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Environmentally induced changes in correlated responses to selection reveal variable pleiotropy across a complex genetic network

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Selection in novel environments can lead to a coordinated evolutionary response across a suite of characters. Environmental conditions can also potentially induce changes in the genetic architecture of complex traits, which in turn could alter the pattern of the multivariate response to selection. We describe a factorial selection experiment using the nematode *Caenorhabditis remanei* in which two different stress-related phenotypes (heat and oxidative stress resistance) were selected under three different environmental conditions. The pattern of covariation in the evolutionary response between phenotypes or across environments differed depending on the environment in which selection occurred, including asymmetrical responses to selection in some cases. These results indicate that variation in pleiotropy across the stress response network is highly sensitive to the external environment. Our findings highlight the complexity of the interaction between genes and environment that influences the ability of organisms to acclimate to novel environments. They also make clear the need to identify the underlying genetic basis of genetic correlations in order understand how patterns of pleiotropy are distributed across complex genetic networks.

KEY WORDS: **Experimental evolution, genetic architecture, G-matrix, natural selection, phenotypic plasticity, stress resistance.**

All phenotypic evolution is dependent on environmental context for at least two reasons. First, the fundamental principle of evolution by natural selection is that the specific circumstances of the environment cause 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 environmental attributes that influence the manifestation of the genetic information in the phenotype. The distinction and interplay between these two roles of the environment is well illustrated by 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). The environment therefore both induces phenotypes and sorts among them.

Over the last four decades, evolutionary quantitative genetics has developed a strong conceptual framework for untangling

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the patterns of natural selection and genetic inheritance for suites of interacting complex traits and for understanding the various roles of the environment in inducing and filtering phenotypes. 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, which is now self-evident in most studies of functional genomics (Westerhoff and Palsson 2004; Bolouri 2014), begs the question of whether this historic approach remains sufficient for understanding the evolution of complex traits. Alternatively, variation in the nature and structure of pleiotropy may have an important influence on evolutionary outcomes that is not fully captured under current approaches. Specifically with respect to our question of the dual role of the environment: how do pleiotropic relationships change under different environmental conditions, and how does this induced pleiotropic variation affect the outcome of filtering by the environment via the action of natural selection?

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:

$$\Delta\bar{\mathbf{z}} = \mathbf{G}\beta \tag{1}$$

where $\Delta\bar{\mathbf{z}}$ is the vector of average phenotypic responses to selection, $\mathbf{G}$ 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 (phenotypic plasticity, e.g., body size at high and low temperatures; Via and Lande 1985; Via 1987). In either case, genetic correlations can produce correlated responses to selection (Lande 1979; Via and Lande 1985). 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 mathematical construct of the G-matrix is by definition symmetric, the naïve expectation is that genetic correlations will result in symmetry in the correlated responses to selection as well (Lande 1979). However, in practice symmetric responses are rarely observed 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). Second, selection can potentially alter the distribution of alleles whose pleiotropic effects underlie the structure of the G-matrix itself (Carrière and Roff 1995; Shaw et al. 1995; Agrawal et al. 2001; Scoville et al. 2009; Stinchcombe et al. 2009; Delph et al. 2011). Third, 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 can also contribute to the unpredictability of the evolutionary response. However, the effect of the environment on correlated responses to selection has very rarely been tested (but see Baker and Cockrem 1970; Fry 2001). Effects that are strongly environmentally dependent will appear to be "cryptic" when observed in another environment. This can hold for pleiotropic effects leading to genetic covariance as well as genetic variance, which is the usual focus of such studies (Paaby and Rockman 2014).

