JOURNAL OF Evolutionary Biology

doi: 10.1111/j.1420-9101.2012.02536.x

A multivariate test of evolutionary constraints for thermal tolerance in *Drosophila melanogaster*

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Keywords:

adaptation;
additive genetic variance covariance;
constraint;
G matrix;
genetic correlation;
heat tolerance.

Abstract

Exposure to extreme temperatures is increasingly likely to impose strong selection on many organisms in their natural environments. The ability of organisms to adapt to such selective pressures will be determined by patterns of genetic variation and covariation. Despite increasing interest in thermal adaptation, few studies have examined the extent to which the genetic covariance between traits might constrain thermal responses. Furthermore, it remains unknown whether sex-specific genetic architectures will constrain responses to climatic selection. We used a paternal half-sibling breeding design to examine whether sex-specific genetic architectures and genetic covariances between traits might constrain evolutionary responses to warming climates in a population of *Drosophila melanogaster*. Our results suggest that the sexes share a common genetic underpinning for heat tolerance as indicated by a strong positive inter-sexual genetic correlation. Further, we found no evidence in either of the sexes that genetic trade-offs between heat tolerance and fitness will constrain responses to thermal selection. Our results suggest that neither trade-offs, nor sex-specific genetics, will significantly constrain an evolutionary response to climatic warming, at least in this population of *D. melanogaster*.

Introduction

Exposure to extreme temperatures is increasingly likely to be a source of strong selective pressure for many organisms. Whereas traits that increase fitness in a warmer climate, such as an increased resistance to heat stress, are predicted to be increasingly favoured (Gienapp *et al.*, 2008; Kingsolver, 2009), the evolutionary factors that underpin the evolution of heat tolerance remain largely unknown. Evolutionary quantitative genetics tells us that phenotypic evolution is fundamentally linked to additive genetic variances and covariances, summarized by the **G** matrix (Lande, 1979; Lande & Arnold, 1983). Genetic covariances are summary statistics that capture the effects of pleiotropy and linkage disequilibrium, and they quantify the extent to which the evolutionary response in one trait will be influenced by selection on another (Falconer & Mackay, 1996; Lynch & Walsh, 1998). As a consequence, traits will not evolve independently of one another. Thus, the ability of a population to respond to selection for increasing heat tolerance will be determined by the patterns of genetic variation and covariation in traits under selection. Specifically, the change in the mean multivariate phenotype across a single generation is given by

$$\Delta z = \mathbf{G}\beta \qquad (1)$$

where $\Delta z = \{\Delta z_1, \Delta z_2, \Delta z_3, \ldots \Delta z_n\}^T$ is a column vector of changes in the means of n traits (the T denotes transpose); **G** is the additive genetic variance–covariance matrix with diagonal elements representing genetic variances and off-diagonal elements representing genetic covariance between traits (element $\mathbf{G}_{ii}$ is the genetic variance of trait i, and element $\mathbf{G}_{ij}$ is the genetic covariance between the traits i and j), and $\beta = \{\beta_1, \beta_2, \ldots \beta_n\}^T$

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is a column vector of directional selection gradients (Lande, 1979; Lande & Arnold, 1983).

Thus, phenotypic evolution might be constrained if genetic variation in a population is lacking in the direction of selection. The prevalence of such absolute genetic constraints, defined by the absence of genetic variance for a particular trait combination (Mezey & Houle, 2005), is not known (Kirkpatrick, 2009). Although univariate estimates of genetic variances are typically greater than zero (Mousseau & Roff, 1987; Lynch & Walsh, 1998; but see Kellermann *et al.*, 2009, 2006), suggesting absolute constraints will be rare, individual trait variances are likely to provide a misleading picture of how multiple traits might respond to selection. Absolute genetic constraints can exist even when genetic variance is present for each individual trait (Agrawal & Stinchcombe, 2009; Walsh & Blows, 2009). Even in the absence of absolute genetic constraints, the uneven distribution of genetic variance among trait combinations in multivariate space (Mezey & Houle, 2005; Hine & Blows, 2006; McGuigan & Blows, 2007) can generate relative genetic constraints, biasing the response for certain directions of selection (Schluter, 1996; Hansen & Houle, 2008; Agrawal & Stinchcombe, 2009; Kirkpatrick, 2009; Walsh & Blows, 2009).

