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The Genetic Covariance Among Clinal Environments After Adaptation to an Environmental Gradient in *Drosophila serrata*

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ABSTRACT

We examined the genetic basis of clinal adaptation by determining the evolutionary response of life-history traits to laboratory natural selection along a gradient of thermal stress in *Drosophila serrata*. A gradient of heat stress was created by exposing larvae to a heat stress of 36° for 4 hr for 0, 1, 2, 3, 4, or 5 days of larval development, with the remainder of development taking place at 25°. Replicated lines were exposed to each level of this stress every second generation for 30 generations. At the end of selection, we conducted a complete reciprocal transfer experiment where all populations were raised in all environments, to estimate the realized additive genetic covariance matrix among clinal environments in three life-history traits. Visualization of the genetic covariance functions of the life-history traits revealed that the genetic correlation between environments generally declined as environments became more different and even became negative between the most different environments in some cases. One exception to this general pattern was a life-history trait representing the classic trade-off between development time and body size, which responded to selection in a similar genetic fashion across all environments. Adaptation to clinal environments may involve a number of distinct genetic effects along the length of the cline, the complexity of which may not be fully revealed by focusing primarily on populations at the ends of the cline.

LATITUDINAL clines are widespread and provide a natural framework within which to examine the operation of natural selection (Endler 1977). Despite their prevalence, the genetic basis of adaptation resulting in clinal variation is poorly understood (Barton 1999). Two main approaches have been applied to determining the genetic basis of adaptation along clines. First, quantitative trait loci (QTL) studies have taken advantage of the large difference in phenotype at the ends of clines that are crossed to generate mapping populations for linkage association scans. This approach has been able to successfully identify loci contributing to the large phenotypic differences at the ends of clines for body size in *Drosophila melanogaster* (Gockel *et al.* 2002), some of which may be involved in replicate clines on different continents (Calboli *et al.* 2003).

A fundamental aspect of the biology of clines is that clines in many traits are usually continuous in nature and may display either linear (*e.g.*, James and Partridge 1995; James *et al.* 1995; Hallas *et al.* 2002; Hoffmann and Shirriffs 2002) or more complex (Azevedo *et al.* 1996; Magiafoglou *et al.* 2002; Sgrò and Blows 2003) associations with latitude or some environmental stress. QTL studies are able to identify loci that contribute to major phenotypic differences. However, if clinal adaptation is controlled by a large number of genes of small effect that respond to selection at various stages along the cline and do not approach fixation (Barton 1999), the genetic basis of clines may be difficult to resolve using the cline-end-QTL approach in isolation. For example, Stratton (1998) found that QTL with large effects on flowering time in *Arabidopsis thaliana* were insensitive to a resource gradient, and that most of the genotype-environment interaction was likely to be caused by many genes of small effect.

A second, and complementary, genetic approach to the study of the genetic basis of clines that is designed explicitly to examine the continuous (or otherwise) nature of the genetic basis of clinal adaptation involves the determination of the pattern of genetic covariation between multiple environments (Kirkpatrick *et al.* 1990; Gomulkiewicz and Kirkpatrick 1992; Cooper and DeLacy 1994). Rather than attempting to identify single loci, this approach endeavors to answer two different questions concerning the genetic basis of clinal adaptation: (1) whether adaptation to environments adjacent along the cline involves more similar genetic responses than adaptation to very different environments such as those at the ends of clines and (2) whether the continuous reaction norms that describe the association between the trait and the particular stress that may charac-

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terize a cline are constrained in their evolution by the available patterns of genetic covariance (Gomulkiewicz and Kirkpatrick 1992; Kingsolver *et al.* 2001).

Although latitudinal clines are widespread and extensively studied, the environmental factors that have shaped the evolution of these clines remain largely unknown. A number of laboratory thermal experiments using *D. melanogaster* have provided some insight into the selective factors underlying latitudinal clines (Cavicchi *et al.* 1989; Partridge *et al.* 1994a,b), but have not specifically examined adaptation to environmental gradients, since they have focused on two or three temperatures at any one time. Yet environmental factors along latitudinal clines are most often expressed as gradients rather than in two or more spatially distinct zones (Endler 1977). In addition, stress resistance traits often display continuous clinal variation (Hallas *et al.* 2002; Hoffmann *et al.* 2002), suggesting that environmental factors along clines may vary in such a way as to form gradients of environmental stress. To understand the role of environmental gradients in the formation of latitudinal clines, it will be necessary to experimentally evaluate how adaptation to environmental gradients occurs.

Here, we have created a gradient of increasing stress to examine the genetic basis of adaptation to an environmental gradient by populations of *D. serrata*. *D. serrata* is a member of the *melanogaster* subgroup, in which genetic clines in body size, weight, and cold resistance (Hallas *et al.* 2002) and development time (Magiafoglou *et al.* 2002; Sgrò and Blows 2003) are exhibited by natural populations along the eastern coast of Australia. Using a laboratory natural selection experiment, we exposed replicates of a single base population to an environmental gradient composed of six environments varying in the frequency of extreme larval temperature stress for 30 generations. We measured the direct and correlated responses to selection of a number of life-history traits. Our experimental design consisted of a reciprocal transplant experiment, in which all populations were assessed for the life-history traits in all environments. This experiment enabled us to extract the realized additive genetic variance-covariance among environments on the basis of the direct and correlated selection responses of the replicate populations to determine the pattern of genetic covariance among the clinal populations.