The G-matrix itself is a composite of myriad 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

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

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 variance and covariance, but strong heterogeneity in these effects has the potential to generate evolutionary responses beyond those predicted by $\mathbf{G}$ alone (Barton and Turelli 1987). We were only beginning to glimpse the molecular underpinnings of pleiotropy when this theory was 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

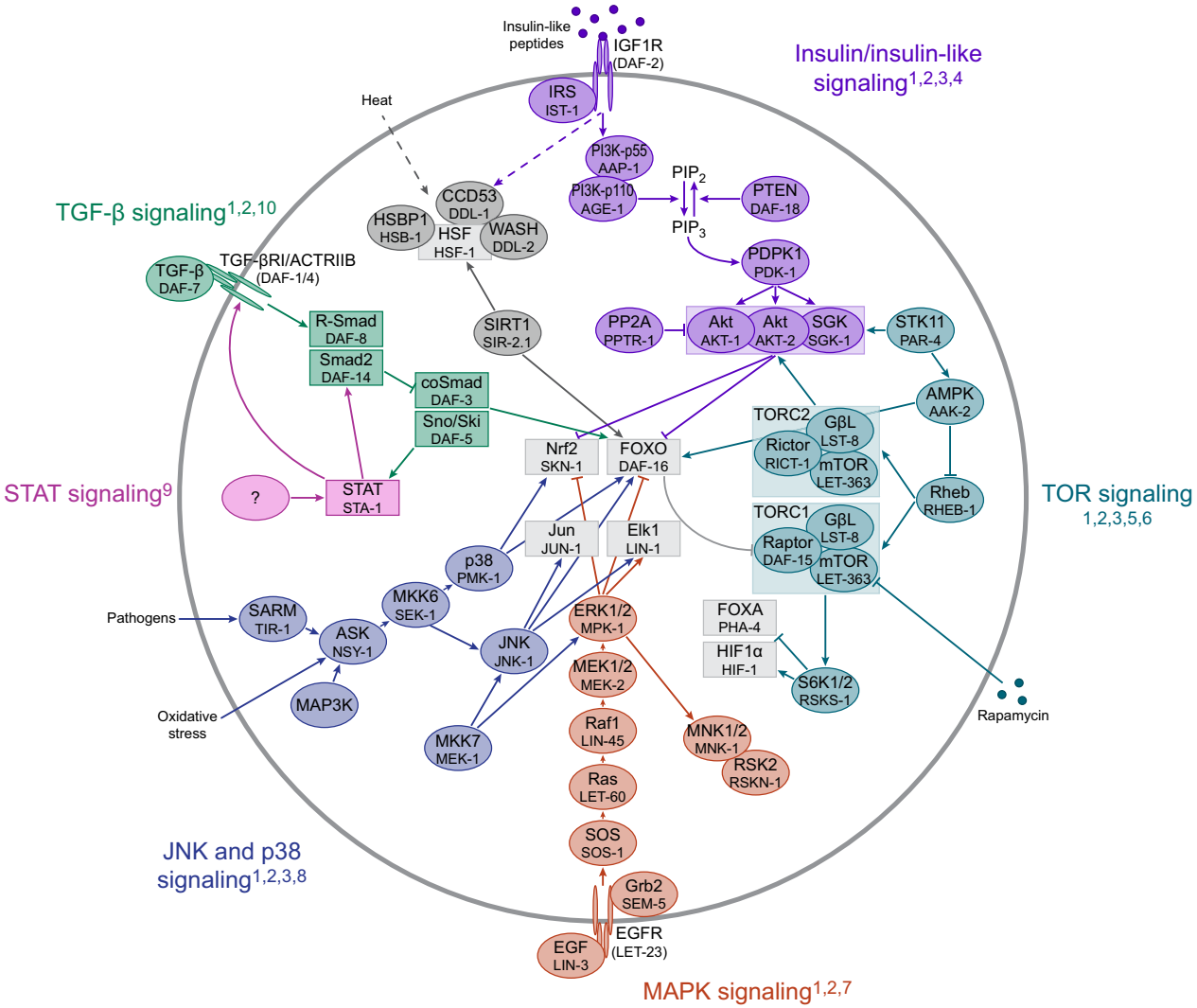

Figure 1. Key components of the stress response signaling network in *C. elegans*. This diagram depicts a summary of our current knowledge of the molecular interactions among six signaling pathways known to play an important role in stress response. These interacting pathways regulate cellular responses induced by specific (e.g., insulin) or general (e.g., heat) exogenous signals, usually via the activation of receptor (e.g., IGF1R), which in turn activates a series of kinases and other proteins (ovals) that maintain specific signaling states within the cell. Ultimately, these signals change either coactivator binding or the protein conformation of transcription factors (rectangles; e.g., FOXO), which are responsible for downstream responses via the transcriptional activation of large suites of genes. The overall pattern of interwoven signaling across the network leads to a strong prediction of pleiotropy within this system, which is usually verified by the phenotypic outcomes of mutations in key pathway components. Protein families or human protein names are given first, with the *C. elegans* gene listed below those names. References (indicated by superscripts on pathway labels): [1]Kanehisa and Goto (2000); [2]Kanehisa et al. (2014); [3]Ivanov et al. (2013); [4]Murphy and Hu (2013); [5]Beauchamp and Plantanias (2013); [6]Honjoh et al. (2009); [7]Sundaram (2013); [8]Inoue (2005); [9]Wang and Levy (2006); [10]Savage-Dunn (2005).

