive control conditions, and identify modules within the network that may contribute to the evolution of phenotypic plasticity.

CHAPTER IV

MODULARITY OF REGULATORY NETWORKS CONTRIBUTING TO THE EVOLUTION OF PHENOTYPIC PLASTICITY FOR STRESS RESISTANCE

The experimental evolution lines described in this chapter were created in collaboration with R. M. Reynolds. C. M. Ituarte and I collected samples and constructed the libraries sequencing for transcriptional profiling, and I performed the analyses of the data. P. C. Phillips and W. A. Cresko were the principal investigators for this study.

INTRODUCTION

When faced with novel and stressful environmental conditions, individual organisms must be able to acclimate in order to survive, and populations of organisms will often need to adapt to flourish in the new conditions. The induction of novel trait values via phenotypic plasticity is one mechanism by which organisms can increase their fitness when faced with an environmental challenge (Bradshaw 1965). Like other complex phenotypes, phenotypic plasticity has a genetic basis, and therefore can evolve in response to selection (West-Eberhard 2003; Moczek et al. 2011). The adaptive response of a population to new, stressful conditions may therefore involve the evolution of novel patterns of phenotypic plasticity (Via and Lande 1985; Gomulkiewicz and Kirkpatrick 1992; Gavrilets and Scheiner 1993; Lande 2009; 2014). Furthermore, adaptation to novel environments in the wild may require the change of myriad characters in response to numerous stresses, and leading to a potential correlated response in both

mean phenotypes in one environment, as well as covariance in patterns of phenotypic plasticity of different traits across environments if the plastic responses share a genetic basis.

Plasticity has been studied in the laboratory and the field for the last century at the phenotypic level (Baldwin 1896a,b; Clausen et al. 1940; Waddington 1953; 1956; Schmitt et al. 1995; Bennett and Lenski 1997; DeWitt 1998; Nussey et al. 2005; Cheviron et al. 2013), and has been shown to be adaptive in many different systems (e.g., Dudley and Schmitt 1996; Agrawal 1998; Aubret et al. 2004; Charmantier et al. 2008). Despite the long body of work on phenotypic plasticity and its documented importance in adaptation to novel environments, little is known about the molecular basis of plasticity, or the adaptive evolution of molecular systems that underlie phenotypic plasticity. Furthermore, little is known about the shared genetic basis of correlated phenotypic plasticity of different traits across environments.

In a few recent cases, the roles of a handful of candidate genes have been characterized (e.g., Gottlieb and Ruvkun 1994; Gibson and Hogness 1996; Ragsdale et al. 2013). In one classic example, genetic assimilation of the ether-induced *Ultrabithorax* phenocopy described by Waddington (1956) was later attributed to allelic variation segregating in the *Ubx* gene (Gibson and Hogness 1996). Even more recently, studies of transcriptional regulation using microarray and RNA-seq approaches have enabled the identification of additional genes that are differentially expressed in response to particular environmental stresses (e.g., Gasch et al. 2000; Swindell et al. 2007; Badisco et al. 2011; Schunter et al. 2014). In such whole-transcriptome studies, the focus has primarily been to identify the specific genes that are most differentially expressed across environments,

and not necessarily to study changes in the networks of transcriptional regulation associated with plastic phenotypes (but see Promislow 2005; Barchuk et al. 2007).

Because research on the regulatory networks that form the basis of phenotypic plasticity is in its infancy, numerous fundamental questions remain (Snell-Rood et al. 2010). It is unclear how many loci regulate phenotypic plasticity, and importantly how segregating alleles at these genes affect the pathways in which they reside. Furthermore, it is not known whether these plasticity loci are central nodes in, and therefore key regulators of, developmental networks, or if they are peripheral to the network, perhaps functioning as specific modifiers. More basically, it is unknown how modular—i.e., nodes within a subnetwork are highly connected, but share fewer connections with adjacent subnetworks—the networks are that underlie plastic responses to the environment. We also do not know to what extent these modular patterns change across environments, and to what extent modules are shared between related, but none-the-less distinct, environments such as temperature and chemical stresses. Most importantly, it is unclear if modules of differentially expressed genes evolve in concert with the evolution of phenotypic plasticity, and if so whether the evolution occurs via the large-scale rewiring of central nodes and modules, changes on the periphery of the network, or some combination of both.

Systems biology approaches, newly enabled by advances in next-generation sequencing and computational analysis strategies, provide a means to more systematically address these questions about the evolution of phenotypic plasticity (Barabási and Oltvai 2004; Alon 2006). While holistic studies of biological systems can occur at a variety of levels, from macromolecular interactions to changes in metabolites

or interactions between hosts and their microbiota, a productive focus for phenotypic plasticity is to examine changes in gene regulatory network (GRN) to determine patterns of covariation across genes in different environments and evolutionary outcomes. A central goal of GRN analyses is to identify coregulated sets of genes, which are likely to have similar functions (Eisen et al. 1998; Wolfe et al. 2005). While network approaches have been applied to understand the basis of plasticity in response to environmental variation (Promislow 2005; Barchuk et al. 2007), this approach has heretofore not been used to ask how GRNs evolve in accord with changes in plasticity in an experimental evolution framework. Performing systems analyses of plasticity within an evolutionary context provides a powerful opportunity to simultaneously identify the loci or pathways responsive to environmental perturbation, as well as the nature of evolution within those pathways over time. Here we present the findings from the first study to take a GRN approach to the evolution of phenotypic plasticity in experimentally evolved populations of nematode worms.

We evolved populations of the nematode *C. remanei* in the laboratory, selecting for resistance to heat stress and oxidative stress under several environmental conditions (Sikkink et al. 2014a,b). As a result of selection, most populations exhibited changes in phenotypic plasticity across environments (Sikkink et al. 2014a). Here we used an RNA-sequencing (RNA-seq) approach to determine the structure and evolution of the GRN in a small sample of populations in this experimental framework. We used deep and highly replicated sampling to obtain transcriptional profiles of the ancestor, and the three selected lines evolved in a permissive environment. Transcription was measured in both permissive and heat stress conditions, providing an estimate of transcriptional plasticity

in response to stress. Using a powerful multivariate statistical approach, we were able to probe the patterns of differentiation in gene regulation across treatments and between evolved populations. We then constructed the coexpression network to identify transcriptional modules associated with the plastic response to stress or the evolution of that response. In particular, we asked whether adaptation to stress involved the same co-regulated modules, and therefore likely the same pathways, in lines selected to withstand different stressors. Additionally, we sought to understand whether gene modules invoked to regulate a plastic response to the environment in the ancestor were the same as those targeted by evolution in the selected lines, and if not, did they differ from the ancestral plasticity modules in their function or regulation.

METHODS

Experimental evolution of *C. remanei*

We used the experimentally evolved populations of *C. remanei* that have previously been described by Sikkink et al. (2014a,b). Briefly, 26 isofemale strains of *C. remanei* were isolated from terrestrial isopods (Family *Oniscidea*) collected from Koffler Scientific Reserve at Jokers Hill, King City, Toronto, Ontario. These strains were crossed in a controlled fashion to promote equal genetic contributions from all strains. The resulting genetically heterogeneous population (PX443) was the ancestral population for the experimental evolution.

A subset of the ancestral population was used for transcriptional profiling. In addition to the ancestor, three experimentally evolved populations were sampled for RNA-sequencing. All selection lines had been evolved at 20°C as described in Sikkink et

al. (2014a,b). One representative control population, one heat-selected population, and one oxidative-selected population were used. The heat-selected line was generated by exposing age-synchronized L1 larval worms to a 36.8°C heat shock approximately every second generation. The oxidative-selected was similarly treated with a 1mM solution of hydrogen peroxide. The control populations received a mock selection treatment, from which worms were selected at random to continue the selected line. All lines were frozen after every two selection events. Final populations for phenotyping and transcriptomics had experienced a total of 10 acute selection events and five freeze-thaw cycles.

Transcriptional profiling of pooled populations

We collected L1 tissue from the ancestral, control, heat-selected, and oxidative-selected populations to use for transcriptional profiling (Fig 4.1). All lines except the oxidative-selected population were previously analyzed in Sikkink et al. (2014b). Briefly, we thawed frozen stocks of worms from each population. Except in the oxidative-selected population, 6 replicates per treatment were collected from a minimum of two independently thawed populations from each line. For the oxidative-selected line, all replicates were collected from a single thawed population of worms. Worms were raised at 20°C until the population was large enough to collect enough individuals for RNA isolation. Age-synchronized L1 larvae were raised for 20 hours in liquid medium at either 20°C or 30°C (Fig 4.1). Prior to tissue collection, larval worms were passed through a 20-μm Nitex screen to remove unhatched eggs and dead adults. Total RNA was isolated from approximately 100,000 pooled individuals using standard TRIzol methods. Sequencing libraries were prepared according to the protocols as previously described (Sikkink et al. 2014b). Samples were sequenced from a single end, to a length of 100

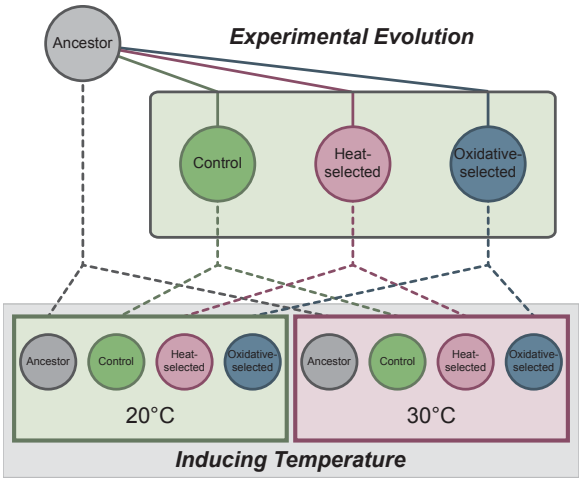


Figure 4.1. Schematic of the transcriptional profiling experiment. Four populations were considered: the ancestor and three experimentally evolved populations selected under different stress conditions. The induction of transcriptional plasticity was assessed in each line across two different thermal environments.

nucleotides in six lanes on an Illumina HiSeq 2000 at the University of Oregon Genomics Core Facility.

Analysis of differential gene expression

Initial quality filtering of raw sequence reads was performed using the *process_shortreads* component of the software Stacks (Catchen et al. 2011; 2013). Reads were discarded if they failed Illumina purity filters, contained uncalled bases, or if sample identity could not be determined due to sequencing errors in the barcode sequence. Reads with ambiguous barcodes were recovered if they had fewer than two mismatches from a known barcode. Using the alignment software GSNAP (Wu and Nacu 2010), we aligned all reads that passed the quality filters to the *C. remanei* genome (C_remanei-15.0.1 assembly) publicly available from Ensembl Metazoa (metazoa.ensembl.org/). We then used the htseq-count tool from the Python package HTSeq (http://www-huber.embl.de/users/anders/HTSeq/) to count all reads unambiguously aligning to gene models.

Multivariate analysis of transcriptional variation

We first normalized the gene counts from all samples to account for differences in library size, using the scaling procedure implemented in the *DESeq2* package (Anders and Huber 2010; Love et al. 2014) in R (R Development Core Team 2013). The expression dataset was next filtered to exclude the lower quartile of genes based on their

average expression across all treatments. Independent filtering of genes with very low expression across treatments generally improves power in subsequent analyses (Bourgon et al. 2010; Anders et al. 2013).

We used non-metric multidimensional scaling (nMDS), which is an unsupervised ordination method that enables highly-dimensional data to be projected onto a few axes for visualization. For RNA-seq data, nMDS may preferable as an ordination method, because it does not assume linear relationships within the data, enabling nMDS algorithms to robustly extract complex patterns from gene expression data (Taguchi and Oono 2005). One drawback of this nonparametric approach, however, is that the scores for variables mapped onto ordination axes can not be easily interpreted (in contrast to principal component scores, for example), and other methods may be required to identify genes contributing to differences between groups.

To carry out the nMDS ordination, a dissimilarity matrix was calculated for the filtered dataset using Bray-Curtis dissimilarities (Bray and Curtis 1957). Using other distance metrics did not substantially alter the ordination plot. Data transformation, ordination, and scaling were performed in 5 dimensions using the *vegan* package (Oksanen et al. 2013). We tested for significant differences among populations and treatments using a permutational analysis of variance performed on the Bray-Curtis dissimilarity matrix. Population, treatment, and the interaction term were included as effects in the model, and 1000 permutations were run.