METHODS

Clinal selection experiment: Previous laboratory thermal selection experiments suggest that average temperature is the most likely factor causing clinal patterns of variation in *D. melanogaster* (Partridge *et al.* 1994a,b). However, average temperature varies linearly with latitude, and previous work with *D. serrata* is suggestive of nonlinear clinal patterns for life-history traits, including development time and size (Hallas *et al.* 2002). Nonlinear patterns for trait means are likely to be the result of adaptation to environmental factors that do not show linear patterns along the latitudinal cline (Loeschcke *et al.* 2000). Examination of climatic data for the east coast of Australia obtained from the Bureau of Meteorology indicated that environmental factors that have nonlinear patterns with latitude similar to those suggested for development time in *D. serrata* do exist: the number of days over 35° is one such factor. After performing a number of pilot studies, we decided to use exposure of larvae to a heat stress of 36° for 4 hr each day for the 1–5 days of larval development as our selective factor. This allowed us to create a gradient in environmental stress that may have some relevance to that experienced by *D. serrata* along its latitudinal distribution from tropical to more temperate areas.

Experimental populations were initiated from the F_7 generation of the cross between two laboratory-adapted populations of *D. serrata* representing northern (tropical) and southern (temperate) areas of the distribution of this species (Sgrò and Blows 2003). Both the northern (Cooktown) and southern (Wollongong) populations had been in the laboratory ~2 years as bottle cultures at 25° and 12-hr light (L):12-hr dark (D) photoperiod, before the commencement of this study under identical culture conditions (three bottles per population, ~300 flies per bottle). The two populations at the cline ends were crossed to ensure that many of the alleles for determining trait differences along the species of the *D. serrata* cline were present within the base population. At the F_7 generation of this cross, replicate lines from this mass-bred population were set up and placed in six environments to evolve in the laboratory natural selection experiment, three replicate lines per environment. Each replicate line consisted of two bottles of 40 females and 40 males per bottle. The selection regime involved exposure of larvae to a heat stress of 36° for 4 hr per day (and then returned to 25°) for 0, 1, 2, 3, 4, or 5 days throughout larval development (hereafter referred to as environments E1, E2, E3, E4, E5, and E6, respectively), starting at the first instar, to generate a gradient of environmental stress. This was done by placing 6-day-old adults in fresh bottles and allowing them to lay for 24 hr at 25° and 12 hr L:12 hr D, after which time the adults were removed from the bottles. The eggs were left to hatch for a further 24 hr at 25° and 12 hr L:12 hr D, and the bottles were then placed in their respective selection environments. Development to the adult stage was completed at 25°, with a 12-hr-L:12-hr-D photoperiod. Selection lines were exposed to the selection regime every second generation, since pilot studies showed the presence of strong carryover effects (in the form of reduced viability in the high-stress treatments) when lines were stressed every generation. For the nonselection generation, all lines were maintained at 25°, with a 12-hr L:12-hr D photope-

riod. Selection continued for 30 generations. The experiments described in this study were performed after 2 generations of relaxed selection.

Response to selection in clinal environments: Since the selection regimes involved exposure of larvae to a gradient of heat stress, we measured the heat resistance of larvae from all selection regimes to characterize the response to selection in the trait that reflected the clinal differences between the environments. Eggs were collected from 5- to 6-day-old flies on plastic caps filled with an agar-treacle-yeast medium and left to hatch at 25° for 24 hr. First instar larvae were then placed into six vials per replicate selection line, at a density of 20 larvae per vial, after which time larvae were exposed to 36° for 4 hr and then returned to 25° to continue development. The number of adults eclosing was used as a measure of larval heat stress resistance. Nested analysis of variance, with selection regime as a fixed factor and replicate selection line nested within selection regime as a random effect, was used to analyze the response to selection in larval heat resistance.

The selection lines were tested in all six selection environments to determine evolved responses to the thermal environments and genotype-environment interactions for three life-history traits: development time, viability, and size (wing area). For each replicate line, six groups of five vials, each containing five eggs, were set up. One group of five vials per line was placed in each of the selection thermal environments and the time taken for these eggs to develop to adults was scored. Viability (proportion of eggs emerging as adults) and size (wing area) were also measured for flies eclosing from these vials in all environments. The right wings of flies were removed using fine forceps and mounted on microscope slides using double-sided tape. Wing images were captured via a video camera and landmarks of captured wings were determined with tpsDig version 1.2 written by F. James Rohlf. Prior to measurement all wings were randomized. We obtained x and y coordinates for eight landmarks, and these were used to calculate wing area (Hallas *et al.* 2002; Hoffmann and Shirriffs 2002). All traits were standardized by environment (*i.e.*, subtracting environment mean and dividing by environment standard deviation) before analysis to remove the macroenvironmental effects (Cooper and DeLacy 1994). Size was also standardized for differences between the sexes (the selection response did not differ between sexes, data not shown), and all analyses were performed on data combined across sex.

Genetic analysis of clinal environments: The genetic correlation between two environments has been used to determine the level of similarity in the genetic basis of a single trait when expressed in two environments (Falconer 1952; Lynch and Walsh 1998). The genetic correlation between environments may be estimated from breeding designs where individuals from a number of families, or alternatively a number of genotypes such as inbred lines, are allowed to develop in each environment (Via 1984; Lynch and Walsh 1998). Here, we used a mixed linear model approach to estimate the genetic variance-covariance matrix for our multiple environments,

$$Y_{ijkl} = \mu + G_i + L_{j(i)} + E_k + \varepsilon_{l(ijk)}, \qquad (1)$$

where G_i is the random effect of the ith genotype that evolved in each selection environment, $L_{j(i)}$ is the effect of the jth replicate line nested within the ith selection environment, and E_k is the fixed effect of the kth rearing environment. Variance components were estimated using restricted maximum likelihood (REML) as implemented by the SAS MIXED procedure. The variance components in each environment and the covariances between environments for the G_i term formed the genetic variance-covariance (**G**) matrix.