environmental stress (Eijkelenboom and Burgering 2013; Rodriguez et al. 2013). For example, in the nematode *Caenorhabditis elegans*, many of the proteins that respond to stressors such as heat, oxidative damage, or starvation are known, and their molecular interactions within the stress response network have been characterized in detail. In most instances, the interactions of the stress response components display strong pleiotropies with one another (Fig. 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; Åkerfelt et al. 2010; Baird et al. 2014), and SKN-1, whose regulatory targets are important for resistance to both oxidative and starvation stress (An and Blackwell 2003; Paek et al. 2012). The IIS pathway clearly plays a central role in determining the response to a variety of stresses, mediated through a core set of regulatory hubs, such as DAF-16, that simultaneously affect resistance to a diverse array of cellular stressors. Each of these systems is also highly conserved across animals, and in some cases across eukaryotes (Nikoletopoulou et al. 2014), suggesting that the relationships among functional systems are highly robust. 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. Yet, nearly every one of these genes have been identified using mutant screens or targeted knock-outs that would be expected to have unusually large effects. We know very little about levels of pleiotropy expressed by alleles segregating within natural populations (Jovelin et al. 2014; Paaby et al. 2014).

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 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 in the nematode *Caenorhabditis remanei* 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. 2014a). 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. 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, which is well below the upper thermal limit for this species, yet still stressful in terms of reproductive output (Sikkink et al. 2014a). To apply a chronic oxidative stress, 160 μM paraquat (methyl viologen), which generates superoxide free radicals, 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.

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. 2, Table 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 this period of early development, 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, 160 μM paraquat was added to the buffer for lines selected 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). Within a given selection line, synchronized L1s were then subject to one of the acute selection regimes described below.