Gene coexpression network analysis

Weighted gene coexpression network analysis was used to identify groups of genes with highly correlated patterns of expression across samples. We used the package

WGCNA (Langfelder and Horvath 2008; 2012) implemented in R to build an undirected network in which each node represents a gene, and each edge describes the correlation in expression patterns between a pair of genes. A variance-stabilizing transformation was performed on the count data using *DESeq2* prior to network analysis (Anders et al. 2013; Giorgi et al. 2013). *WGCNA* utilizes a soft thresholding strategy to infer network topology, which emphasizes strong correlations between pairs of genes by raising the correlation coefficient to a power (Langfelder and Horvath 2008). We used a soft threshold power of 10, which maximized the scale-free fit of the network topology.

Modules containing a minimum of 30 coexpressed genes were identified using the Dynamic Tree Cut method (Langfelder et al. 2008). In an unsigned network, these modules contain both positively and negatively correlated genes. The eigengene for each module, defined as the first principal component of the expression of all the genes in the module, was calculated to represent the general pattern of expression seen within each module. We then performed an analysis of variance on module eigengenes to test for effects of population, temperature, and population-by-temperature interactions on the overall module expression. Since statistical tests were performed for each module in the network, we corrected for multiple testing using the Benjamini-Hochberg method (Benjamini and Hochberg 1995). For modules that had a significant effect of population, we used Tukey HSD to identify pairwise differences between lines.

Gene ontology enrichment analysis

We tested for over-representation of Gene Ontology (GO) terms (The Gene Ontology Consortium 2000) within each module using the software program Blast2GO (Conesa et al. 2005; Conesa and Götz 2008). Blast2GO computes a Fisher's exact test

with a FDR correction to test for significant enrichment of GO terms in a test set. The Blast2GO database was created previously for genes that were passed the more stringent criteria for expression in used in Sikkink et al. (2014b). This database includes functional information for 15937 (67%) of the genes used in the current dataset, which should provide a representative sample for enrichment tests. We tested for over-representation of generic GOSlim ontology terms for the genes within each module using a one-tailed test.

Enrichment of transcription factor targets

Coexpressed gene modules may share expression patterns because they share a common regulatory basis. We examined each module for enrichment of known regulatory targets of 23 transcription factors for which binding data is available for the related nematode *C. elegans*. Binding targets for all transcription factors except for the FOXO transcription factor DAF-16 were obtained from the *C. elegans* modENCODE project (Niu et al. 2011). These targets were all identified from chromatin immunoprecipitation sequencing (ChIP-seq). Putative target genes bound by DAF-16 have been previously identified using two different approaches: ChIP (Oh et al. 2006) and DNA adenine methyltransferase identification (DamID; Schuster et al. 2010). In addition, several microarray studies have identified genes with DAF-16 dependent expression patterns (McElwee et al. 2003; Murphy et al. 2003; McElwee et al. 2004; 2007). Genes with DAF-16 dependent expression could be either direct targets of DAF-16, or could be indirect targets in the same pathway. We therefore considered two separate gene sets for DAF-16: genes known to be bound by DAF-16 within their promoter region, and genes with DAF-16 dependent expression. Putative target genes

could be included multiple gene sets, if they are bound by more than one transcription factor.

C. remanei homologs for each of the *C. elegans* transcription factor targets were determined based on the annotations that have been curated in the WS220 release of WormBase (Harris et al. 2009). Homologous genes identified by any method were included as possible transcription factor targets in *C. remanei*. In cases where multiple *C. remanei* genes were matched to a single gene in *C. elegans*, all possible homologous genes were included in the gene set, since no information was available to determine whether transcription factor binding was preserved preferentially in either possible homolog.

Modules were tested for significant enrichment of target genes bound by each transcription factor using a one-tailed Fisher's exact test. In addition, we tested for enrichment of the *C. remanei* heat shock proteins previously identified (Sikkink et al. 2014b), and the genes with DAF-16 dependent expression. *P*-values were adjusted to account for multiple testing using the Benjamini-Hochberg method (Benjamini and Hochberg 1995).

RESULTS

Divergence occurs in transcriptional regulation across temperatures and between evolved populations

We first sought to determine whether samples from different populations or the different temperatures could be differentiated based on global patterns of gene expression. To do this, we used non-metric multidimensional scaling (nMDS), a powerful

ordination method that does not assume linear relationships among variables. We observed distinct separation between the two temperature treatments primarily on the second ordination axis (Fig. 4.2). We tested for significant differentiation between temperatures using a permutational analysis of variance on the dissimilarity matrix. The differences in gene expression encapsulated by nMDS2 in response to temperature were highly statistically significant ($F_{1,40} = 11.02$, $P = 0.001$).

The four populations differed from one another mainly on the third nMDS axis (Fig. 4.2). The control and heat populations both diverged from the ancestor in the same direction. Unsurprisingly, the heat-selected population was more different from the

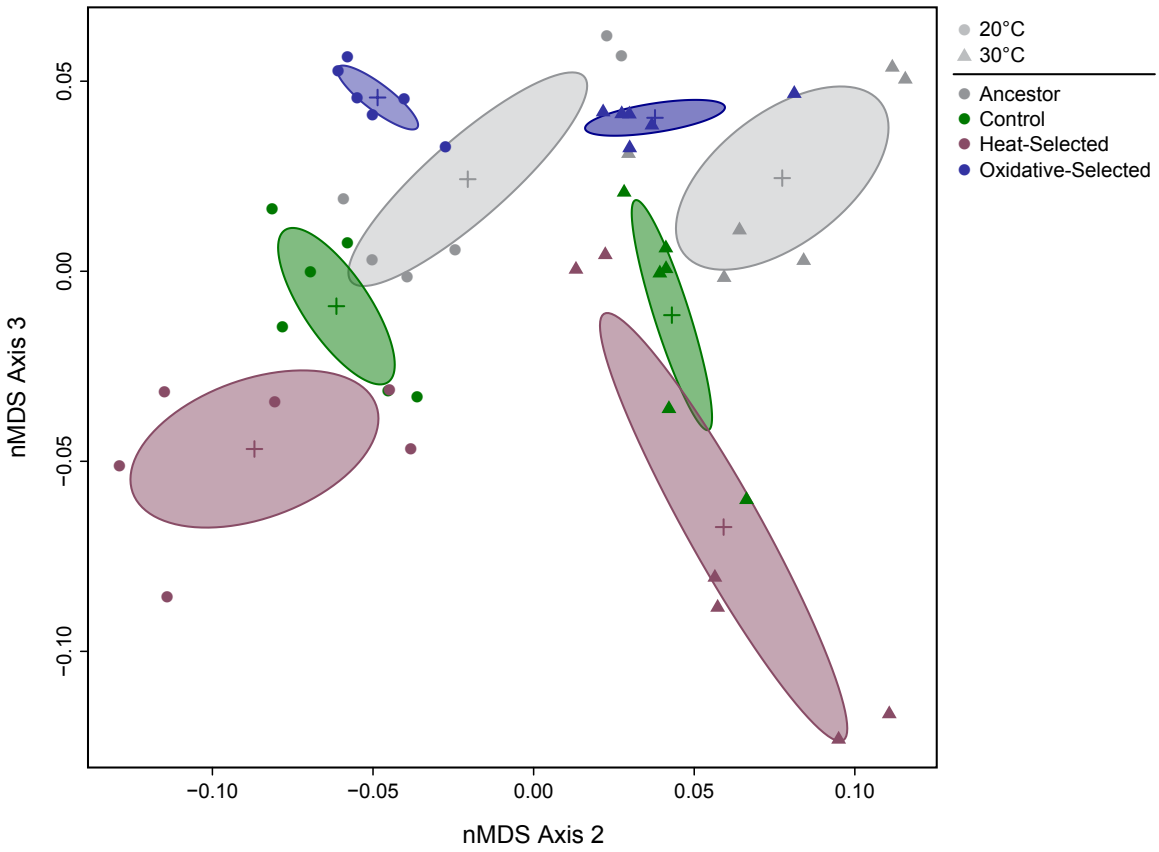


Figure 4.2. Non-metric multidimensional scaling plot of RNA-seq samples based on the filtered set of all expressed transcripts. Axes 2 and 3 from the ordination are shown. Crosses and ellipses indicate the centroid and 95% CI for each treatment group, respectively.

ancestor than the control population was. The oxidative-selected population and the ancestral population were separated more completely on nMDS4 (Fig. 4.3), indicating an at least partially different genetic basis to adaptation to oxidative stress than heat stress and general lab adaptation. The differences we observed among populations were statistically significant ($F_{3,40} = 3.00$, $P = 0.004$). Notably, though, all lines appeared to be responding to the temperature treatment in much the same manner, as evidenced by the roughly parallel change between temperature treatments on nMDS2 (Fig. 4.2). Likewise, there was no support for a line-by-temperature interaction in the permutational ANOVA ($F_{3,40} = 0.7703$, $P = 0.648$). This does not, however, preclude the possibility that some subsets of genes show significant interaction effects corresponding to the evolved phenotypic differences between selected lines.

Network modules are differentially associated with line- and temperature-specific variation in expression

Because nMDS is a non-metric method, the contribution of specific genes to divergence on each axis is not readily interpretable. To get around this limitation, we next

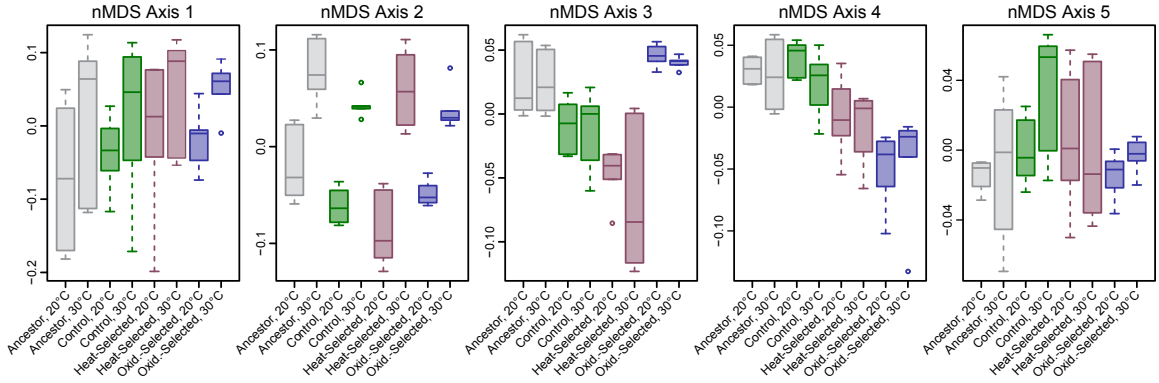


Figure 4.3. Scores on each nMDS axis at 20°C and 30°C for the ancestor (grey), control (green), heat-selected (red), and oxidative-selected (blue) populations. Boxplots show median and interquartile range of sample nMDS scores.

used weighted gene co-expression network analysis (Langfelder and Horvath 2008) to identify modules—sets of genes with strongly correlated expression patterns that are more loosely connected to other such modules. We sought to identify modules that were important in the differential regulation of stress resistance in our evolved populations of *C. remanei*, because members of a gene module often share a common function (Eisen et al. 1998; Wolfe et al. 2005), and highly correlated genes sets may share transcriptional regulators (Allocco et al. 2004, but see also Marco et al. 2009). Network analysis can therefore provide unique and useful insights into GRNs.

Network analysis identified 22 co-expressed modules containing a total of 16,463 genes (Table 4.1). An additional 7,175 genes could not be assigned to any module, and were designated as "Unassigned". For each module, we calculated the eigengene, defined as the first principal component of the module (Appendix C, Fig. S4.1). An eigengene's expression is representative of the expression of the combined set of genes within the module. We performed an analysis of variance on each eigengene to test for differences in expression attributable to either divergence between evolved populations or inducible responses to temperature, as well as interactions between the population and temperature. Significant temperature differences were observed in seven modules, while three showed significant line effects (Table 4.1). An additional seven modules differed by both population and temperature. Only Module 19 showed a significant population-by-temperature interaction effect ($F_{3,40} = 6.87$, FDR = 0.006).