The interpretation of the genetic effects from an analysis using (1) will depend on the genetic relationships between the genotypes that enter the analysis. Here, our experimental design consisted of genotypes (*i.e.*, selection lines) having evolved from a common genetic base, which were then tested in all environments in the fashion of a complete reciprocal transplant experiment. Genotypes (G_i) therefore differed solely in each environment as a consequence of the direct and correlated responses to selection, which may be used to estimate the additive genetic correlation between environments (Falconer 1989, Eq. 19.7). Therefore, the genetic variance-covariance matrix we present may be interpreted as the realized additive genetic relationships between our experimental environments.

Although our experimental design has the advantage of estimating realized additive genetic relationships among environments, a number of genetic mechanisms may contribute to the observed variances and covariances. First, differential changes in allele frequency under selection at the same loci in different environments may contribute to the genetic correlations among those environments. Therefore, a low genetic correlation between two environments may not solely reflect different genes underlying the responses to selection in each environment. Second, changes in genetic variances and covariances may also have occurred in our experiment as a consequence of genetic drift (Bohren *et al.* 1966) or through the generation of linkage disequilibrium by selection (Bulmer 1971). It is unlikely that genetic drift greatly influenced our estimates of **G** as the effects of genetic drift have been isolated and are contained in the replicate line ($L_{j(i)}$) term. Although linkage disequilibrium may be generated by selection (Bulmer 1971), the relaxation of selection every second generation during the experiment, and for two generations before life-history measures were taken at the end of the experiment, suggests limited opportunity for linkage disequilibrium to be maintained in our populations.

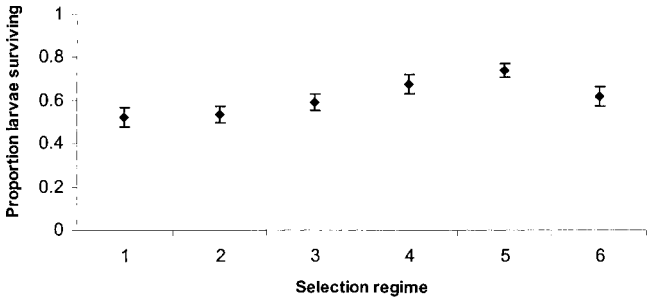


FIGURE 1.—Mean proportion of larvae surviving a heat stress of 36° for 4 hr at the first larval instar for all six selection regimes. Error bars are standard errors calculated across replicate selection line means.

RESULTS

Response to selection in clinal environments: Larval heat stress resistance showed a significant response to selection (nested ANOVA; $F_{5,12} = 9.98$, $P < 0.001$), with larval heat stress increasing with increasing exposure to heat stress up to those populations that had evolved in E4, after which there was a drop in viability in the E5 populations (Figure 1). There was no effect of replicate line nested within selection treatment ($F_{12,17} = 1.23$, $P = 0.277$). A significant linear regression of larval survival against selection regime ($b = 0.034$, $P < 0.001$, $R^2 = 0.58$) indicated that the response to selection in this trait reflected the gradient of differences among the environments.

Reaction norms for standardized mean development time, viability, and body size for all selection lines measured in all six environments are shown in Figure 2. The effect of selection on development time was not a simple linear relationship, whereby increasing stress increased development time. Instead, a complex selection response is evident from the reaction norm for development time (Figure 2A) with populations that had evolved in E4 having the slowest development time across all local environments and the remaining selection regimes showing complex changes in their reaction norms for development time. In general, viability decreased with an increasingly stressful environment; however, the ranking of reaction norms for viability changed with environment (Figure 2B). Exceptions to this trend were evident for the viability reaction norms of selection lines that had evolved in E3, E4, and E6, which tended to increase with increasing stress. As with development time, these changes along the gradient of stress did not involve a simple linear change in the reaction norm for viability. Similarly, the response of body size to selection along a gradient of stress was complex with nonlinear reaction norms, although size did tend to decrease with an increase in stress. Exceptions to this trend were again evident, this time for selection lines that had evolved in E2, where size tended to increase with increasing stress.