When additive genetic variance is present for individual traits, absolute and relative multivariate genetic constraints arise as a consequence of the genetic covariance among traits. Such genetic constraints have traditionally been inferred through interpretation of pairwise genetic correlations, particularly in a life-history context (Houle, 1991; Roff & Fairbairn, 2007; Poissant *et al.*, 2010). However, such an approach fails to recognize the influence of the broader context of the multivariate phenotype (Pease & Bull, 1988; Fry, 1993). In addition, whereas interpretation of genetic correlations that are ± 1 and 0 is clear, it is not clear how correlations less than one might affect evolutionary trajectories (Hansen & Houle, 2008; Agrawal & Stinchcombe, 2009). Even genetic correlations that are less than one can prevent adaptation when there are more than two traits under selection (Blows & Hoffmann, 2005), if the genetic variance present in all n traits is structured (as described by covariances) such that there is no genetic variation in certain directions of multivariate space (i.e. the rank of $\mathbf{G}$ is $< n$; Hine & Blows, 2006). In addition, a reliance on pairwise genetic correlations to understand evolutionary constraints misses the point that the important factor in making such inferences is not the genetic correlations *per se*, but rather the amount of genetic variation that exists in multivariate space in the direction of selection (Agrawal & Stinchcombe, 2009; McGuigan & Blows, 2010). Finally, a focus on genetic correlations to understand constraint runs the risk of ignoring the fact that genetic correlations alone do not dictate the pattern of genetic variance in phenotypic space; genetic variances are also important (Agrawal & Stinchcombe, 2009). The effect of genetic correlations on evolution is always dependent on trait variances because correlations can only be considered in multivariate space through genetic covariances (e.g. $\mathbf{G}_{12} = r_{12}\sqrt{(\mathbf{G}_{11}\mathbf{G}_{22})}$, where $\mathbf{G}_{12}$ = genetic covariance between traits 1 and 2, r_{12} = the genetic correlation between traits 1 and 2, and $\mathbf{G}_{11}$ and $\mathbf{G}_{22}$ are the genetic variances of traits 1 and 2, respectively). Thus, multivariate approaches are required to identify evolutionary constraints.

Despite a strong theoretical framework describing the evolution of multiple traits, the extent to which the covariance structure among traits constrains or facilitates multivariate phenotypic evolution remains largely unknown, in part because of a lack of analytical tools for testing the importance of genetic constraints due to genetic covariances and the availability of genetic variance in the multivariate direction of selection (Agrawal & Stinchcombe, 2009; McGuigan & Blows, 2010). To do so involves testing hypotheses concerning $\mathbf{G}$ matrix properties, particularly size, shape and orientation (McGuigan & Blows, 2010). Several approaches have been developed for analysis of these properties of $\mathbf{G}$ (Schluter, 1996; Hansen & Houle, 2008; Agrawal & Stinchcombe, 2009; Calsbeek & Goodnight, 2009; Kirkpatrick, 2009). Many of these methods combine information on $\mathbf{G}$ with information about the pattern of natural selection, β, to examine the extent to which evolution might be constrained (Agrawal & Stinchcombe, 2009; Simonsen & Stinchcombe, 2010). However, not all methods of analysing $\mathbf{G}$ necessarily require information on β.