To determine how the lines differed with respect to one another, we examined pairwise differences among lines using Tukey's HSD (honest significant differences) for modules that showed a significant population effect (Fig. 4.4). Evolved lines that

Table 4.1. Modules identified in gene coexpression network analysis

Module	Number of Genes	Eigengene Effects (FDR)	Evolved[a]	Functional annotation (Biological process)
Unassigned	7175			
1	5092	Temperature (<0.001)		signal transduction ion transport
2	3657			embryonic development reproduction cellular component organization
3	1451	Temperature (0.016)		embryonic development cell differentiation epigenetic regulation of expression
4	1420	Temperature (<0.001) Population (<0.001)	●	response to stress response to biotic stimulus
5	980			embryonic development cell differentiation cellular homeostasis
6	686	Population (0.006)		
7	597			
8	451	Temperature (<0.001)		
9	429			
10	383	Temperature (<0.001) Population (0.001)	●	cell signaling
11	234	Temperature (0.020)		multicellular organism development growth anatomical structure morphogenesis
12	202	Temperature (<0.001) Population (<0.001)	●	
13	174	Temperature (<0.001) Population (0.001)		peroxisome component[b]
14	128	Temperature (<0.001) Population (<0.001)	●	
15	127	Temperature (0.042) Population (<0.001)	●	DNA metabolic process
16	116	Population (<0.001)	●	

Table 4.1. (continued).

Module	Number of Genes	Eigengene Effects (FDR)	Evolved[a]	Functional annotation (Biological process)
17	99	Temperature (0.009)		
18	54	Temperature (<0.001)		
19	47	Temperature (<0.001) Population (<0.001) Interaction (0.006)	●	
20	47	Population (0.024)		
21	45			
22	44	Temperature (<0.001)		

[a]Significant difference (P<0.05) between ancestor and any evolved line determined by Tukey HSD test
[b]Cellular component ontology term

diverged from the ancestral population are of particular interest, as these could indicate a set of genes that are adaptive for stress resistance. The expression of Module 4 was significantly different from the ancestral population only in the lab-adapted control population. Similarly, Modules 10 and 19 indicated heat-adapted genes, while Module 16 was unique in the oxidative-selected line. Module 12 differed from the ancestor in both the control and heat populations, but the two evolved populations were not significantly different from each other, suggesting that Module 12 may have contributed to lab adaptation in these two lines. In contrast, Module 14 evolved in both the heat and oxidative-selected lines, but the heat and oxidative populations also differ from each other. In fact, the direction of the evolutionary response in these two lines was in opposite directions, such that the oxidative line exhibited higher expression of the eigengene than

the ancestor and the heat line had lower expression (Fig 4.4). Similarly, Module 15 was different among all four lines.

Regulatory targets of stress-responsive transcription factors are enriched in network modules

In *C. elegans*, several transcription factors are known to be critical regulators of cellular responses to stress. However, these regulators may not be differentially expressed in response to stress themselves, but rather undergo protein modifications to activate them under certain conditions. For example, the FOXO transcription factor DAF-16 is a major target of the insulin/insulin-like growth factor signaling (IIS) pathway in worms, and is responsible for mediating responses to heat and oxidative stress, among others (Honda and Honda 1999; Hsu et al. 2003). DAF-16 is normally localized in the cytoplasm, but in stress conditions, DAF-16 is activated and transported to the nucleus, where it regulates transcription of many target genes (Lee et al. 2001; Lin et al. 2001). We identified *C. remanei* homologs of known binding targets of 23 transcription factors and tested for significant

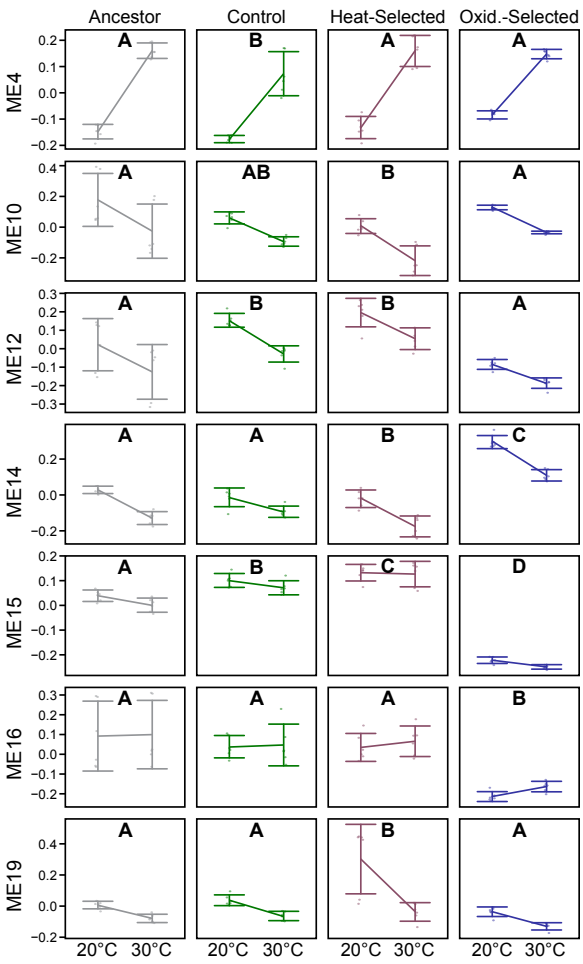


Figure 4.4. Eigengene expression across temperatures for each population. Only modules which differ significantly between the ancestor and at least one evolved population as determined by a Tukey test (P<0.05) are shown.

enrichment in each of the network modules. We also examined enrichment of two other gene sets, the heat shock protein families previously examined in Sikkink et al. (2014b), and genes with DAF-16 dependent effects on expression. The latter group included both genes that are known to be bound by DAF-16, and other genes that may occur downstream in the pathway.

We observed significant enrichment (FDR < 0.05) of regulatory targets for all but three of the available transcription factors (Fig. 4.5). Modules 1, 2, 3, and 5 in particular share many regulators in common. Targets of three HOX transcription factors—LIN-39, MAB-5, and EGL-5—were enriched in these modules. Several transcription factors that regulate stress responses also showed enrichment of their target genes in two or more of these modules. PHA-4, a developmental regulator necessary for formation of the pharynx (Mango et al. 1994; Horner et al. 1998), has also been implicated in regulating heat shock response through HSP90 (van Oosten-Hawle et al. 2013). Targets of PHA-4 were enriched in Modules 1, 2, 3, and 5. Genes regulated by DAF-16 and SKN-1, another target of IIS that is critical for oxidative stress resistance (An and Blackwell 2003), were also enriched in two and four of these same modules, respectively.

Module 4, which was significantly divergent in the control population, was enriched for targets of ELT-3, a GATA transcription factor that functions during hypodermal development in *C. elegans* (Gilleard et al. 1999) and may also function downstream of IIS to influence longevity (Budovskaya et al. 2008), pathogen resistance (Pujol et al. 2008), and osmotic stress response (Rohlfing et al. 2010). This module was also significantly enriched for heat shock proteins and genes with DAF-16 dependent expression. However, direct DAF-16 target genes were not enriched in this module.

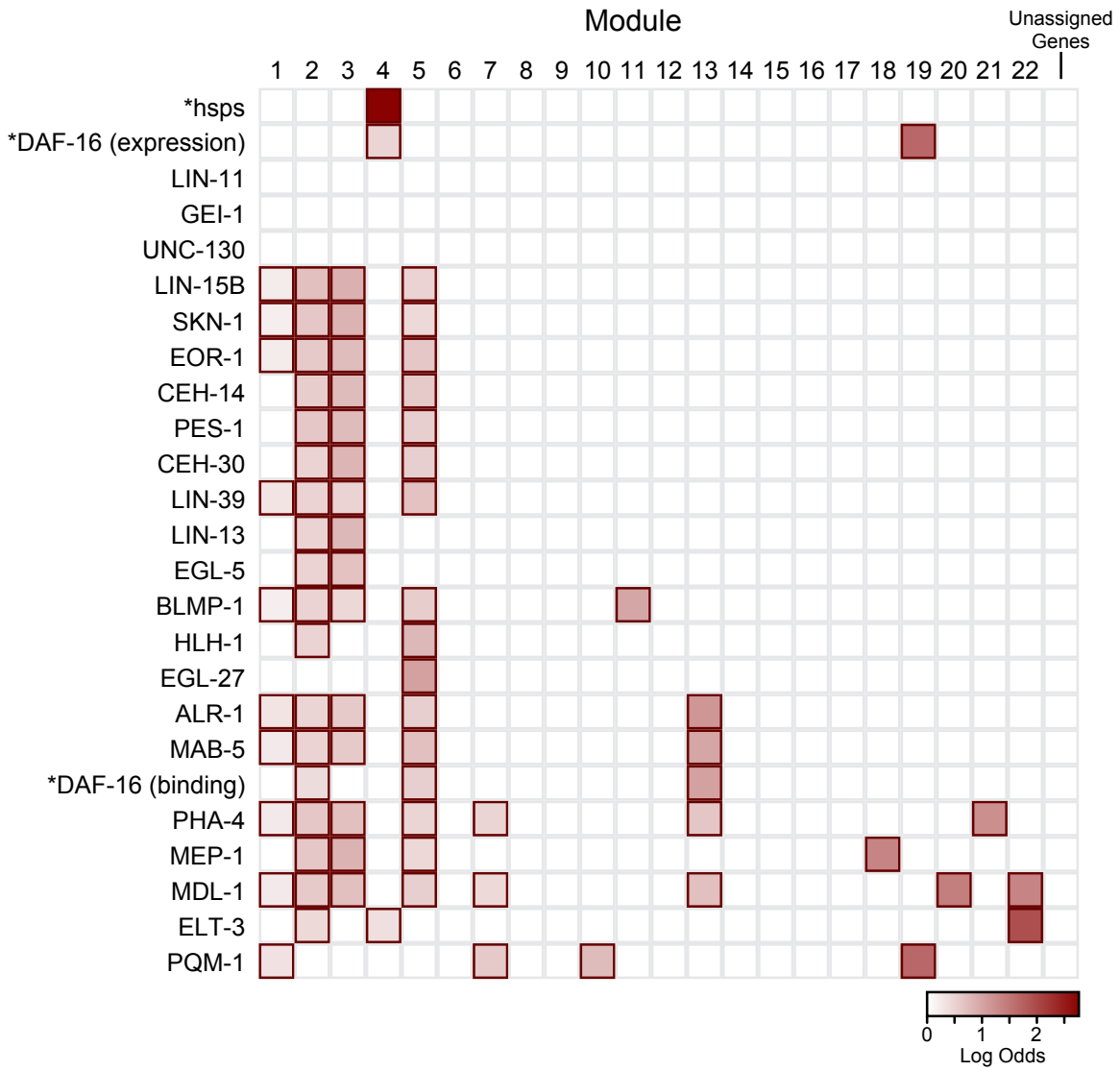


Figure 4.5. Enrichment of transcription factor target genes in coexpression modules. Red outlines signify enrichment of target genes in the module (FDR<0.05). Intensity of shading indicates the log odds ratio for the set.

Modules 10 and 19, which contributed to adaptation in the heat-selected populations, were both enriched for targets of PQM-1, a C2H2 zinc finger and leucine zipper-containing protein (Tawe et al. 1998). In *C. elegans*, PQM-1 is responsive to certain types of oxidative stress (Tawe et al. 1998), and is a key regulatory target of IIS, in addition to DAF-16 (Tepper et al. 2013). Module 19 was also enriched for genes with

DAF-16 dependent expression, which is expected for regulatory targets of PQM-1 (Tepper et al. 2013).

Other modules that were significantly different between the ancestor and evolved populations did not exhibit significant enrichment of target genes for the available transcription factors. However, ChIP binding data from *C. elegans* was not available for some key transcription factors involved in stress response, particularly HSF-1 and HIF-1. Heat shock proteins are known to be regulated by HSF-1 in response to heat stress (Wu 1995; Åkerfelt et al. 2010), therefore enrichment of hsps in Module 4 may indicate a role for HSF-1 in regulation of that module.

Gene expression modules are enriched for functionally related genes

We also examined the functional relationships among genes in identified modules by looking for enrichment of Gene Ontology terms within each module, specifically terms in the biological process ontology. Several modules (2, 3, 5, and 11) were enriched for genes regulating embryonic development. Unsurprisingly, genes responding to stress or biotic stimulus were enriched in Module 4. Module 5 also contained genes that maintain cellular homeostasis. Modules 1 and 10 both contained cell-signaling genes. Module 13 was not significantly enriched for any biological process, however, peroxisome components were significantly enriched.