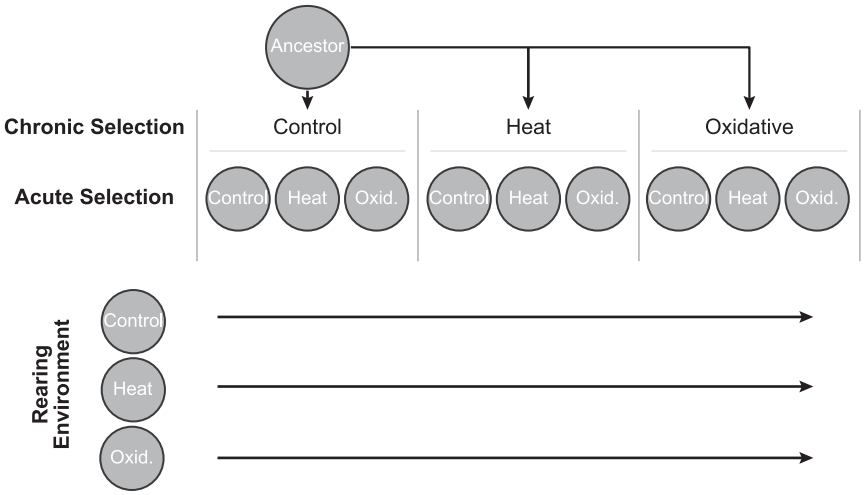

Figure 2. Schematic representation of the experimental evolution design. Lines derived from a single outbred ancestral population recently isolated from the wild were raised throughout selection (~30–40 generations total) in one of three chronic selective environments: standard lab conditions (control), high heat (30°C), and an oxidative environment (160 μM paraquat). Within each environmental treatment, selection lines were generated by selecting individuals at random (Control), or by selecting survivors of an acute heat shock (~37°C) or oxidative shock (1 mM H_2O_2). The response to selection was determined by first rearing individuals from each of these nine selection environments (plus the ancestor) in each of the three different environmental treatments (control, high heat, high oxidative stress), and then determining individual resistance to either heat or oxidative shock. Thus, there were a total of 27 different combinations of selection treatments and rearing environments (plus the ancestors), each tested in replicate for their influence on two different resistance phenotypes.

Table 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 + 160 μM paraquat	Control	—	2
Oxid.	20°C, NGM + 160 μM paraquat	Heat	36.4–36.8°C	2
Oxid.	20°C, NGM + 160 μM 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.

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. (2014a, b).

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 1 mM 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, 1 mM 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 control environment are the same as those analyzed in Sikkink et al. (2014a, b).

For each selection line, we propagated two independently evolved replicates (Table 1), each derived from independently thawed ancestral stocks, except for the lab-adapted lines from the chronic control environment in which four replicate populations were maintained. 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 three times during selection, while this occurred five 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 (160 μM 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. Within this context, then, we define phenotypic plasticity as the change in phenotypic outcome (response to acute heat or oxidative stress) averaged over the whole selected population. Because each population was selected in only one environment, any change in phenotypic plasticity is therefore perforce an indirect response to selection.

After being raised in a specific rearing environment, worms in L1 diapause suspended in liquid buffer were then exposed to either an acute heat stress or an acute oxidative stress. Survival in either of these two conditions thus constituted the measured phenotypes for each line. 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 1 mM 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 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 in 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. 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 rearing environment were tested in separate models, with the ancestor included each time. In this case, we defined the direct response to selection as the response when the rearing environment matched the chronic environment experienced during evolution. A correlated response to selection could occur in either of the two other rearing environments.

To estimate the power to reject the null hypothesis of no evolutionary change given our data, we used a simulation approach. For each simulated dataset, we created the same number of replicates for each selection line as existed in the original data. Each simulated replicate was populated with a total number of individuals drawn from a negative binomial distribution, with mean and dispersion index matching the observed values in the experiment. The number of survivors for each replicate was drawn from a beta-binomial distribution, with a size (n) equal to the simulated total population. Here, the probability of success (p) in the binomial is itself a beta distribution. The shape parameters of the beta distribution were selected such that the mean and variance of the distribution for each acute selection line was appropriate for the effect size being examined. For each comparison, 1000 simulated datasets were generated and fit with the generalized linear model described above. Power was taken to be the proportion of simulations in which the tested effect was significant ($P < 0.05$).

Results

DIRECT RESPONSE TO SELECTION FOR STRESS RESISTANCE PHENOTYPES

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, Table 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. 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. 2014a, b).

Similarly, acute oxidative selection increased resistance to hydrogen peroxide stress (Fig. 3, Table 2). This direct response to selection occurred in 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. (The individual responses for each replicate are provided in Supplementary Fig. S1).