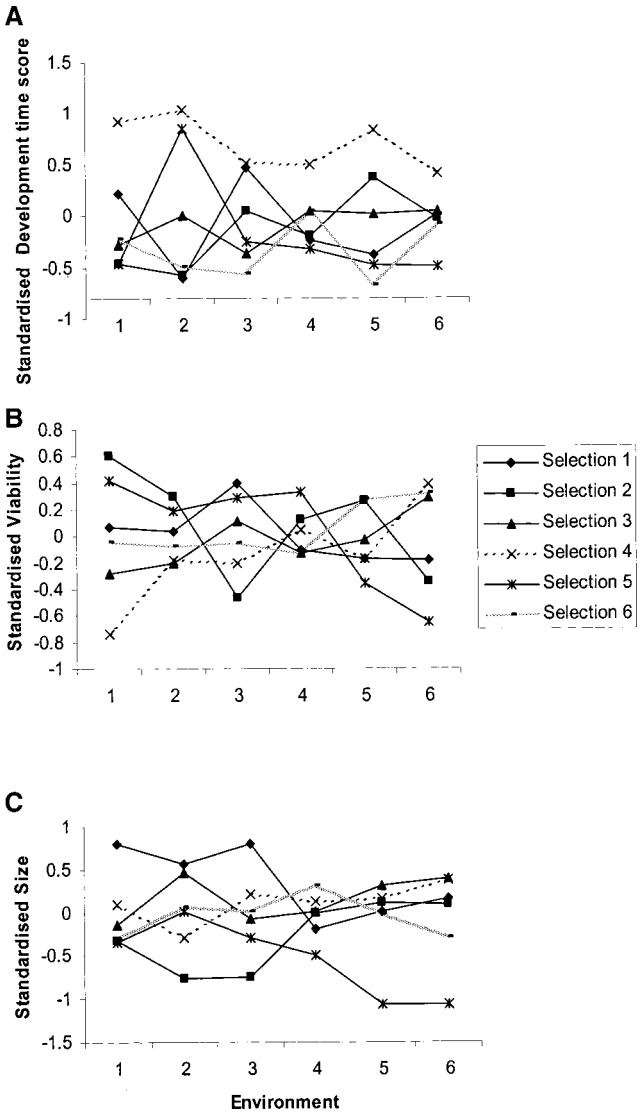


FIGURE 2.—Reaction norms for the three life-history traits standardized for macroenvironmental effects for all six selection regimes across all six environments. (A) Standardized development time score. (B) Standardized viability. (C) Standardized size.

From the visual inspection of reaction norms for individual trait means, it was clear that a combination of the three life-history traits may have responded to selection, but the favored combination may have differed among environments, a finding that has been observed previously in Drosophila (CORTESE *et al.* 2002). We therefore conducted a principal components analysis (PCA) of the three traits (on the correlation matrix corrected for the mean), resulting in three new variables that reflected the relationships between the three life-history traits. The PCA also resulted in three normally distributed and uncorrelated variables (principal components) that were better suited to multivariate analysis (particularly REML variance component estimation) than the original three variables. The PCA found three principal components that explained similar amounts

TABLE 1

Principal components analysis of the three life-history traits

Trait	PC1 (37.7%)	PC2 (33.6%)	PC3 (28.7%)
Development time	0.761	−0.093	0.643
Viability	−0.152	0.955	0.255
Body size	0.728	0.297	−0.618

of the variation among the three traits (Table 1), which did not represent simply the original three life-history traits. The first principal component (PC1) reflected a positive association between development time and body size, PC2 represented primarily viability with a smaller contribution from body size, and PC3 contrasted body size with development time and to a lesser extent viability.

Reaction norms of the three PCs for all selection regimes across all six environments are shown in Figure 3. The reaction norm for PC1 (Figure 3A) is complex and similar in form to the reaction norms for standardized development time and size, reflecting the positive contributions that both traits make to this new variable. The reaction norm for PC2 mirrors that for the standardized mean viability (Figure 3B), reflecting the fact that PC2 primarily represents viability. Finally, the reaction norm for PC3 (Figure 3C) again reflects the complexity of the relationship among the three life-history traits across the environmental gradient.

The response of the PCs to selection was tested using MANOVA followed by univariate ANOVAs using model (1) as implemented by PROC GLM in SAS. We used the PCs here, rather than the original traits, as viability in particular displayed a highly skewed distribution. MANOVA indicated that there had been a significant response to selection in the life-history traits (Wilks' $\lambda = 0.030$, $F_{15,25.2} = 4.28$, $P < 0.001$). PC1 and PC2 displayed significant interactions between rearing environment and the environment they had evolved in (Table 2), indicating that adaptation to one clinal environment affected the expression of these life-history traits in another environment. PC3 responded to selection, but did not display an interaction with rearing environment (Table 2).

Genetic analysis of clinal environments: Visual inspection of the genetic variance-covariance matrix of PC1 among the clinal environments (Table 3) suggested that the response to selection varied considerably in its genetic basis among the six environments, with genetic correlations ranging from above the theoretical limit of 1 in two cases (E1–E3, E5–E6) to negative genetic correlations of −0.575 (E2–E6) and −0.400 (E2–E5). None of the three variance-covariance matrices in Table 3 were positive definite, probably as a consequence of estimation error. While Falconer (1952) first proposed