DISCUSSION

For many organisms phenotypic plasticity is a vital adaptation to cope with environmental stress. However, we still know little about the molecular mechanisms contributing to plastic traits. In particular, we know very few of the genes that are

involved in phenotypic plasticity, and only a couple of case studies (Promislow 2005; Barchuk et al. 2007) have begun to define the genetic regulatory networks that underlie a plastic phenotypic response. Even less is known about how the evolution of phenotypic plasticity is related to the rewiring of GRNs that are environmentally sensitive, particularly when adaptation to more than one environment is occurring. Here, we present the first study of global changes in GRN involved in the evolution of phenotypically plastic responses. We used RNA-sequencing and network analysis in a powerful experimental evolution system to identify sets of coexpressed genes, or modules, which are associated with the evolution of phenotypic plasticity in *C. remanei* in two related, but distinct, evolutionary stresses.

Global patterns of gene expression describe evolutionary divergence

Based on the global profiles of expression among our filtered set of genes, we observed clear differentiation attributable to the induction of a response to temperature (i.e., plasticity), as well as evolved differences between populations (Fig. 4.2). Exposure to the inducing temperature resulted in very pronounced changes in the global patterns of gene expression. However, every line responded to the treatment in a parallel fashion on nMDS2, and there was no support for a line-by-temperature interaction effect at this scale. Changes in plasticity in the evolved populations are therefore not a result of global changes in gene regulation, at least to the extent that these global patterns are captured by commonly used multivariate statistics such as nMDS. Interactions may still be important in the evolution of plasticity; however, they will likely be localized to modules within the larger GRN. We have previously described this lack of change in global transcriptional regulation for the heat-selected line (Sikkink et al. 2014b), and noted that the apparent

genetic assimilation of heat stress resistance was not a loss of plasticity, but rather a shift in the threshold temperature at which the plasticity becomes apparent. Here we used a more comprehensive multivariate statistical framework to confirm our previous results and extend the observation to the transcriptome of the oxidative-selected line as well.

Notably, the heat-selected and oxidative-selected populations seem to have diverged from the ancestor primarily along different axes – nMDS3 and nMDS4 respectively (Fig. 4.3). This pattern suggests that at least partially different GRNs contribute to adaptation in each case, and likely act in a modular fashion. These findings are consistent with the observations we have previously made—that there is no genetic correlation between heat and oxidative resistance under the environmental conditions in which these populations evolved (Sikkink et al. 2014a). In short, although one might reasonably hypothesize a correlated selective response to heat and oxidative stresses that acts through a generic stress response pathway, our data support the alternative hypothesis that evolution results from changes in different GRNs, or least different modules within a GRN, for these two related stresses.

Modularity of stress GRN evolution

The pattern of expression differences that we observed in our data indicates a high degree of modularity within the gene regulatory network. Despite a relatively small number of experimental treatments, we were able to identify 22 transcriptional modules with highly correlated patterns of expression. Furthermore, the eigengenes that describe expression patterns within each module are differentially associated with the experimental treatments.

A test of our ability to draw meaningful inferences from our RNA-seq data is to examine a well-known pathway. Heat shock proteins (HSPs) are molecular chaperones known to be a critical component of response to heat stress (Lindquist and Craig 1988). Therefore, we expect these genes to form one or more modules that covary strongly with temperature. Module 4 seems to capture many of the expected elements of the generalized HSP response. This module was strongly enriched for the set of heat shock proteins (Fig. 4.5), and was also significantly regulated by temperature (Fig. 4.4). GO analysis also indicated that this module was enriched for genes that function in stress response, further supporting this role for the module. Our data clearly help us identify this module, and surprisingly show that it is localized primarily to a single module.

In this stress response module, significant expression differences attributable to line were observed (Fig. 4.4). On closer examination, however, the control population was the only selected line to show divergence from the ancestral population. If the generalized heat stress response contributed to evolution of stress resistance, then the heat or oxidative populations would be expected to evolve, rather than the control population. A plausible explanation is that the selection pressure for maintaining a strong generalized stress response was reduced in the control populations as they evolved in the benign laboratory environment. There is a precedent for this observation from *C. elegans*, in which thermoregulatory behaviors have decayed in a highly laboratory-adapted strain (Anderson et al. 2007).

Although Module 4 did not appear to contribute to evolution of heat or oxidative stress resistance, a few other modules are of particular interest as candidates to fulfill this role. Modules 10 and 19, for example, exhibited differences in eigengene expression

among lines that were specific to the heat-selected line and consistent with adaptation to heat shock (Fig. 4.4). Module 16 shows a pattern in gene expression that is uniquely associated with adaptation to oxidative stress (Fig. 4.4). The lack of overlap between the modules evolving in response to each stress provides further support for the hypothesis that different GRN modules contribute to adaptation to these two stresses.

The GRN basis of the evolution of phenotypic plasticity

In other modules, the eigengenes show patterns of gene expression that could be interpreted as the evolution of phenotypic plasticity. The best candidate genes contributing to the evolution should show differences in the degree of plasticity of genes in different populations, i.e., a population-by-environment interaction. Module 19 was the only module with a consistent interaction effect (Table 4.1). The heat-selected population generally has increased plasticity for this set of genes across environments relative to the other populations. Specifically, in the heat-selected population, expression levels in the 20°C condition seem to be different from other lines at the same temperature (Fig. 4.4). Since the phenotypic changes in heat resistance between the ancestor and heat-selected line were more apparent at 20°C (Sikkink et al. 2014a,b), the genes in this module are strong candidates for the evolution of plasticity in the heat-selected line.

Reduced plasticity at the phenotypic level, however, does not necessarily require a corresponding change in transcriptional plasticity resulting in population-by-temperature interactions. For example, in a threshold trait a loss of phenotypic plasticity might be observed if the basal level of gene expression was increased above the threshold for induction, even if the transcriptional plasticity of the causal gene did not change. Module 10 is comprised of genes that fit this pattern in the heat-selected population (Fig.

4.4), and may therefore contribute to adaptation to heat. Similarly, Module 16 has a large change in expression in the oxidative-selected line, although in this case there is no inducible expression across temperatures (Fig. 4.4). Alteration of threshold responses often contributes to the evolution of polyphenisms (e.g., Moczek and Nijhout 2003; Suzuki and Nijhout 2006), and the patterns observed in these two modules indicate a similar role for some portions of the GRN in regulating response to stress.

Although other modules do show evidence of evolved change in regulation in both the heat- and oxidative-selected lines (Fig. 4.4), our data do not support the evolution of a generalized stress response pathway contributing to adaptation in both heat and oxidative stress resistance in the evolved populations. In cases where the same module responds in both stress selection lines, selection for heat resistance typically results in an overall change in gene expression in one direction, while selection for oxidative resistance occurs in the opposite direction, as observed in Modules 14 and 15 (Fig. 4.4). The independence of the evolved responses to each stressor provides further support for the hypothesis that the GRN underlying the evolution of plasticity is highly modular.

Regulation and function of the evolved plasticity GRN modules

Genes that are co-regulated by a common transcription factor are likely to have highly correlated expression (Marco et al. 2009), and therefore should be classified as part of the same module. Identifying the transcriptional regulators of each module can provide important insight into which pathways contribute to the evolution of plasticity. In this study, we tested for enrichment of known targets of 23 transcription factors within each of the identified gene modules. Most of these transcription factors have vital roles in

regulating developmental processes, but a few also have well-characterized roles in mediating stress responses.

Many of the tested transcription factors are enriched in Modules 2, 3, and 5 as well as Module 1 to a lesser extent (Fig. 4.5). Given that the tested factors are key regulators of development, it is not surprising that Modules 2, 3, and 5 are also functionally annotated as involved in growth, embryonic development, and reproduction. It is likely that these large modules contain many developmental and housekeeping genes. Consistent with that role, these modules appear to have canalized patterns of expression, and are invariant among the experimental populations. None of these three modules showed significant differences between lines, and although Module 3 does show a temperature effect (Fig. 4.4), it is relatively weak compared to other modules in the broader network. We therefore do not expect these modules, or the core developmental pathways they represent, to contribute strongly to the evolution of plasticity.

An intriguing result from this study is the enrichment of PQM-1 targets in candidate heat-evolved modules, specifically Modules 10 and 19, which both exhibit regulatory changes specific to the heat-evolved lines (Fig. 4.5). PQM-1 is known to respond to environmental stress, although previous studies describe a response to the oxidative stressor paraquat (methyl viologen; Tawe et al. 1998) and infection by the pathogen *Pseudomonas* (Shapira et al. 2006). However, PQM-1, like DAF-16, is a major target of the IIS pathway, and the two transcription factors appear to function in opposition to one another (Tepper et al. 2013). Further study will be required to determine whether PQM-1 and its targets are important contributors to evolution of the heat stress response.

An important caveat to note is that not all transcription factors have binding data available. Two major regulators of stress response, HSF-1 and HIF-1, are missing from this dataset, but almost certainly play a role in the induction of the plastic response, if not the evolutionary response. Given that HSF-1 is known to regulate many of the heat shock proteins (Wu 1995; Åkerfelt et al. 2010), we might speculate that HSF-1 contributes in some way to the regulation of the generalized stress response described by Module 4. However, given the lack of available data, we are not able to test that hypothesis at this time. In the future, network analyses using genomic data will continue to become richer, as projects like modENCODE provide better functional annotation of the genome.

Conclusion

We have identified transcriptional modules with patterns of expression consistent with evolutionary response to selection in two different, but related phenotypes. Notably this response did not occur in the major generalized stress response module we identified, nor did we identify any shared stress response module that adapted in both selective environments in the same way. However, modules with significant responses, particularly to heat stress, were enriched for targets of a key transcription factor known to be connected to stress response pathways, indicating that plasticity evolution likely occurred within the existing stress resistance network. This is the first study to investigate the structure of GRNs underlying phenotypic plasticity in an experimental evolution framework, where the evolutionary history of the evolved changes is known. The observations made here provide important insight into the evolution of phenotypic plasticity within the regulatory networks, and valuable candidates to direct future study in this system.

BRIDGE

In Chapter IV, we described the first transcriptional network analysis undertaken in an experimental evolution framework to understand the genetic basis of phenotypic plasticity. We showed that at a global level, transcriptional responses to temperature are largely similar across all lines. However, analysis of the GRN indicated key modules that could contribute to the evolution of plasticity observed in Chapter II and III. In Chapter V, I conclude with a summary of the findings presented in this dissertation, and their significance to understanding the evolution of phenotypic plasticity.

CHAPTER V

CONCLUSION

Phenotypic plasticity is a widespread phenomenon in nature (Bradshaw 1965; West-Eberhard 2003), and is a fundamentally important adaptation for many species to cope with an unpredictable, changing world (Parejko and Dodson 1991; Schmitt et al. 1995; Agrawal 1998; Aubret et al. 2004; Justice et al. 2006; Charmantier et al. 2008; Muschick et al. 2011; Cheviron et al. 2013). Phenotypic plasticity is itself a heritable trait that can evolve. There is strong interest in the field to understand how phenotypic plasticity evolves, and whether this evolution contributes to the emergence of novel phenotypes (Pfennig et al. 2010; Snell-Rood et al. 2010; Moczek et al. 2011). However, previous studies have been hampered by the fact that natural populations can only be studied in the present, without knowledge of the historical circumstances that have influenced their evolution. Experimental evolution allows this history to be observed and recorded (Rose et al. 1990), providing a powerful alternative strategy to study the evolution of plasticity. This approach has yielded many useful insights into how plasticity evolves (Waddington 1953; 1956; Scheiner and Lyman 1991; Rutherford and Lindquist 1998; Suzuki and Nijhout 2006), but suffers in that the connection to the ecology and fitness of the organisms are lost.

The experimental selection lines described in this dissertation were created to address these limitations. By selecting on resistance (i.e., survivorship) to ecologically relevant stressors, heat and oxidative stress, we have developed a system in which the

fitness consequences are clearer. At the same time, by performing selection under tightly controlled laboratory conditions, we were able to make strong inferences about the role of a variable environment in the evolution of phenotypic plasticity.

Genetic assimilation, or the evolutionary loss of environmental sensitivity in a previously plastic phenotype, is of great interest as possible driver of evolutionary innovation (Pfennig et al. 2010; Snell-Rood et al. 2010; Moczek et al. 2011). Genetic assimilation is notoriously difficult to demonstrate, and the best examples still come from Waddington's classic selection lines in *Drosophila melanogaster* (Waddington 1953; 1956). In natural populations, it is next to impossible to demonstrate genetic assimilation except in very rare cases (Aubret and Shine 2009) because the evolutionary history is often lost to time.