McGuigan & Blows (2010) recently developed an analytical approach to the study of genetic constraint, building on the work of Hansen and colleagues (Hansen, 2003; Hansen *et al.*, 2003; Hansen & Houle, 2008). This approach first involves recognizing that, whereas the multivariate context in which a trait exists is crucial for understanding evolutionary constraints, it might often be the case that the evolutionary potential of one (focal) trait, rather than a suite of traits, is of particular interest. Hansen and colleagues suggested that estimating the genetic variance in a trait that is independent of other traits provides an informative approach for studying genetic constraints (Hansen, 2003; Hansen *et al.*, 2003; Hansen & Houle, 2008). The genetic variance in traits that is independent of genetic variance in other traits is the trait-specific variance.

The portion of genetic variation that is shared among traits, the common genetic variance, is the portion of genetic variance for which any selection will illicit correlated responses across traits. The impact of this genetic covariation among traits on their evolutionary trajectories will depend on the orientation of selection relative to the genetic variation (Hansen & Houle, 2008; Agrawal & Stinchcombe, 2009; Calsbeek & Goodnight, 2009; Walsh & Blows, 2009). The genetic variance that is independent of other traits, the trait-specific genetic variance, is available for traits to respond independently

to selection and will not drive correlated evolution. McGuigan & Blows (2010) developed an analytical approach for estimating the independence of genetic variance in individual traits within a hypothesis testing, mixed-model framework.

Trait-specific genetic variation might be particularly important when the focal trait is under directional selection, whereas other, correlated traits are under stabilizing selection; in this case, evolution will be dependent on the trait-specific variance (Hansen & Houle, 2008). Such a scenario is highly relevant in the context of selection for increasing heat tolerance as a consequence of ongoing warming. With ongoing climate change predicted to impose selection for increased heat tolerance, the persistence of populations will depend on their ability to evolve higher levels of heat tolerance. Numerous studies have examined genetic variation for heat tolerance in *Drosophila* (e.g. Coyne *et al.*, 1983; Jenkins & Hoffmann, 1994; Cavicchi *et al.*, 1995; McColl *et al.*, 1996; Bubliy *et al.*, 1998; Gilchrist & Huey, 1999; Krebs & Thompson, 2006; Sorensen *et al.*, 2007; Mitchell & Hoffmann, 2009; Sisodia & Singh, 2010) and other taxa (e.g. Bennett & Lenski, 1993; Neargarder *et al.*, 2003; Elderkin & Klerks, 2005; Winne & Keck, 2005; Willett, 2010; Doyle *et al.*, 2011; Kelly *et al.*, 2012), yet these studies have relied on univariate or bivariate approaches to examining evolutionary constraints on heat tolerance. Thus, perhaps surprisingly, we still know very little of the extent to which the evolution of heat tolerance in natural populations might be constrained by covariances between traits or the expression of trait-specific variance.

In particular, few studies have explicitly examined how the genetic variances for, and covariances between, fitness-related traits and heat tolerance might influence the evolution of high levels of heat tolerance in nature. Whereas some work (Bennett & Lenski, 1993) indicates that adaptation to new thermal environments will not be constrained by fitness trade-offs, more recent work suggests that evolution to high, stressful temperatures might indeed come at the cost of reduced performance (fitness) at lower temperatures (Willett, 2010). However, neither of these studies has dissected the multivariate genetic basis of thermal adaptation and the extent to which thermal adaptation might be constrained by genetic variances and covariances.

Our understanding of constraints acting on thermal evolution is further limited by the fact that we still know very little about how males and females of the same species might adapt to a warming environment. Although males and females of the same species essentially share a genome, they typically have very different strategies for maximizing their fitness (Arnqvist & Rowe, 2005). However the extent to which these differences might lead to sex-specific constraints on the ability to evolve higher levels of heat tolerance is not known. The possibility that sex-specific trade-offs between reproductive output (e.g. fertility, fecundity) and heat tolerance might constrain an evolutionary response to selection within one sex, and at the same time constrain the evolution of heat tolerance in the other sex due to genetic covariances, needs testing. Although several studies have previously examined evolutionary potential for upper thermal limits in both sexes in *Drosophila* (e.g. Jenkins & Hoffmann, 1994; McColl *et al.*, 1996; Bubliy *et al.*, 1998; Gilchrist & Huey, 1999; Mitchell & Hoffmann, 2009), none have explicitly dissected the underlying genetic architecture of heat tolerance between the sexes, nor the extent to which the evolution of heat tolerance might be constrained either by covariances with other traits, within or between the sexes. Finally, despite increasing interest in the evolution of upper thermal limits (Angilletta, 2009), the genetic relationship between thermotolerance and components of fitness in both sexes has seldom been directly estimated (Willett, 2010).