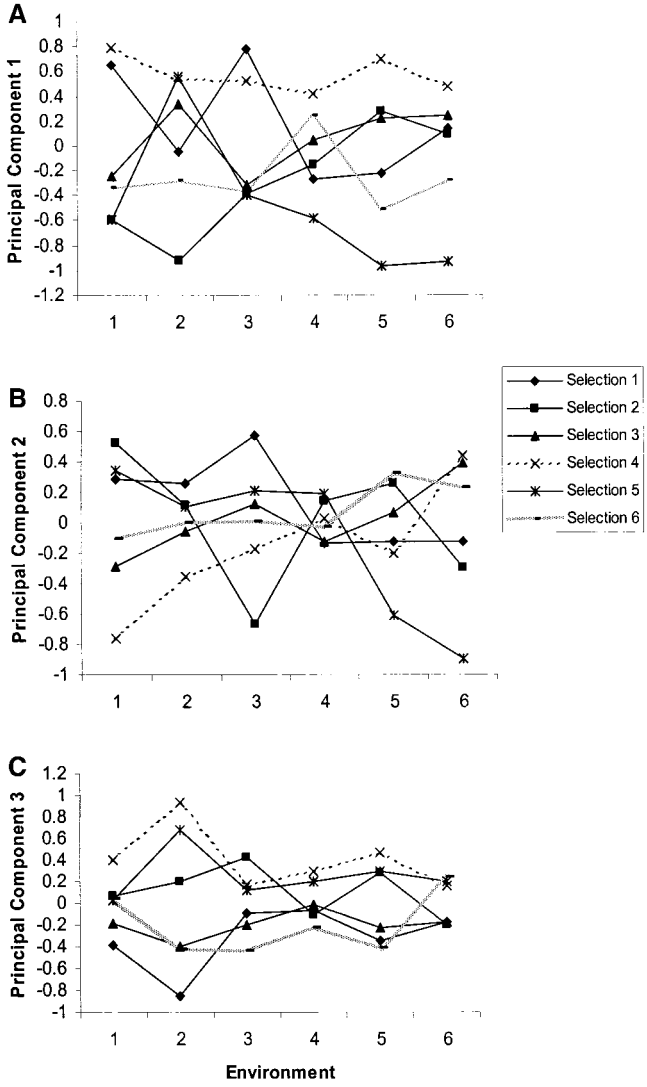


Figure 3.—Reaction norms for the three principal components (PCs) obtained from the principal components analysis on the three life-history traits, for all six selection regimes across all six environments. (A) Principal component 1. (B) Principal component 2. (C) Principal component 3.

the genetic correlation for the study of genotype-by-environment interaction using standard quantitative genetic techniques (Lynch and Walsh 1998), its application to multiple or continuous environments requires a multivariate analytical approach to the study of pattern in genetic variance-covariance (**G**) matrices. A relatively straightforward approach is to visualize the genetic relationships between environments by conducting a principal components analysis (on the uncorrected covariance matrix) of the genetic variance-covariance matrix, and to then plot the resulting first two genetic principal components on a biplot (Cooper and DeLacy 1994). This approach allows the genetic relationships among the environments to be visualized by the similarity in direction of six vectors (one for each environment) in the two-dimensional space defined by the first two

TABLE 2

Analysis of variance of the three life-history principal components

	PC1		PC2		PC3	
	MS	*F*	MS	*F*	MS	*F*
Environment	0.11	0.14	0.05	0.05	0.02	0.02
Selection regime	10.69	3.20*	0.85	0.59	6.50	8.06**
$E \times S$	1.99	2.59***	1.75	1.84**	0.98	1.03
Line (selection)	3.36	4.36***	1.44	1.51	0.81	0.85
Error	0.77		0.95		0.95	

*$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$. MS, mean square.

principal components of the variance-covariance matrix. The biplot for PC1 effectively shows that most of the genetic variance among environments for PC1 is a consequence of the response to selection in E2 that appears to have a very different genetic basis from either environment closer to it in the level of stress (E1 and E3) and, in particular, from the more extreme environments E4, E5, and E6 (Figure 4A).

Alternatively, Kirkpatrick *et al.* (1990) proposed that the continuous nature of clinal environments might best be modeled genetically by determining the genetic covariance function from **G** using smooth curves. A number of alternatives are available to generate the genetic covariance function, the relative merits of which have yet to be established (Kirkpatrick and Bataillon 1999). While Kirkpatrick *et al.* (1990) favored the use of orthogonal polynomials, this method assumes that the genetic variance and covariances change in a continuous fashion, which did not appear likely from the reaction norms presented in Figure 3 or from the estimates of genetic variance in Table 3 for PC2 and PC3. We therefore employed the nonparametric approach of cubic splines to generate the genetic covariance function. The cubic spline representation of the genetic covariance function for PC1 (Figure 4D) again emphasized a general decline in genetic correlation among environments as they became more different, but also the lack of genetic correlation between E2 and the other environments. The other major feature of the genetic covariance function was the decline in genetic variance in middle environments, particularly E4 (Table 3).

The genetic analysis of PC2 was limited by the zero values for genetic variance in E2, E4, and E5 returned by the REML analysis, suggesting that the selection lines did not vary substantially when reared in these three environments. Although few genetic correlations could be estimated for this trait, most of the genetic covariances are negative, and the genetic correlation between the two most different environments (E1 and E6) was just over the theoretical limit of −1. The biplot (Figure 4B) suggested that populations that evolved in more benign environments (E1 and E2) responded to selection in a very different way from populations experiencing extreme environments (E5 and E6). This trend is more striking in the representation of the genetic covariance function (Figure 4E) where there is a relatively smooth and rapid decline in genetic correlation between environments as they become more different. The peak in the center of this surface represented the large estimate of genetic variance in the E3 environment.

PC3 was the only one of the three life-history principal components that did not display an interaction between selection regime and rearing environment (Table 2). All but one of the genetic covariances were positive, but two genetic variances were set to zero by the REML analysis, which did not allow the estimation of all genetic correlations. The biplot (Figure 4C) confirmed that populations evolving in all environments responded to selection in a similar fashion for this trait, although populations that evolved in E5 appeared to diverge along the second genetic principal component to some extent. The genetic covariance function (Figure 4F) displayed a rapid decline in genetic variance from the control to the most extreme environments and, in contrast to the covariance functions for PC1 and PC2, depicted relatively uniform genetic correlation among environments.

DISCUSSION

Response to selection in clinal environments: Laboratory natural selection along a gradient of heat stress resulted in an increase in larval heat stress resistance. The response in larval stress resistance increased in a roughly linear clinal fashion. Our laboratory environments therefore appear to have been successful in generating an abiotic cline that the populations responded to in a fashion similar to that seen in natural clines of stress resistance traits in *D. serrata* (Hallas *et al.* 2002; Hoffmann *et al.* 2002).