Patterns consistent with genetic assimilation of stress resistance were observed in the stress selection lines. In Chapter I, we described the genetic assimilation of heat stress resistance in the lines selected to withstand heat stress. However, when survival was measured across a broader range of temperatures, plasticity was still present. The maintenance of transcriptional plasticity in both the heat selected (Sikkink et al. 2014b; Chapters II and IV) and the oxidative-selected (Chapter IV) lines suggest that such shifts in the threshold for phenotypic plasticity may be common, especially in physiological traits like stress response. These observations further complicate the search for examples of genetic assimilation in nature, as organisms may be constantly moving through environments that are outside the "zone of canalization" (Waddington 1942) where genetic assimilation might have occurred.

Environmental complexity can also affect the evolutionary trajectory of populations. In Chapter III, we examined the consequences of changing environments on the patterns of multivariate selection. Notably, we observed changes in correlated responses to selection when evolution occurred in a different environment. It seems likely that this pattern resulted from a change in the patterns of pleiotropy underlying heat and oxidative stress resistance in the two environments. In our carefully controlled experimental system, we were able to probe the past environmental contingencies contributing to the observed phenotypes in these populations.

Two possible hypotheses explain the changes in pleiotropy described in Chapter III. “Cryptic genetic covariation” might be uncovered as a result of direct effect of the environment modifying linkages between genes in the network. Alternatively, if selection favors alleles with varying pleiotropic linkages, evolution of the G-matrix could produce similar patterns. Distinguishing these possibilities requires knowledge of the molecular pathways underlying stress resistance. In Chapter IV, we examined the structure of the regulatory network under one of the evolutionary environments, the control environment. This study was the first of its kind to use a systems biology approach to investigate the gene regulatory network underlying phenotypic plasticity in an experimental evolution framework.

The network that was identified appeared to be highly modular in structure. Furthermore, the regulation of some modules responded to temperature or was correlated with the evolutionary response. Importantly the patterns that we observed in the evolution of these modules—that adaptation to heat and to oxidative stress involve changes in the

regulation of different modules—support the lack of correlated phenotypic response to selection between these two traits (Sikkink et al. 2014a; Chapter III).

In Chapter IV, we established that network approaches could provide powerful insights into the molecular basis of the evolved stress response. These methodologies will enable the other questions raised by Chapters II and III to be more fully addressed in future studies. Of most pressing concern, the network described in Chapter IV only represents the patterns in the control environment. Do populations that have evolved in other environments show the same network structure, or are the modules composed of entirely different sets of genes? If the network structure is the same, do different modules encapsulate the response to selection than those observed in the control environment? The answers to these fundamental questions will go far to explain why genetic correlations are contingent on the environment, and how the regulation of phenotypic plasticity evolves.

In conclusion, the historical influences of both long-term and transient environments can have important effects on the evolution of phenotypic plasticity and other complex traits, but are difficult to measure outside of an experimentally evolved model system such as the one presented here. Careful consideration of the environmental context in which selection occurs is vitally important to understanding the nature of the evolutionary response, as is knowledge of the underlying molecular networks. The work described herein provides a foundation for understanding these two properties in evolved populations, and provides valuable insight into the evolution of phenotypic plasticity.

APPENDIX A

SUPPORTING INFORMATION FOR CHAPTER II

Table S2.1 Summary of differential expression results by line.

Line	Genes expressed above threshold	Differential expression (FDR 5%)
Ancestor	15,347	6431
Control	15,141	4286
Heat	14,784	2769
Combined	15,963	8377

Table S2.2 List of the 200 most differentially expressed genes across environments from the RNA-seq analysis.

HSP	Gene ID	Gene Name	Gene Description	Ancestor Log_2 FC	Ancestor FDR	Control Log_2 FC	Control FDR	Heat Log_2 FC	Heat FDR
	CRE09388		C-type lectin	35.31	>0.0001	--	--	4.61	>0.0001
	CRE24573		Protein kinase	-5.31	>0.0001	-34.57	>0.0001	-33.46	>0.0001
	CRE08067			32.35	>0.0001	--	--	--	--
	CRE23800		Hydrolase	8.99	>0.0001	7.00	>0.0001	n.s.	n.s.
◆	CRE24849		HSP70 protein	8.66	>0.0001	7.31	0.0011	5.64	0.0017
	CRE19381			8.22	>0.0001	8.57	0.0024	5.33	0.0344
	CRE18157			6.14	>0.0001	8.52	0.0059	--	--
	CRE18318			8.50	>0.0001	--	--	6.22	0.0198
◆	CRE04868		HSP70 protein	8.05	>0.0001	6.83	0.0116	5.78	0.0006
	CRE26773		Integrase	8.03	>0.0001	6.09	0.0222	6.09	>0.0001
◆	CRE01097		HSP70 protein	7.90	>0.0001	6.50	0.0192	5.73	0.0035
◆	CRE18319		Small hsp (HSPB)	7.90	>0.0001	6.93	0.0035	5.01	0.0002
◆	CRE18317		Small hsp (HSPB)	7.86	>0.0001	5.61	>0.0001	4.97	0.0013
◆	CRE04869		HSP70 protein	7.81	>0.0001	6.79	0.0166	5.87	0.0043
◆	CRE01029		HSP70 protein	7.72	>0.0001	6.86	0.0052	5.79	0.0008
	CRE26772		Reverse transcriptase	7.57	>0.0001	n.s.	n.s.	5.99	>0.0001
◆	CRE19380		Small hsp (HSPB)	6.99	>0.0001	7.38	0.0005	5.03	>0.0001
◆	CRE18323		Small hsp (HSPB)	7.36	>0.0001	6.48	0.0048	5.56	>0.0001
◆	CRE04666		HSP70 protein	7.33	0.0017[a]	--	--	4.59	0.0188
◆	CRE19334		Small hsp (HSPB)	7.30	>0.0001	6.47	0.0081	5.11	>0.0001
◆	CRE26901		HSP70 protein	6.49	>0.0001	7.28	0.0043	5.54	>0.0001
◆	CRE18316		Small hsp (HSPB)	6.65	>0.0001	7.15	>0.0001	5.38	>0.0001
◆	CRE18322		Small hsp (HSPB)	7.01	>0.0001	6.96	0.0003	5.10	>0.0001
◆	CRE19384		Small hsp (HSPB)	7.01	>0.0001	6.64	0.0017	4.83	>0.0001

Table S2.2 (continued).

HSP	Gene ID	Gene Name	Gene Description	Ancestor Log_2 FC	Ancestor FDR	Control Log_2 FC	Control FDR	Heat Log_2 FC	Heat FDR
	CRE23104			6.95	>0.0001	--	--	--	--
◆	CRE19383		Small hsp (HSPB)	6.93	>0.0001	6.64	0.0006	5.44	>0.0001
◆	CRE25393		HSP70 protein	6.93	>0.0001	6.43	0.0035	5.50	0.0005
◆	CRE19333		Small hsp (HSPB)	6.61	>0.0001	6.91	0.0009	5.31	>0.0001
◆	CRE18315		Small hsp (HSPB)	6.63	>0.0001	6.75	0.0007	4.91	>0.0001
◆	CRE19335		Small hsp (HSPB)	6.74	>0.0001	6.68	>0.0001	4.75	0.0001
	CRE01030			6.62	>0.0001	--	--	6.08	>0.0001
◆	CRE18321		Small hsp (HSPB)	6.61	>0.0001	6.46	0.0004	4.93	>0.0001
◆	CRE27162		Small hsp (HSPB)	6.50	>0.0001	6.37	0.0077	5.23	0.0016
	CRE05591			n.s.	n.s.	-6.47	>0.0001	--	--
◆	CRE20780	*hsp-70*	HSP70 protein	6.42	>0.0001	6.26	0.0003	5.27	>0.0001
	CRE24278		C-type lectin	--	--	--	--	-6.39	>0.0001
◆	CRE27471		Small hsp (HSPB)	6.27	>0.0001	6.27	0.0027	5.16	0.0001
	CRE21296		Reverse transcriptase	6.13	>0.0001	n.s.	n.s.	6.18	>0.0001
	CRE16108		CUB-like domain	--	--	--	--	-6.07	>0.0001
	CRE05459	*end-3*	GATA zinc finger transcription factor	6.05	>0.0001	--	--	--	--
	CRE20711		UDP-glucose:glycoprotein glucosyltransferase	5.87	>0.0001	n.s.	n.s.	4.61	0.0010
◆	CRE19382		Small hsp (HSPB)	5.80	>0.0001	5.22	>0.0001	4.99	0.0018
	CRE27833		Helitron helicase-like domain	5.69	>0.0001	--	--	n.s.	n.s.
	CRE19026		Metridin-like ShK toxin domain	-5.06	>0.0001	-5.51	>0.0001	-5.62	>0.0001
	CRE06466	*sre-42*	Serpentine receptor, class E	-3.61	0.0001	-2.54	0.0028	-5.45	>0.0001
	CRE10142		CUB-like domain	--	--	--	--	-5.39	>0.0001
	CRE11034			5.38	>0.0001	3.81	>0.0001	1.33	>0.0001

Table S2.2 (continued).

HSP	Gene ID	Gene Name	Gene Description	Ancestor Log_2 FC	Ancestor FDR	Control Log_2 FC	Control FDR	Heat Log_2 FC	Heat FDR
	CRE28993		Serpentine receptor, class W	--	--	5.38	>0.0001	--	--
	CRE08692		Serpentine receptor, class Z	--	--	--	--	-5.37	>0.0001
	CRE22721			5.34	>0.0001	--	--	5.10	0.0022
	CRE16387			3.95	>0.0001	3.37	>0.0001	5.24	0.0055
	CRE28585			4.66	>0.0001	n.s.	n.s.	4.19	0.0016
	CRE27404	*gcy-13*	Guanylate cyclase	-1.46	0.0301	--	--	-5.14	0.0015
	CRE24995	*cdh-7*	Cadherin	--	--	4.99	0.0314	--	--
	CRE08101			4.68	>0.0001	--	--	--	--
	CRE09372			3.44	>0.0001	4.64	0.0489	2.87	>0.0001
	CRE20636	*phy-2*	Prolyl 4-hydroxylase	4.52	>0.0001	4.61	>0.0001	2.03	>0.0001
	CRE01098			1.77	>0.0001	n.s.	n.s.	4.59	>0.0001
	CRE10141			--	--	--	--	-4.57	>0.0001
	CRE03576		Hydrolase	3.77	>0.0001	4.55	>0.0001	2.01	>0.0001
	CRE12322			--	--	--	--	4.53	0.0016
	CRE14503		C-type lectin	n.s.	n.s.	-1.38	>0.0001	-4.53	>0.0001
	CRE14636	*clec-140*	C-type lectin	n.s.	n.s.	-1.23	0.0004	-4.51	>0.0001
	CRE09419			4.45	>0.0001	2.64	0.0224	4.50	>0.0001
	CRE08905			-3.46	0.0009	-4.48	>0.0001	--	--
	CRE20697		Hexosyltransferase	n.s.	n.s.	-1.26	0.0003	-4.46	>0.0001
	CRE13476		Thaumatin-like protein	-4.46	>0.0001	-3.03	>0.0001	-3.64	>0.0001
	CRE29499			4.44	>0.0001	--	--	3.55	0.0004
	CRE08033			4.43	>0.0001	--	--	--	--
	CRE23366			4.38	>0.0001	--	--	--	--
	CRE11169			4.36	>0.0001	2.78	0.0011	2.12	0.0411

Table S2.2 (continued).