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. 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, Table 2; 80% power to detect a response of ~0.15). A similar lack of correlated responses was observed when selection occurred in the chronic heat environment (80% power = ~0.15). 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, because the response is essentially identical to that observed in the control treatment.

A very different pattern was observed under the chronic oxidative selection environment generated with paraquat, which is a related, but likely distinct, source of oxidative stress compared to hydrogen peroxide (Schaar et al. 2015). In the acute heat-selected line evolved under these conditions, there was a significant negative correlated response in oxidative stress resistance (Fig. 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 reversed in sign, leading to increases in both heat and oxidative stress resistance under acute oxidative selection (Fig. 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 (Supplementary Fig. S1; also note that population sizes after selection were always well in excess of 1000 individuals). Overall, the realized genetic covariance between these phenotypes is highly contingent on the environment in which selection occurs and can

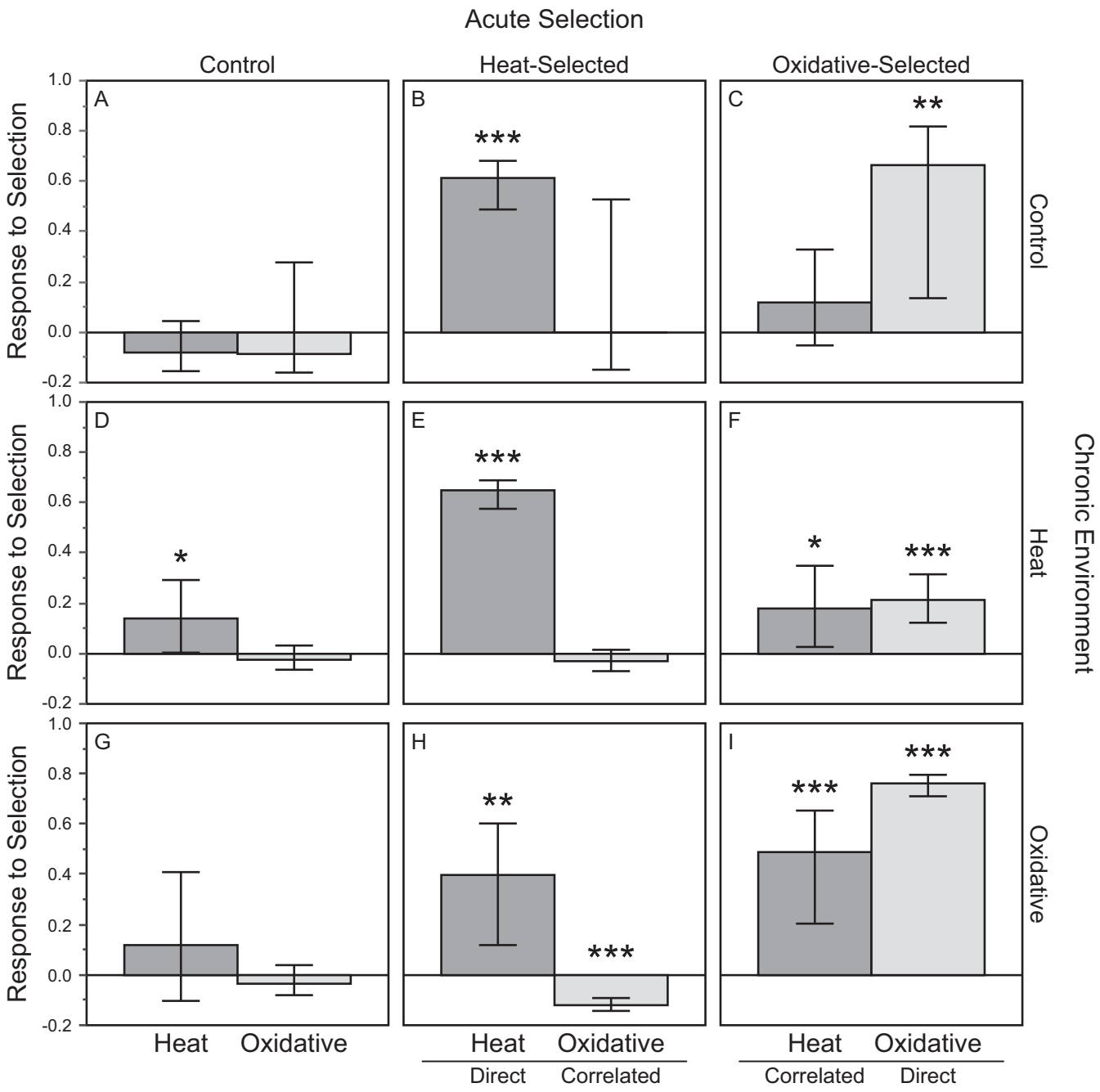

Figure 3. **Direct and correlated responses to selection between traits in experimentally evolved lines. Response to selection is defined as the mean difference in survival of the selected line relative to the ancestral population. Responses in both heat shock resistance (dark gray) and oxidative shock resistance (light gray) 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 *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.**

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, individuals raised under a mild heat stress (30°C) prior to acute stress exhibited an induction of a plastic response that protected against both heat and oxidative stress,

Table 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	Regression coefficient (SE)	*z* value	*P*-value	Regression coefficient (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 $P < 0.05$.**

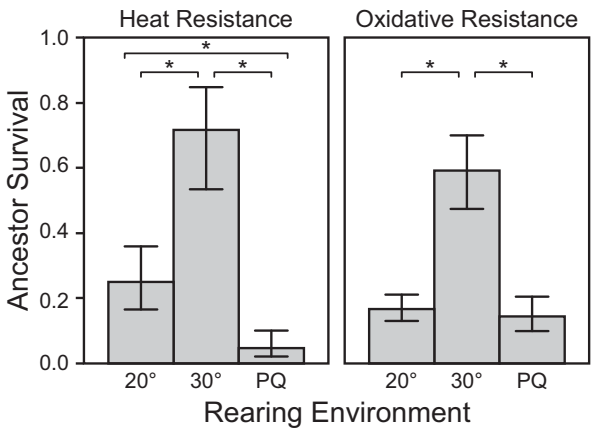