Life-history traits measured on these populations displayed strong correlated responses to selection for larval heat resistance. Costs associated with the evolution of

TABLE 3

Genetic variance-covariance matrix of the six clinal environments for the three life-history principal components

		E1	E2	E3	E4	E5	E6
PC1	E1	0.315*	0.138	0.255**	0.043	0.145	0.153
	E2	*0.515*	0.230	0.014	0.017	−0.088	−0.088
	E3	*1.095*	*0.072*	0.173*	0.011	0.101	0.059
	E4	*0.309*	*0.139*	*0.103*	0.061	0.099	0.077
	E5	*0.565*	*−0.400*	*0.529*	*0.873*	0.209	0.249**
	E6	*0.852*	*−0.575*	*0.443*	*0.966*	*1.701*	0.102
PC2	E1	0.206*	0.095	−0.029	0.014	0.031	−0.220**
	E2	—	0	0.020	−0.009	−0.051	−0.071
	E3	*−0.159*	—	0.162	−0.024	−0.103	−0.055
	E4	—	—	—	0	−0.007	−0.015
	E5	—	—	—	—	0	0.074
	E6	*−1.084*	—	*−0.306*	—	—	0.200*
PC3	E1	0.065	0.187*	0.038	0.040	0.111	0.022
	E2	*1.028*	0.505***	0.161*	0.137	0.238*	0.114
	E3	*0.370*	*0.993*	0.052	0.030	0.099	−0.050
	E4	—	—	—	0	0.046	0.026
	E5	*2.750*	*2.116*	*2.755*	—	0.025	0.063
	E6	—	—	—	—	—	0

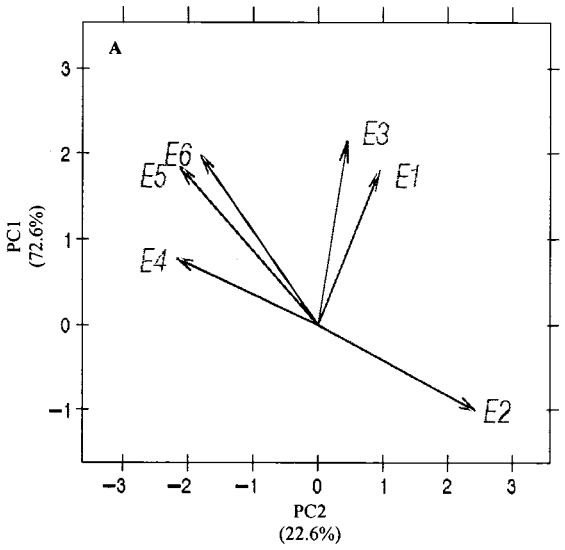
In each section, genetic correlations are in italics below the diagonal, genetic variances are on the diagonal, and genetic covariances are above the diagonal. Dashes occur where genetic correlations could not be calculated because of zero genetic variance. Significance of (co)variance components was determined by a change in the −2 log-likelihood, evaluated in a chi-square test within 1 d.f., as each individual genetic variance or covariance was constrained to equal zero.

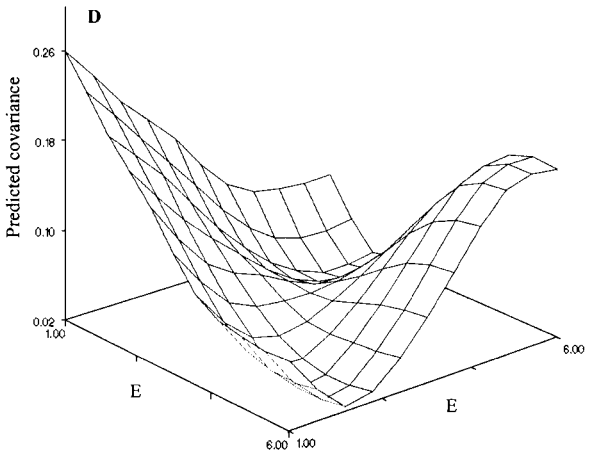
many forms of stress resistance, expressed in the form of trade-offs between stress resistance and life-history traits, are common in animals (Hoffmann and Parsons 1991; Hoffmann *et al.* 2003) and plants (Bergelson and Purrington 1996). In particular, laboratory natural selection experiments have previously been used to examine thermal evolution in *D. melanogaster* (Cavicchi *et al.* 1989; Huey *et al.* 1991). All of these studies have shown significant correlated responses to selection in adult and pre-adult life-history traits.

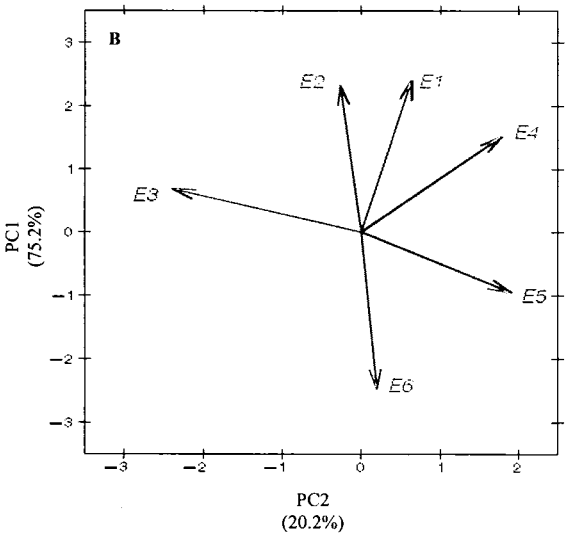
Although previous studies examining thermal evolution have used relatively simple experimental conditions, considering environments that differed only in average (nonextreme) temperature using two, or at a maximum three, different temperatures (but see Loeschcke and Krebs 1996), significant environment-dependent responses were seen. For example, genotype-environment interactions were shown for pupal period, larval competitive ability, and critical weights for pupariation, but not for larval period or larval growth rate (Partridge *et al.* 1994b) or for body size (Partridge *et al.* 1994a). Cavicchi *et al.* (1989) examined thermal adaptation to three temperatures (18°, 25°, and 28°) and found genotype interactions for a range of size-related and fitness traits. As our results illustrate, even more complex and environment-specific selection responses are evident when several stressful environments are considered. Although the primary stress resistance trait responded to selection in a linear clinal fashion, the gradient of temperature stress resulted in complex selection responses in development time, viability, and body size. Combinations of these life-history traits responded strongly to selection, but there was no indication of simple linear changes in any individual trait along the gradient of stress.