HSP	Gene ID	Gene Name	Gene Description	Ancestor Log_2 FC	Ancestor FDR	Control Log_2 FC	Control FDR	Heat Log_2 FC	Heat FDR
	CRE24133		Collagen	n.s.	n.s.	-4.36	>0.0001	-2.32	>0.0001
	CRE02480		Nuclear hormone receptor	--	--	-1.70	0.0219	-4.35	0.0001
	CRE16040			2.38	>0.0001	4.28	>0.0001	2.55	>0.0001
	CRE03584			4.24	>0.0001	--	--	3.38	0.0061
	CRE31291			4.22	>0.0001	3.03	0.0043	3.01	0.0053
	CRE24819			4.21	>0.0001	2.84	0.0062	2.04	>0.0001
	CRE01842	*clec-60*	C-type lectin	3.71	>0.0001	4.20	>0.0001	n.s.	n.s.
	CRE07273		Lipocalin-related protein	n.s.	n.s.	4.13	0.0461	1.69	0.0140
	CRE26387			--	--	4.11	0.0054	--	--
	CRE13045		Metridin-like ShK toxin domain	-4.09	>0.0001	-4.10	>0.0001	--	--
	CRE15564		Prolyl 4-hydroxylase	3.97	>0.0001	4.08	>0.0001	2.31	>0.0001
	CRE23551			3.28	>0.0001	--	--	4.07	>0.0001
	CRE06358			--	--	-4.07	>0.0001	--	--
	CRE20525		Serpentine receptor, class E	--	--	--	--	4.07	0.0104
	CRE13953		Fatty acid CoA synthetase family	2.49	>0.0001	2.06	0.0001	4.06	0.0001
	CRE08193			--	--	2.15	0.0266	4.04	0.0130
	CRE21837			2.36	>0.0001	2.52	0.0025	4.01	0.0001
	CRE01268			4.01	>0.0001	1.35	0.0356	2.46	0.0345
	CRE18121	*mlt-10*		4.01	>0.0001	3.68	>0.0001	1.42	>0.0001
	CRE06617		Serpentine receptor, class I	3.98	>0.0001	--	--	--	--
	CRE01964			3.96	>0.0001	3.50	>0.0001	2.23	>0.0001
	CRE01322			3.95	>0.0001	--	--	--	--
	CRE01264			3.95	>0.0001	--	--	--	--
	CRE30275			-2.09	0.0018	-3.88	>0.0001	-2.54	>0.0001

Table S2.2 (continued).

HSP	Gene ID	Gene Name	Gene Description	Ancestor Log_2 FC	Ancestor FDR	Control Log_2 FC	Control FDR	Heat Log_2 FC	Heat FDR
	CRE18513			-3.88	>0.0001	-2.10	>0.0001	-3.19	>0.0001
	CRE13168			--	--	--	--	-3.86	>0.0001
	CRE01279			3.86	>0.0001	1.96	0.0010	n.s.	n.s.
	CRE01306			3.86	>0.0001	n.s.	n.s.	--	--
	CRE22946			-3.84	>0.0001	-2.11	0.0017	--	--
	CRE18158			3.21	>0.0001	n.s.	n.s.	n.s.	n.s.
	CRE08879	*srh-129*	Serpentine receptor, class H	-3.83	0.0003	--	--	-2.50	0.0265
	CRE11248	*srx-85*	Serpentine receptor, class X	3.81	>0.0001	--	--		
	CRE02474			-2.87	>0.0001	-2.74	>0.0001	-3.81	>0.0001
	CRE09165			-3.77	>0.0001	-1.71	0.0006	--	--
	CRE30010	*ech-9*	Enoyl-CoA hydratase	1.91	0.0003	3.76	>0.0001	n.s.	n.s.
	CRE05592			n.s.	n.s.	-3.49	>0.0001	-3.74	>0.0001
	CRE30392			3.73	>0.0001	n.s.	n.s.	n.s.	n.s.
	CRE21032		Flavin monooxygenase	-3.73	>0.0001	-2.79	>0.0001	-1.64	>0.0001
	CRE18358			3.03	>0.0001	--	--	3.73	0.0001
	CRE09421			3.69	>0.0001	3.11	>0.0001	3.11	>0.0001
	CRE03133			-3.69	>0.0001	-1.36	>0.0001	-2.38	0.0226
	CRE17248	*aagr-4*	Acid alpha glucosidase related	2.40	>0.0001	3.68	>0.0001	2.18	>0.0001
	CRE01319			3.68	>0.0001	2.31	0.0023	n.s.	n.s.
	CRE09022			-3.45	>0.0001	-3.66	>0.0001	-2.64	0.0007
	CRE03585			3.65	>0.0001	n.s.	n.s.	3.60	0.0027
	CRE03432		Serpentine receptor, class W	-2.33	0.0002	n.s.	n.s.	-3.64	>0.0001
	CRE21838			1.91	0.0022	2.25	0.0001	3.63	0.0002
	CRE00936	*npax-2*	N-terminal PAX (PAI domain only) protein	2.13	0.0004	3.63	>0.0001	2.68	0.0018

Table S2.2 (continued).

HSP	Gene ID	Gene Name	Gene Description	Ancestor Log_2 FC	Ancestor FDR	Control Log_2 FC	Control FDR	Heat Log_2 FC	Heat FDR
	CRE09422			3.63	>0.0001	2.85	0.0005	3.19	0.0009
	CRE25745			3.30	>0.0001	3.60	>0.0001	3.11	>0.0001
	CRE02652			3.57	>0.0001	1.38	0.0403	2.13	0.0004
	CRE17298			-3.56	0.0001	--	--	--	--
	CRE12966		Serpentine receptor, class W	-3.56	>0.0001	--	--	-2.77	0.0008
	CRE27097			n.s.	n.s.	3.54	0.0066	n.s.	n.s.
	CRE10586		Dehydrogenase	-1.22	>0.0001	-1.05	>0.0001	-3.52	>0.0001
	CRE04420			3.51	>0.0001	--	--	--	--
	CRE18856			-3.50	>0.0001	-2.28	>0.0001	-2.17	>0.0001
	CRE23575		MAM (Meprin, A5-protein, PTPmu) domain protein	3.36	>0.0001	3.48	>0.0001	3.09	0.0013
	CRE12497			--	--	--	--	-3.48	0.0015
	CRE03438		C-type lectin	2.47	0.0002	3.47	>0.0001	--	--
	CRE05780			-3.06	>0.0001	-3.47	0.0004	--	--
	CRE02894			3.46	>0.0001	1.36	0.0231	--	--
	CRE21368			3.45	>0.0001	2.79	0.0041	--	--
	CRE16306		PAN domain-containing protein	--	--	3.44	0.0014	--	--
	CRE23687		Threonine dehydratase catabolic-like protein	-3.43	>0.0001	-3.20	>0.0001	-1.45	0.0105
	CRE00199	*ptr-4*	Patched-related family	--	--	3.43	0.0181	--	--
	CRE08830			3.42	>0.0001	--	--	--	--
	CRE28521			2.80	>0.0001	--	--	3.42	0.0154
	CRE08701		Serpentine receptor, class Z	-3.42	>0.0001	-3.06	>0.0001	--	--
	CRE01255			3.41	0.0004	--	--	--	--
	CRE01261			3.41	0.0001	--	--	--	--

Table S2.2 (continued).

HSP	Gene ID	Gene Name	Gene Description	Ancestor Log_2 FC	Ancestor FDR	Control Log_2 FC	Control FDR	Heat Log_2 FC	Heat FDR
◆	CRE04918	*daf-21*	HSPC (HSP90)	3.40	>0.0001	n.s.	n.s.	2.52	0.0008
	CRE30954			-3.40	>0.0001	-3.15	>0.0001	-2.37	>0.0001
	CRE08915			-2.95	0.0336	-3.40	>0.0001	--	--
	CRE01263			3.39	0.0009	--	--	--	--
	CRE06458			3.38	>0.0001	2.74	>0.0001	2.47	>0.0001
	CRE26886		Myosin light chain kinase	3.38	>0.0001	3.05	0.0103	n.s.	n.s.
	CRE08273			3.37	>0.0001	--	--	n.s.	n.s.
	CRE17339			-1.49	0.0449	-2.97	0.0001	-3.37	>0.0001
◆	CRE00198	*hsp-3*	HSP70 protein	3.36	>0.0001	1.92	0.0384	2.44	>0.0001
	CRE00152	*clec-266*	C-type lectin	3.36	>0.0001	n.s.	n.s.	3.11	0.0478
	CRE09072			-3.35	>0.0001	-3.30	>0.0001	-1.93	0.0396
◆	CRE26138		HSP70 protein	3.34	>0.0001	n.s.	n.s.	2.15	0.0302
	CRE11953			1.74	>0.0001	1.75	0.0023	3.32	0.0003
	CRE10900	*fmo-2*	Flavin monooxygenase	-3.31	>0.0001	-2.79	>0.0001	-1.41	>0.0001
	CRE10649		Zinc finger protein	2.33	>0.0001	3.29	0.0197	2.04	0.0441
	CRE09656		AMP deaminase	1.38	0.0156	--	--	3.27	0.0006
	CRE13074	*str-96*	7-transmembrane receptor	--	--	--	--	-3.26	0.0003
	CRE06193		Ribonucleotide reductase	-3.26	0.0304	-3.19	>0.0001	-2.10	0.0016
	CRE24981			1.09	0.0011	3.25	0.0058	n.s.	n.s.
	CRE09429			3.24	>0.0001	2.39	>0.0001	2.37	0.0002
	CRE09190	*cyp-34A5*	Cytochrome p450 family protein	--	--	-3.24	0.0022	--	--
	CRE06003	*fat-5*	Fatty acid desaturase	2.14	>0.0001	3.23	0.0012	1.23	>0.0001
	CRE05507		Aspartyl protease	n.s.	n.s.	n.s.	n.s.	-3.22	>0.0001
	CRE30887			3.22	>0.0001	1.30	0.0132	n.s.	n.s.

Table S2.2 (continued).

HSP	Gene ID	Gene Name	Gene Description	Ancestor Log_2 FC	Ancestor FDR	Control Log_2 FC	Control FDR	Heat Log_2 FC	Heat FDR
	CRE08991			3.21	>0.0001	n.s.	n.s.	2.34	>0.0001
	CRE01717			2.19	>0.0001	2.46	0.0057	3.21	0.0009
	CRE29186			-3.20	>0.0001	-2.24	>0.0001	-1.85	0.0134
	CRE15658			-3.20	>0.0001	-1.20	0.0037	--	--
	CRE01661			-2.99	>0.0001	-2.59	>0.0001	-3.20	0.0005
	CRE12737			1.25	0.0036	3.20	0.0001	n.s.	n.s.
	CRE17300	*cyp-23A1*	Cytochrome p450 family protein	2.97	>0.0001	2.59	0.0451	3.20	0.0013
	CRE13167			--	--	--	--	-3.19	>0.0001
	CRE01678			--	--	-3.19	>0.0001	--	--
	CRE19390		Ani s 1 allergen	-2.53	>0.0001	-3.18	>0.0001	-1.88	0.0028
	CRE05593			-3.01	>0.0001	-2.89	>0.0001	-3.18	>0.0001
	CRE24499			3.16	>0.0001	2.17	0.0014	2.21	0.0035
	CRE09281			-3.16	>0.0001	-2.57	>0.0001	-2.49	>0.0001
	CRE16493			-3.04	>0.0001	-2.40	>0.0001	-3.15	>0.0001
	CRE04919	*gasr-8*	Growth-arrest-specific-protein 8	3.15	>0.0001	n.s.	n.s.	n.s.	n.s.
	CRE12600			3.14	>0.0001	1.99	0.0020	n.s.	n.s.
	CRE28556			3.13	>0.0001	n.s.	n.s.	n.s.	n.s.
	CRE01305			3.13	>0.0001	--	--	--	--
	CRE10806		Cysteine-rich intestinal protein-related	-1.83	>0.0001	-1.60	0.0178	-3.12	>0.0001
	CRE17641			3.11	>0.0001	--	--	--	--
	CRE12674			-1.67	0.0356	n.s.	n.s.	-3.11	>0.0001
	CRE16136			-1.04	0.0486	-3.11	>0.0001	n.s.	n.s.
	CRE06373		Serpentine receptor, class I	3.10	>0.0001	--	--	--	--
	CRE03436		C-type lectin	2.54	>0.0001	3.09	>0.0001	1.80	0.0083

Table S2.2 (continued).

HSP	Gene ID	Gene Name	Gene Description	Ancestor Log_2 FC	Ancestor FDR	Control Log_2 FC	Control FDR	Heat Log_2 FC	Heat FDR
	CRE19316			--	--	3.09	0.0015	--	--
	CRE20157		Ribonucleotide reductase	--	--	-3.08	>0.0001	-1.73	0.0289
	CRE03421	*sru-7*	Serpentine receptor, class U	-3.08	>0.0001	n.s.	n.s.	--	--
	CRE01311			3.07	>0.0001	--	--	--	--
◆	CRE26406		HSP70 protein	3.07	>0.0001	2.33	0.0044	2.05	0.0075
	CRE15096		Cytochrome p450 family protein	-1.91	0.0007	-3.06	>0.0001	n.s.	n.s.
	CRE08150			3.06	>0.0001	n.s.	n.s.	n.s.	n.s.
	CRE10669			-3.05	>0.0001	-1.96	>0.0001	-3.05	>0.0001
	CRE02873			3.04	>0.0001	1.31	0.0283	2.90	0.0037
	CRE31451			3.03	>0.0001	--	--	--	--

[a]Model did not converge

Table S2.3 List of genes differentially expressed over evolutionary time (20°C environment).