In this study, we examined the distribution of additive genetic variances and covariances among male and female heat tolerance and life-history traits as a first step in analysing multivariate constraints for the evolution of heat tolerance in *Drosophila melanogaster*. We used a combination of analytical methods to examine **G** for these traits. Specifically, we asked whether there is independent (trait-specific) genetic variation associated with individual traits in male and female *D. melanogaster* that might allow them to respond independently to selection for increased heat tolerance. We also asked whether the evolution of heat tolerance might be constrained by covariances between traits, within or between the sexes.

Materials and methods

Experimental stocks and data collection

Field collection took place at Coffs Harbour, NSW, in February, 2010. There is extensive gene flow between populations of *D. melanogaster* from eastern Australia (Kennington *et al.*, 2003), and therefore, a mid-latitude population was sampled in order to capture as much of the genetic variation that is present within the entire Australian range of this species as possible. Individuals were collected from three locations within Coffs Harbour. A banana and yeast mixture was used to attract individuals to the first two collection sites, a public park (30°19′10′S 153°05′20′E) and a caravan park (30°17′33′S 153°08′13′E). No bait was used at the third site, a fruit shop (30°18′18′S 153°07′48′E).

The offspring of 20 field-inseminated females from each collection site were used to establish a single mass bred laboratory population. Ten males and ten females from each of these 20 isofemale lines were allowed to mate and were then mixed together to form the single mass bred population. The mass bred population was kept at a constant temperature of 25 °C with a 12:12 h light: dark cycle for seven generations prior to the

experiments described below. The mass bred population was maintained at a population size of at least 2000 individuals. Throughout the study, flies were reared on a potato dextrose media (37.32% yeast, 31.91% dextrose, 23.40% potato medium and 7.45% agar combined with 98.48% H_2O, 0.97% ethanol, 0.45% propionic acid and 0.11% nipagen) with live yeast added to the media surface to stimulate oviposition.

A paternal half-sibling design was used to estimate the level of additive genetic variance underlying heat tolerance and several fitness-related morphological traits (Lynch & Walsh, 1998). Generation seven individuals – the parents of the focal flies – were collected over 2 days as virgins. Males and females were sexed under light CO_2 anaesthesia and held in separate vials by sex, at a density of approximately 20–30 individuals per vial until 4 days old.

One hundred virgin males (sires) were randomly selected from all possible holding vials for the breeding design. Each sire was placed in a vial containing 6 mL of food media and *ad libitum* live yeast, and with three virgin females (dams). Sires and dams were allowed 4 days to mate. Each dam was then placed individually in a separate vial and allowed to lay eggs for 6–8 h, then moved to a fresh vial and allowed to lay eggs for a further 6 h. This was performed to control larval density to no more than 20 larvae per vial. There were thus two vials of offspring per dam to control for any effects of larval (common) environment on the measured offspring phenotypes.

Generation eight individuals – the focal offspring – were moved to fresh vials after eclosion and allowed to mature and mate over 2 days before being moved to separate holding vials by sex.

Heat tolerance data were collected between five and 6 days after the focal flies had eclosed. At least one focal fly from each laying vial per dam (up to a maximum of four males and four females focal offspring per sire-dam pair) was scored for heat tolerance. The remaining focal flies were stored at −80 °C so that male and female fitness-related morphological traits could be scored at a later date.