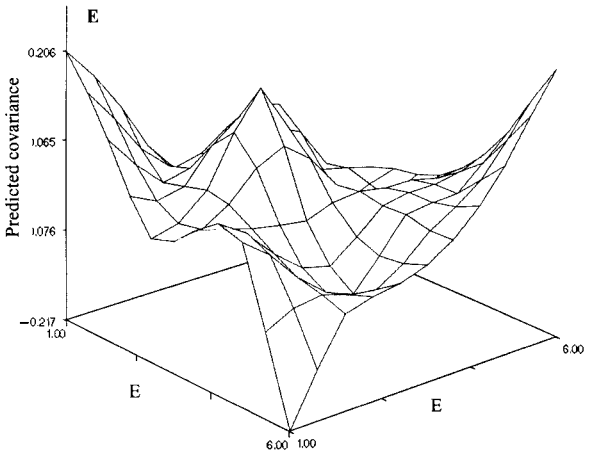
Therefore, our laboratory clinal selection experiment has reproduced a prominent feature of natural *D. serrata* clines; stress resistance tends to display linear clinal patterns, while life-history traits tend to display more complex associations with environmental gradients. Unfortunately, we cannot make direct comparisons between the results from our clinal selection experiment and those obtained from clinal studies of natural populations of *D. serrata* (Magiafoglou *et al.* 2002; Sgrò and Blows 2003) since we have examined only one environmental factor under controlled laboratory conditions. However, our clinal selection experiment suggests that the complex patterns of life-history clines in nature (Hallas *et al.* 2002; Magiafoglou *et al.* 2002; Sgrò and Blows 2003) may result at least in part from adaptation to gradients of environmental stress.

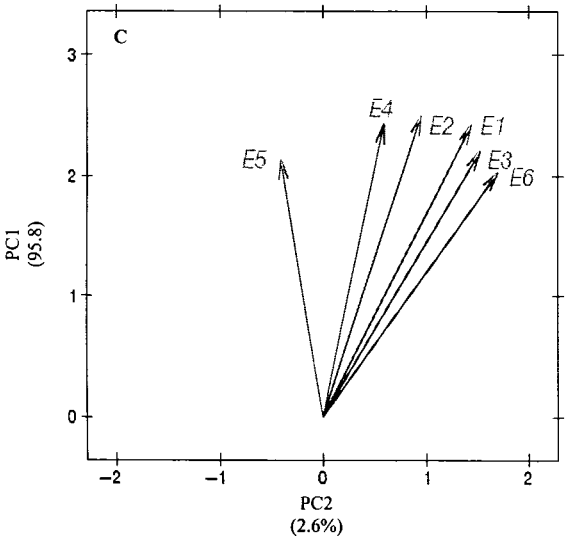
Genetic analysis of clinal environments: Using a novel experimental design to estimate the genetic variance-covariance matrix among clinal environments, we have been able to show how the responses to selection in a number of clinal environments are genetically related.

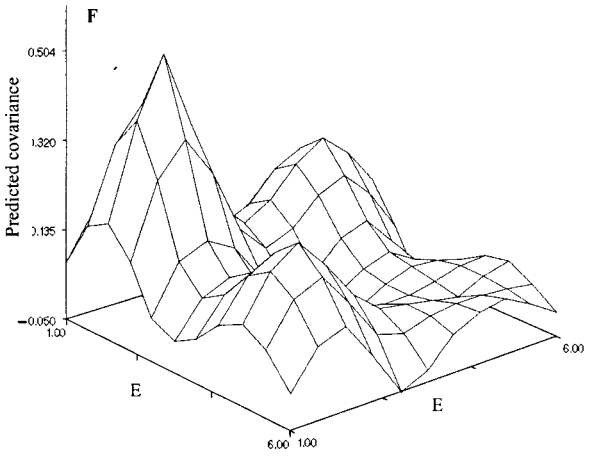

A
E6
E5
E3
E1
E4
E2
PC1
(72.6%)
PC2
(22.6%)


D
Predicted covariance
0.26
0.18
0.10
0.02
1.00
6.00
E
E


B
E2
E1
E4
E3
E5
E6
PC1
(75.2%)
PC2
(20.2%)


E
Predicted covariance
0.206
1.065
1.076
−0.217
1.00
6.00
E
E


C
E4
E2
E1
E5
E3
E6
PC1
(95.8)
PC2
(2.6%)