	GeneID	Gene Name	Gene Description	FC: Heat/ Ancestor	FDR (Heat)	FC: Ctrl/ Ancestor	FDR (Control)
DE in Heat-Selected Line	CRE23514			8.49	0.0013	--	--
	CRE24278		C-type lectin	7.78	0.0002	--	--
	CRE13167		CUB-like domain	4.32	0.0316	--	--
	CRE16108		CUB-like domain	4.03	0.0156	--	--
	CRE10142		CUB-like domain	3.19	0.0395	--	--
	CRE20697		UDP-glucuronosyl transferase	3.12	0.0316	n.s.	n.s.

Table S2.3 (continued).

	GeneID	Gene Name	Gene Description	FC: Heat/ Ancestor	FDR (Heat)	FC: Ctrl/ Ancestor	FDR (Control)
DE in Heat-Selected Line	CRE18453		Molybdenum cofactor sulfurase	2.84	0.0000	n.s.	n.s.
	CRE30538		Helitron helicase-like domain	-2.79	0.0037	n.s.	n.s.
	CRE15658			-2.62	0.0012	n.s.	n.s.
	CRE10586		Short-chain dehydrogenase	2.59	0.0366	n.s.	n.s.
	CRE02477		Short-chain dehydrogenase	2.55	0.0137	n.s.	n.s.
	CRE09885			2.49	0.0034	n.s.	n.s.
	CRE24807			-2.42	0.0000	n.s.	n.s.
	CRE12164	*glb-1*	Globin-related protein	2.06	0.0000	n.s.	n.s.
	CRE28721	*lact-6*	Beta-lactamase related protein	1.94	0.0357	n.s.	n.s.
	CRE09484		C-type lectin	1.83	0.0013	n.s.	n.s.
	CRE09800		Zinc finger protein	1.79	0.0041	n.s.	n.s.
	CRE25687			-1.79	0.0026	n.s.	n.s.
	CRE11848		Glutathione S-transferase	1.73	0.0050	n.s.	n.s.
	CRE10310		SCP-like extracellular protein	-1.67	0.0318	n.s.	n.s.
	CRE18035		aminoglycoside phosphotransferase	-1.66	0.0019	n.s.	n.s.
	CRE30855		NADH oxidase	1.63	0.0006	n.s.	n.s.
	CRE10033			-1.61	0.0437	n.s.	n.s.
	CRE22864	*lipl-2*	Lipase-like protein	-1.58	0.0051	n.s.	n.s.
	CRE12163		Cytochrome b5	1.58	0.0169	n.s.	n.s.
	CRE00804	*amt-1*	Ammonium transporter homolog	-1.50	0.0312	n.s.	n.s.
	CRE09420			1.37	0.0342	n.s.	n.s.
	CRE18856			-1.30	0.0211	n.s.	n.s.
	CRE13371	*nit-1*	Nitrilase	1.20	0.0301	n.s.	n.s.

Table S2.3 (continued).

	GeneID	Gene Name	Gene Description	FC: Heat/ Ancestor	FDR (Heat)	FC: Ctrl/ Ancestor	FDR (Control)
	CRE23798		Integrase	n.s.	n.s.	-6.11	0.0000
	CRE12053		DDE endonuclease	--	--	3.29	0.0001
	CRE16136		Methyltransferase	n.s.	n.s.	3.08	0.0479
	CRE06358			--	--	2.90	0.0014
	CRE07402		Integrase	n.s.	n.s.	-2.73	0.0000
	CRE24828			n.s.	n.s.	2.33	0.0104
	CRE19091			--	--	2.27	0.0003
	CRE00568			n.s.	n.s.	-2.26	0.0034
	CRE30234			n.s.	n.s.	2.09	0.0012
	CRE12487			n.s.	n.s.	-2.04	0.0072
	CRE08705		Glutathione S-transferase	n.s.	n.s.	2.04	0.0012
DE in Control Line	CRE10692		Cytochrome p450 family protein	n.s.	n.s.	1.97	0.0000
	CRE18381			n.s.	n.s.	-1.94	0.0179
	CRE09193	*cyp-34A10*	Cytochrome p450 family protein	n.s.	n.s.	-1.93	0.0104
	CRE19314		Protein kinase	n.s.	n.s.	-1.89	0.0151
	CRE28667			--	--	1.88	0.0059
	CRE23839			n.s.	n.s.	1.83	0.0012
	CRE25599		Lipase-like protein	--	--	1.78	0.0381
	CRE15096		Cytochrome p450 family protein	n.s.	n.s.	1.72	0.0214
	CRE11440		Integrase	n.s.	n.s.	1.57	0.0003
	CRE25834		Integrase	n.s.	n.s.	1.50	0.0434
	CRE06192			n.s.	n.s.	1.50	0.0034
	CRE28296		Calponin	n.s.	n.s.	-1.46	0.0071
	CRE27735		Prion-like (Q/N-rich) domain protein	n.s.	n.s.	1.38	0.0068

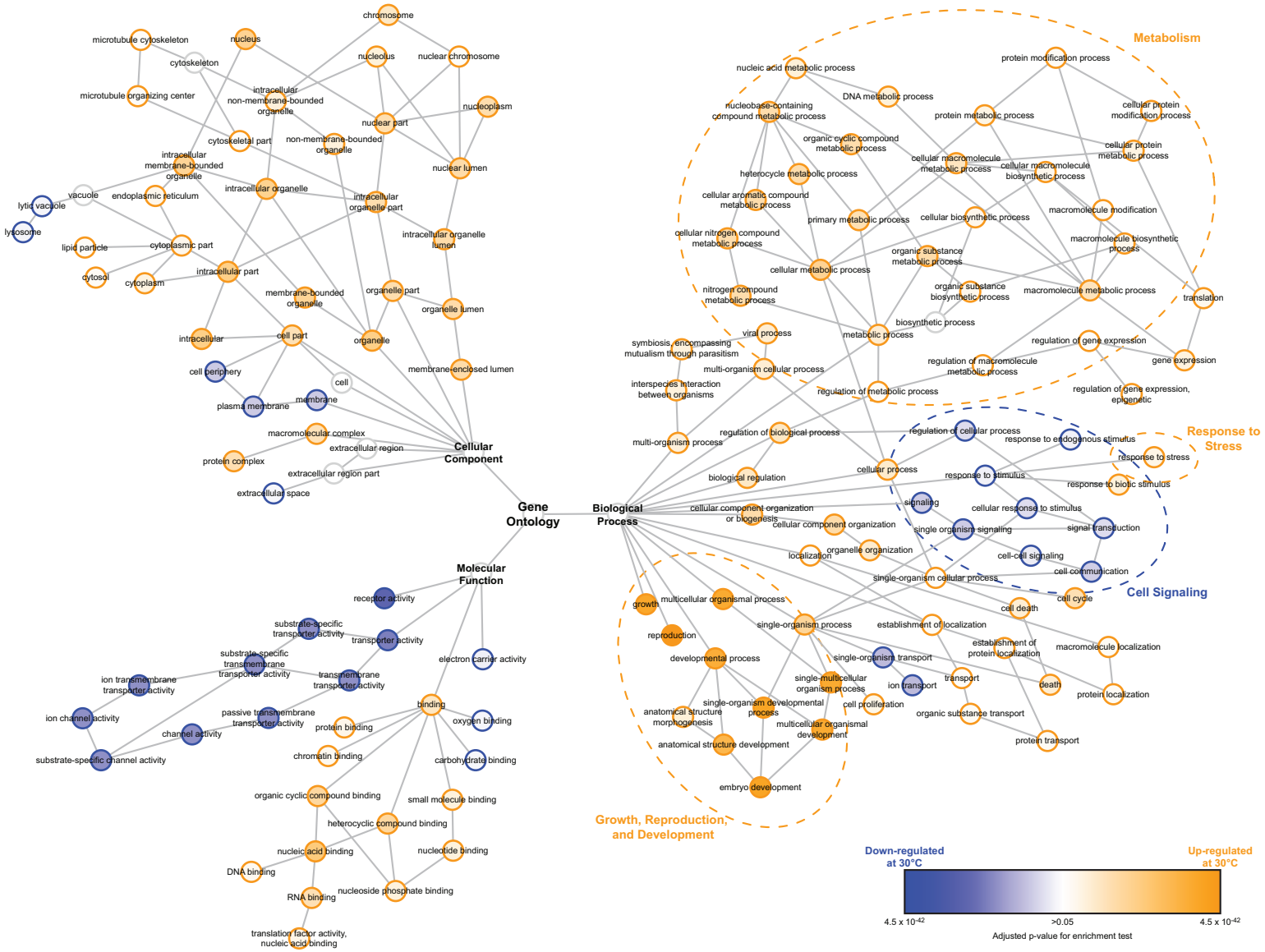
Table S2.3 (continued).

	GeneID	Gene Name	Gene Description	FC: Heat/ Ancestor	FDR (Heat)	FC: Ctrl/ Ancestor	FDR (Control)
DE in Control Line	CRE17915		Acyl-CoA thioesterase	n.s.	n.s.	-1.36	0.0028
	CRE20531			n.s.	n.s.	1.32	0.0242
	CRE29200			n.s.	n.s.	-1.32	0.0063
	CRE09559		5'-nucleotidase	n.s.	n.s.	1.29	0.0095
	CRE29277		SCP-like extracellular protein	n.s.	n.s.	-1.28	0.0154
	CRE10669			n.s.	n.s.	-1.26	0.0120
	CRE14147			n.s.	n.s.	1.26	0.0298
	CRE06222		Integrase	n.s.	n.s.	1.24	0.0124
	CRE09560			n.s.	n.s.	1.16	0.0179
	CRE26779		Integrase	n.s.	n.s.	-1.13	0.0428
	CRE24146			n.s.	n.s.	1.13	0.0104
	CRE29479			n.s.	n.s.	1.10	0.0154
	CRE14067			n.s.	n.s.	-1.10	0.0283
	CRE08770		Glutathione S-transferase	n.s.	n.s.	1.01	0.0136
	CRE14333		5-oxoprolinase	n.s.	n.s.	0.99	0.0198
DE in Both Selected Lines	CRE14636	*clec-140*	C-type lectin	6.10	0.0006	1.95	0.0000
	CRE14503		C-type lectin	5.72	0.0008	1.85	0.0000
	CRE01641	*chil-8*	Chitinase	-3.41	0.0421	-5.27	0.0001
	CRE21731			-2.84	0.0037	1.68	0.0000
	CRE05881			-1.75	0.0328	-2.67	0.0000
	CRE02474		Dehydrogenase	2.58	0.0143	1.72	0.0000
	CRE13743			2.52	0.0000	2.33	0.0000
	CRE21610		C-type lectin	-2.42	0.0136	-1.55	0.0150
	CRE09194	*nlp-34*	Neuropeptide-like protein	-2.38	0.0003	-1.74	0.0059

Table S2.3 (continued).

	GeneID	Gene Name	Gene Description	FC: Heat/ Ancestor	FDR (Heat)	FC: Ctrl/ Ancestor	FDR (Control)
DE in Both Selected Lines	CRE13741		Integrase	2.36	0.0000	2.09	0.0000
	CRE09886			2.27	0.0006	1.73	0.0179
	CRE13742			2.26	0.0026	1.95	0.0006
	CRE13476	*thn-5*	Thaumatin-like protein	-2.19	0.0000	-2.02	0.0000
	CRE29705		Integrase	2.10	0.0000	1.95	0.0000
	CRE07706			2.07	0.0000	1.40	0.0014
	CRE25992		NADH oxidase	2.03	0.0019	1.13	0.0059
	CRE14226		UDP-glucuronosyl transferase	1.86	0.0001	1.44	0.0005
	CRE13701			1.80	0.0006	1.40	0.0136
	CRE29704			1.77	0.0036	1.66	0.0005
	CRE29212	*gst-1*	Glutathione S-transferase	1.75	0.0001	1.33	0.0012
	CRE29481			1.56	0.0026	1.42	0.0001
	CRE07709			1.52	0.0019	1.18	0.0028

Figure S2.1 (next page) Gene ontology enrichment network for genes differentially expressed by environment. Shown are GOSlim term with significant enrichment (FDR < 5%) for the upregulated genes (orange) or downregulated genes (blue) in the ancestral population. The intensity of shading is proportional to the significance value from the Fisher's Exact Test for the ontology term.