Figure 4. Phenotypic plasticity for stress resistance in the ancestral population. Mean survival (± 95% CI) is shown for individuals raised in each of the three rearing environments. Pairwise comparisons across environments that show significant plastic effects on survival are indicated by * (Tukey HSD; $P < 0.05$).

which made these individuals up to twice as likely to survive subsequent acute stress (Fig. 4). Mild heat stress is therefore a form of hormesis that increases resistance to subsequent, more severe stressors (Sikkink et al. 2014a). However, raising worms in the oxidative environment did not have a hormetic effect for either subsequent heat or oxidative stress. In fact, prior exposure to paraquat decreased resistance to heat stress in the ancestral population.

Within any given selection regime, a direct response to selection occurred if the rearing environment matched the chronic selective environment for that particular line. We predicted that the presence of a genetic correlation across environments should lead to correlated increases or decreases in survival in the other (novel) rearing environments as well. For the environments tested here, cross-environment correlations for the same trait were observed more frequently than between-trait correlations within the same environment (Fig. 5). In acute heat-selected lines, we observed a significant direct response to selection for heat stress resistance in lines selected at 20°C or 30°C, but not on paraquat (Table 3). In addition, a correlated increase in heat resistance was detected in lines selected under each of the three environmental conditions. However, similar to the findings presented above, the precise pattern of the correlated response was complex and 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 30°C environment, but not in the paraquat environment (Fig. 5A). In contrast, if selection occurred in the chronic heat selective environment, correlated improvements in heat resistance are apparent in all three rearing environments. 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. 5A). The full pattern of responses across all combinations of selective and environmental treatments is shown in Supplementary Fig. S2.