F
Predicted covariance
0.504
1.320
1.135
−0.050
1.00
6.00
E
E

The main pattern to emerge from our genetic analysis was a general tendency for the genetic correlations between environments to decline (PC1), even to the extent that they became substantially negative (PC2) as the environments became more different. Such differences in genetic response may be a consequence of different mechanisms of heat stress resistance being selected for at different points along our environmental gradient of stress. One such possible mechanism could involve changes in hsp70 expression in our selection treatments. Hsp70 levels appear to be downregulated during adaptation to high but not extreme temperatures (Bettencourt *et al.* 1999; Sorensen *et al.* 1999; Lansing *et al.* 2000). This downregulation has been interpreted as an evolutionary response to reduce the costs of repeated heat exposure. When frequently exposed, the cost of stress resistance, in terms of reduced fecundity and increased development time, is thought to outweigh the benefits of increased thermotolerance, and a fixed basal level of resistance is thought to be favored (Hoffmann *et al.* 2003). For example, environment E4, which was exposed to the larval heat stress for 3 days of the larval period and had the most consistently slow development time across all experimental environments, may have evolved a heat resistance mechanism involving higher hsp70 levels (thus the increase in development time), while the selection regimes either side of this treatment may have evolved heat stress responses involving lower levels of hsp70. Assays specifically examining changes in hsp70 expression during adaptation to the environmental gradient would be required to determine if this is the case. Experimental evaluation of potentially different mechanisms underlying the selection response is particularly important here as differential allele frequency change at the same loci in different environments may also contribute to the low genetic correlations detected to some extent.

There were two exceptions to the general trend of decline in genetic correlation along the cline. First, PC3, which represented the classic trade-off between development time and body size (Cortese *et al.* 2002), did not display this trend. For this trait, all environments appeared to elicit similar genetic responses as indicated by the consistently positive genetic covariances and experienced a spike in genetic variance in the E2 environment, which was exposed to the smallest level of heat stress. One possible explanation for such a pattern may be that a single mechanistic trait was under selection whereby genetic variance increases as rare alleles increase for stress resistance (E1–E2) and then declines again as the same alleles are pushed to high frequency in more extreme environments (E3–E6). The E2 environment, with the lowest frequency of larval temperature stress, may not have pushed alleles past the symmetrical frequencies.

Second, in not all cases did environments that are adjacent along the gradient of stress respond to selection in a similar fashion. This was particularly clear in the response of the populations that evolved in the E2 environment, which appeared to find a genetically distinct way of responding to selection on PC1. Therefore, similarity in environmental stress may not always be a good predictor of similarity in genetic response, at least when levels of stress are relatively low. Under extreme stress, however, the genetic responses always appeared to be more consistent across the very stressful environments (E4, E5, and E6), particularly for PC1. More highly genetically correlated phenotypes under extreme conditions has been observed before by Kingsolver *et al.* (2001, Figure 6) in relation to temperature effects on relative growth rate of caterpillars.

Periods of environmental stress may result in changes in the expression of genetic variation for life-history traits, as well as in the genetic correlations within and between environments, resulting in complex patterns of evolutionary responses under stress (Hoffmann and Parsons 1991; Hoffmann and Merilä 1999). While many attempts have been made to determine the extent to which genetic correlations acting across environments may constrain evolution under changing environmental conditions (Hoffmann *et al.* 1995), few studies have specifically addressed the effects of extreme environmental conditions on genetic correlations for traits in different environments. We have shown that, depending on the traits involved, genetic correlations for life-history traits across environments either may change in sign and/or magnitude as the environment becomes more different (PC1 and PC2) or may actually remain constant across a range of environments (PC3). Previous work examining the effect of thermal stress on the expression of genetic variation for life-history traits in *D. melanogaster* has also shown that responses may be trait specific (Sgrò and Hoffmann 1998). In addition, more than one genetic response may be available to a population during selection along a gradient of environmental stress. Diverse evolutionary responses are well known when insects evolve in response to insecticides, where several different mechanisms may confer resistance to the same insecticide (Hoffmann and Parsons 1997), although most comparisons of differences in evolution-

Figure 4.—Biplots (A–C) and cubic spline representations of genetic covariance functions (D–F) among the six clinal environments for life-history principal components (A and D) PC1, (B and E) PC2, and (C and F) PC3. Principal components analyses to generate the biplots were conducted on the genetic variance-covariance matrices in Table 3. Cubic splines to generate the genetic covariance functions were conducted by first finding the value of the smoothing parameter that minimized the cross-validation score using the SAS TPSPLINE procedure.

ary responses have been between species, as opposed to differences between populations within a species (this study). The complex responses shown in this study could be due to a number of factors, including differential gene expression and gene-environment interaction, as well as changes in epistasis (Blows and Hoffman 1996) along the environmental gradient of stress. As with most quantitative genetic analyses, we are unable to distinguish between these possibilities without recourse to more complex and logistically demanding experimental designs.

In summary, we have shown that adaptation to an environmental gradient of stress, such as can be expected to occur along latitudinal gradients in nature, may involve multiple and complex evolutionary responses of life-history traits at different points along the gradient. Such complex evolutionary responses to clinal adaptation are excluded from studies that use only the cline-end QTL approach. While QTL studies using populations from cline ends may be able to isolate genes with large effects on quantitative traits that also show clinal variation, they may not provide a complete representation of the genetic basis of adaptation that occurs at different points along the length of a cline and of how natural populations adapt to environmental gradients. Ideally, a combination of QTL, gene expression, or candidate gene approaches in multiple environments, in conjunction with experiments designed to examine the change in genetic basis of clinal traits along the environmental gradient, would provide a comprehensive understanding of the genetic architecture underlying adaptation to environmental gradients.

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