Gene Ontology
Cellular Component
chromosome
microtubule cytoskeleton
nucleus
cytoskeleton
nucleolus
nuclear chromosome
microtubule organizing center
intracellular non-membrane-bounded organelle
nucleoplasm
nuclear part
cytoskeletal part
non-membrane-bounded organelle
intracellular membrane-bounded organelle
nuclear lumen
intracellular organelle
intracellular organelle part
vacuole
endoplasmic reticulum
lytic vacuole
lysosome
intracellular organelle lumen
lipid particle
cytoplasmic part
cytosol
cytoplasm
intracellular part
membrane-bounded organelle
organelle part
organelle lumen
intracellular
cell part
organelle
membrane-enclosed lumen
cell periphery
cell
plasma membrane
membrane
macromolecular complex
extracellular region
protein complex
extracellular region part
extracellular space
Molecular Function
receptor activity
substrate-specific transporter activity
transporter activity
electron carrier activity
substrate-specific transmembrane transporter activity
ion transmembrane transporter activity
transmembrane transporter activity
binding
ion channel activity
passive transmembrane transporter activity
oxygen binding
protein binding
channel activity
substrate-specific channel activity
chromatin binding
carbohydrate binding
organic cyclic compound binding
small molecule binding
heterocyclic compound binding
nucleic acid binding
nucleotide binding
DNA binding
RNA binding
nucleoside phosphate binding
translation factor activity, nucleic acid binding
Biological Process
Metabolism
nucleic acid metabolic process
protein modification process
DNA metabolic process
nucleobase-containing compound metabolic process
protein metabolic process
cellular protein modification process
organic cyclic compound metabolic process
cellular protein metabolic process
cellular macromolecule metabolic process
cellular macromolecule biosynthetic process
heterocycle metabolic process
cellular aromatic compound metabolic process
primary metabolic process
cellular biosynthetic process
macromolecule modification
cellular nitrogen compound metabolic process
macromolecule biosynthetic process
organic substance metabolic process
cellular metabolic process
organic substance biosynthetic process
macromolecule metabolic process
translation
nitrogen compound metabolic process
biosynthetic process
viral process
metabolic process
regulation of gene expression
symbiosis, encompassing mutualism through parasitism
regulation of macromolecule metabolic process
gene expression
multi-organism cellular process
interspecies interaction between organisms
regulation of metabolic process
regulation of gene expression, epigenetic
regulation of biological process
regulation of cellular process
response to endogenous stimulus
Response to Stress
response to stress
multi-organism process
biological regulation
cellular process
response to stimulus
response to biotic stimulus
signaling
cellular response to stimulus
cellular component organization or biogenesis
Cellular component organization
single organism signaling
signal transduction
organelle organization
localization
cell-cell signaling
cell communication
single-organism cellular process
Cell Signaling
growth
multicellular organismal process
cell death
cell cycle
single-organism process
establishment of localization
reproduction
establishment of protein localization
macromolecule localization
developmental process
single-organism transport
single-multicellular organism process
ion transport
transport
death
protein localization
single-organism developmental process
cell proliferation
organic substance transport
anatomical structure morphogenesis
multicellular organismal development
protein transport
anatomical structure development
embryo development
Growth, Reproduction, and Development
Down-regulated at 30°C
Up-regulated at 30°C
4.5 x 10-42
>0.05
4.5 x 10-42
Adjusted p-value for enrichment test

APPENDIX B

SUPPORTING INFORMATION FOR CHAPTER III

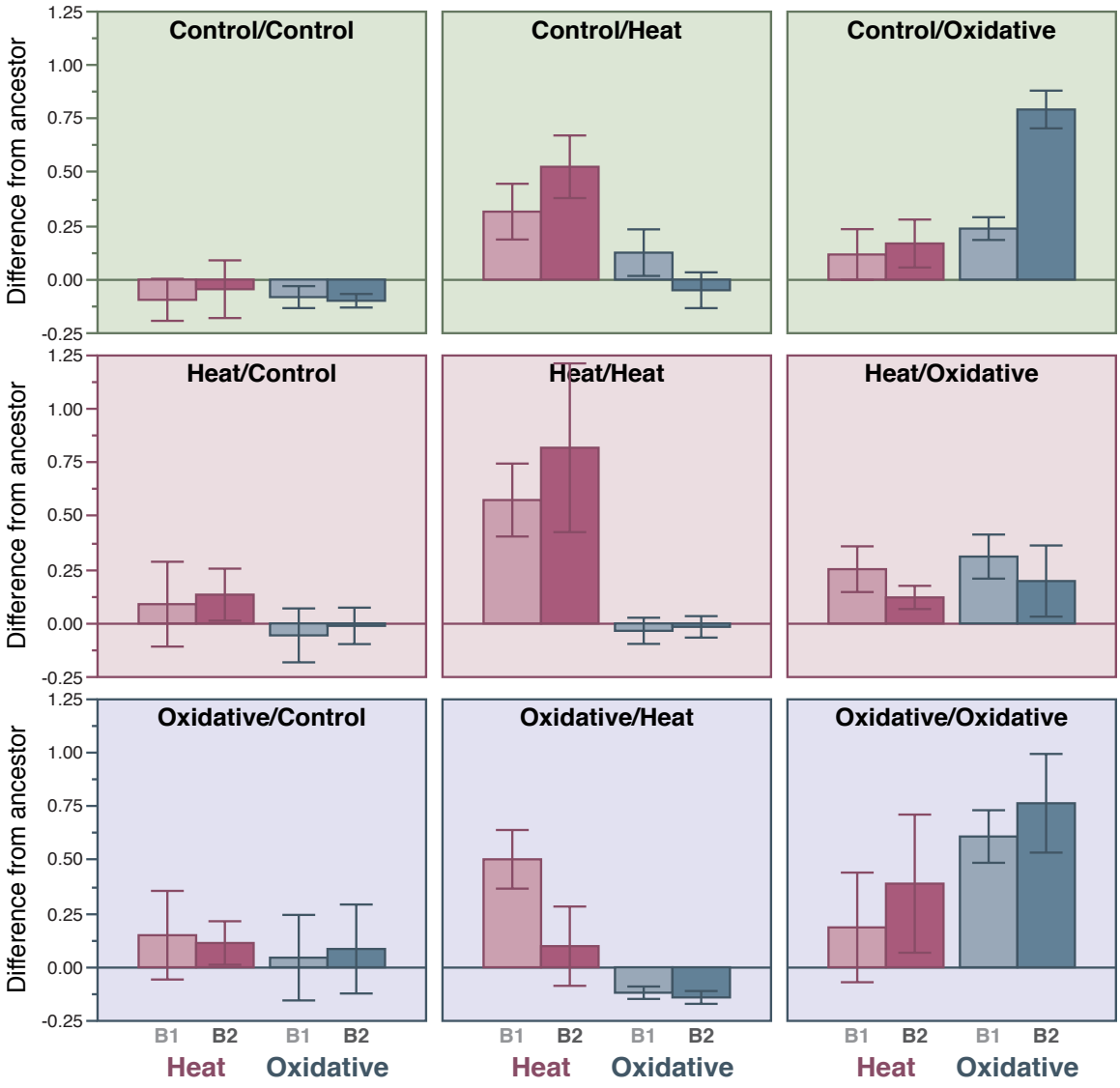
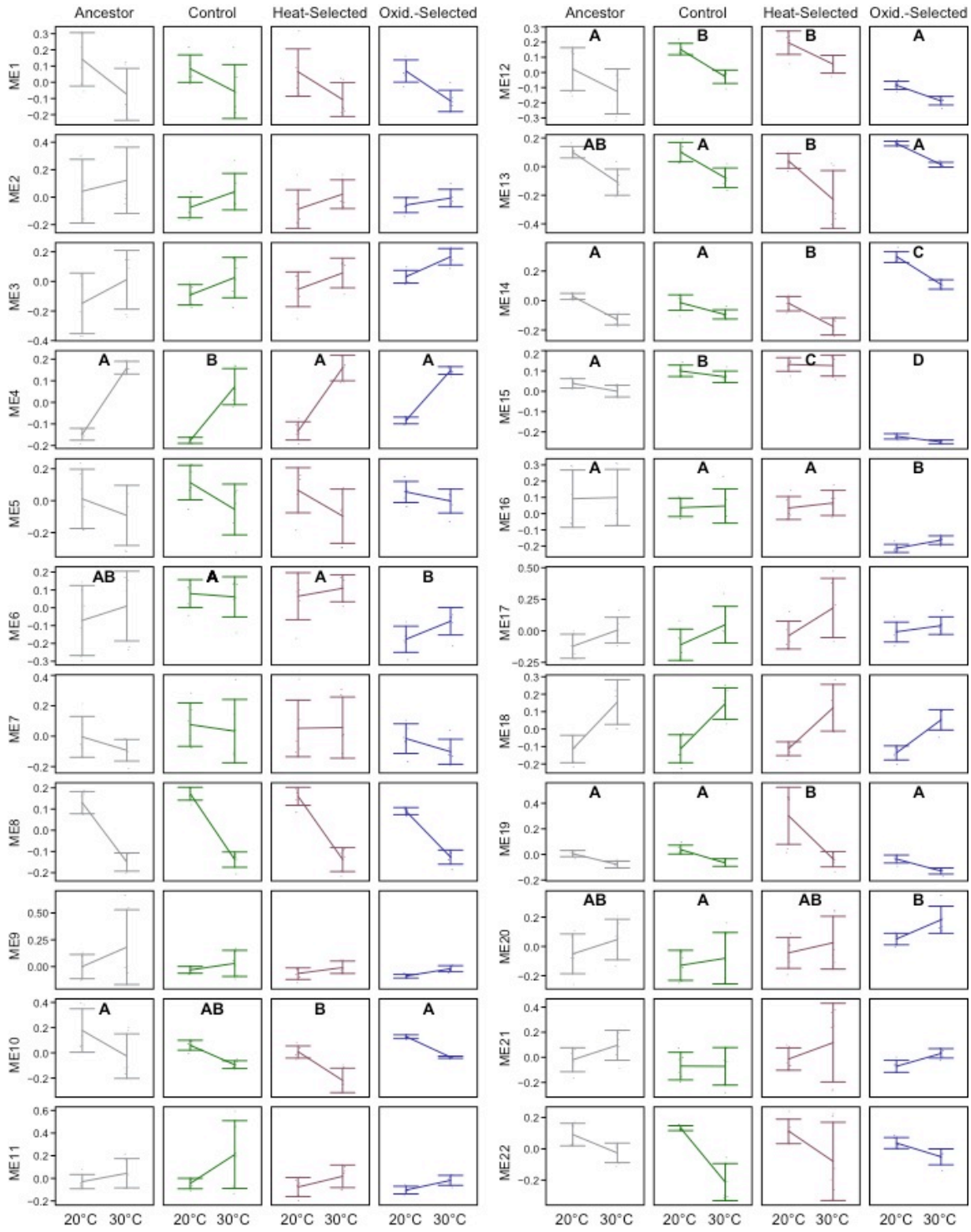


Figure S3.1. Direct and correlated responses to selection between traits in experimentally evolved lines for each evolutionary replicate. Responses in both heat shock resistance (red) and oxidative shock resistance (blue) are shown. Data are mean difference from the ancestor, measured at 20°C in all lines (±2 SEM).

APPENDIX C

SUPPORTING INFORMATION FOR CHAPTER IV

Figure S4.1. (next page) Reaction norms showing average eigengene expression in each evolved population for all modules identified in weighted gene coexpression network. For modules that significantly differed among experimentally evolved populations, we tested for pairwise differences among lines using a Tukey HSD test. Letters indicate homogenous groups from the Tukey test for those modules. Average eigengene expression (±2 S.E.M.) for each module is plotted.


Ancestor
Control
Heat-Selected
Oxid.-Selected
ME1
ME2
ME3
ME4
ME5
ME6
ME7
ME8
ME9
ME10
ME11
ME12
ME13
ME14
ME15
ME16
ME17
ME18
ME19
ME20
ME21
ME22
20°C 30°C

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