The pattern of cross-environment correlations also varied depending on the trait under selection. Unlike the heat-selected lines, for the oxidative-selected lines we observed significant direct responses to selection in every selective environment (Table 3). Additionally, the correlated responses were much more consistent and appeared in nearly every rearing condition, regardless of

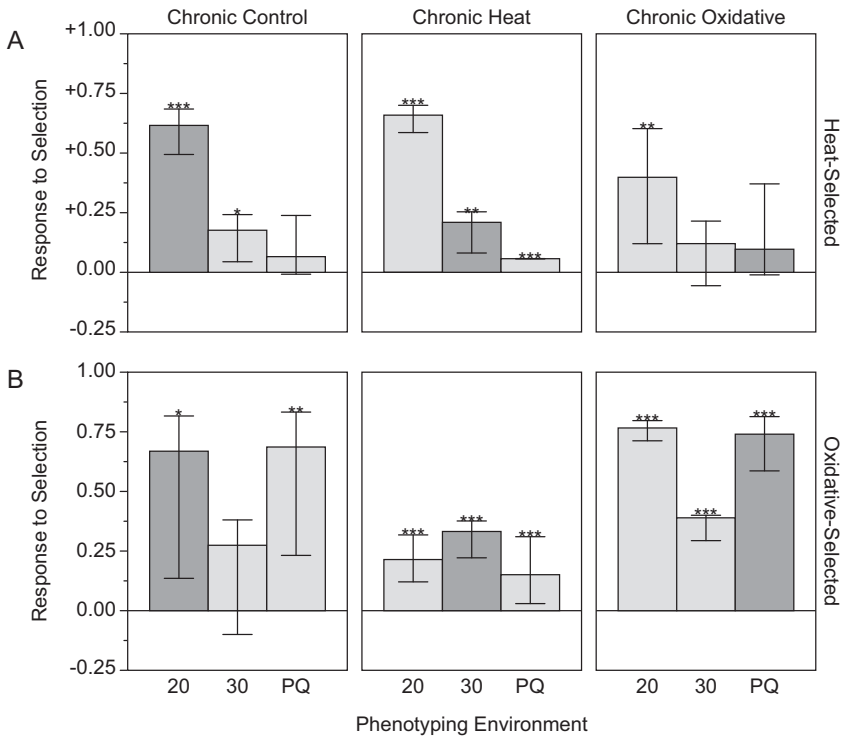

Figure 5. **Direct and cross-environment correlated responses to selection for acute stress-selected lines. Differences in survival from the ancestral population for (A) the acute heat-selected lines or (B) oxidative-selected lines are plotted (± 95% CI). Dark gray bars indicate the effects of direct selection, i.e., the rearing environment matches the chronic selective environment. Light gray 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 ***$P < 0.001$**

Table 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	Rearing environment	Regression coefficient (SE)	z value	P-value	Regression coefficient (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	1.15 (0.47)	2.44	0.01*	1.53 (0.98)	1.55	0.121
	PQ	0.95 (0.59)	1.62	0.104	3.37 (1.07)	3.16	0.002*
Heat	20°C	3.41 (0.34)	9.92	<0.001*	1.12 (0.22)	5.19	<0.001*
	30°C	1.72 (0.64)	2.67	0.008*	2.21 (0.55)	3.97	<0.001*
	PQ	0.78 (0.002)	293.39	<0.001*	0.91 (0.35)	2.59	<0.001*
Oxidative	20°C	1.71 (0.58)	2.94	0.003*	4.22 (0.33)	12.91	<0.001*
	30°C	0.71 (0.49)	1.43	0.153	4.02 (1.18)	3.42	<0.001*
	PQ	1.19 (0.74)	1.62	0.106	3.81 (0.52)	7.38	<0.001*

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

the selection environment (Fig. 5B). In general, the patterns of correlated responses to selection 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 in a trait-specific manner.

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 survive and multiply. Phenotypic plasticity can provide immediate, short-term acclimation of individuals to shifting conditions, while natural selection enables adaptation of populations

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 from the second half of the twentieth century 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). Both are seen as central drivers of this integration and indeed must almost certainly be the central agents that structure these systems. 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 by 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 noncanonical 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. 1), we generally did not find correlated responses to selection. When we did observe correlated responses to selection within specific environments, they were asymmetrical and of opposite sign (Fig. 3 and Fig. 5). We explore 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:

$$\begin{aligned}\Delta\bar{z}_H &= G_{HH}\beta_H + G_{HO}\beta_O \\ \Delta\bar{z}_O &= G_{OO}\beta_O + G_{HO}\beta_H\end{aligned} \tag{3}$$

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 terms in equation (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** (eq. (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 genetic correlation classes 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 a different pattern of correlational selection operating on other 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 in turn are 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:

$$\begin{aligned}\Delta\bar{z}_H &= G_{HH}\beta_H + G_{HO}\beta_O + G_{HU}\beta_U \\ \Delta\bar{z}_O &= G_{OO}\beta_O + G_{HO}\beta_H + G_{OU}\beta_U\end{aligned} \tag{4}$$

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 has measured 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), 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.

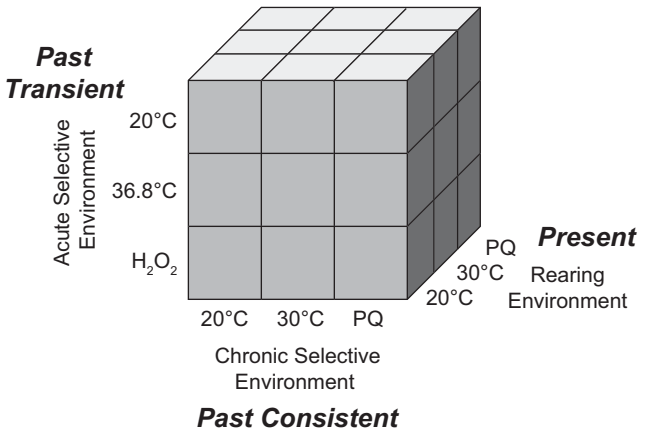

Figure 6. The three types of environmental perturbation experienced by the experimental evolutionary populations, represented as a section from a phenotypic hypercube. Various aspects of the past and present environmental influences on evolution are depicted on each face. In general, the response observed within each segment of the cube is different and highly dependent on interactions between all three factors. Temperature values represent temperature treatments, H_2O_2 represents the high oxidative stress environment via hydrogen peroxide (liquid), PQ represents the high oxidative stress environment via treatment with paraquat (plates).

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 because these alleles in turn display different patterns of pleiotropy across the genetic network. This can create 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). 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 are the primary determinants of the results we observe here. Determining whether one or both of these possibilities is correct will require determining the alleles that are actually 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 $3 \times 3 \times 3$ response hypercube (Fig. 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 rearing environment then represents the present environmental circumstances (Fig. 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 ephemeral, shifts in the environment. It is sobering to consider that the responses in this hypercube, as complex and varied as they may be, still only represent 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, 1984; Lande and Arnold 1983; 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 this contingency is manifested—through changes in

genetic covariance structure via direct environmental perturbations or via the response to multivariate selection—is unknown. Indeed, we believe that it is 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.

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The authors declare no competing interests with the results of this work.

DATA ARCHIVING

Data reported in this article are available at the Dryad Digital Repository, doi:10.5061/dryad.rq964.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Supplementary Figure S1. Direct and correlated responses to selection between traits for each replicate of the experimentally evolved lines.
Supplementary Figure S2. Direct and cross-environment correlated responses to selection in both phenotypes for all populations.