Heat tolerance was assessed over 2 days, with five runs performed each day. For each heat tolerance run, 200–250 flies, spread evenly across families resulting in a balanced design, were placed individually in 5-mL glass vials and submerged in a water bath heated to 38.5 °C by a digital thermo-regulator (Model: TH5; Ratek, Melbourne, Victoria, Australia). Heat knockdown time was recorded as the time (to the nearest second) until a fly was unable to stand (Berrigan, 2000; Hoffmann *et al.*, 2002).

At least one focal female and one focal male from each laying vial (to a maximum of eight females and eight males per sire-dam pair) were scored for reproductive morphologies. The number of ovarioles present within the female reproductive tract has been shown to be positively correlated with female reproductive output in this species (Cohet & David, 1978; Bouletreau-Merle *et al.*, 1982). Previous studies have shown that this trait is not affected by body size (Wayne *et al.*, 1997; Telonis-Scott *et al.*, 2005). Reproductive organs were removed from each focal female, and the total number of ovarioles present within each female counted. Accessory gland and testis size were scored in males. *Drosophila* species comparisons have shown that investment in reproductive tissue is positively associated with sperm quality and quantity (Pitnick & Markow, 1994; Pitnick, 1996), and it is known from other species that testes size increases under post-copulatory sexual selection (Hosken & Ward, 2001; Gay *et al.*, 2009). Furthermore, larger accessory glands are associated with greater sex peptide production and reproductive success in *D. melanogaster* (Wigby *et al.*, 2009). Reproductive organs were removed from each focal male and photographed at ×400 magnifications under a stereomicroscope. The area of accessory glands and testes within each male was measured from these photographs using ImageJ, version 1.38 (Rasband, 2007). The average of the two accessory glands and two testes was determined for each individual measured.

In order to determine whether testes and/or accessory gland size were related to overall body size, the wing size of all focal males assayed for accessory and testes size was also measured. Wing size has been shown to be a good proxy for body size in *Drosophila* (Misra & Reeve, 1964; Azevedo *et al.*, 1998). One wing was removed from each focal male, mounted on a slide with double-sided tape and protected by a glass cover slip. Wings were photographed under a stereomicroscope. The left wing was used to determine wing size unless it had been damaged. Wing images were landmarked at eight standard points (Kellermann *et al.*, 2006) using tpsDig2, version 2.16 (Rohlf, 2006). Wing area was then calculated from the relative distances between landmarks using CoordGen6 (Sheets, 2003).

Estimating the additive genetic variance covariance matrix, G

Our data were generated from a standard paternal half-sibling breeding design (Lynch & Walsh, 1998). The mixed model used to analyse the data was

$$y = \alpha + \mathbf{XB} + \mathbf{YS} + \mathbf{Z}_s\delta_s + \mathbf{Z}_d\delta_d + \varepsilon \qquad (2)$$

where $\mathbf{X}$ is the design matrix for the fixed effect of run, $\mathbf{B}$ (described below), $\mathbf{Y}$ is the design matrix for the fixed effect of sex, $\mathbf{S}$, and $\mathbf{Z}_s$ and $\mathbf{Z}_d$ are the design matrices for the random effects of sire and dam, respectively. The total phenotypic variance for the breeding design for the purpose of estimating genetic parameters was represented by

$$\sigma^2_P = \sigma^2_S + \sigma^2_D + \sigma^2_W \qquad (3)$$

We first estimated the additive genetic variance for each trait using a univariate model. Log likelihood ratio tests

were performed to determine whether we were able to detect significant levels of additive genetic variance for each trait. We then estimated the unconstrained **G** matrix. In both cases, the variance at both the sire, δ_s, and the dam, $\delta_{d,}$ levels was modelled using an unstructured covariance matrix. The additive genetic variance and covariance components of **G** were individually tested for significance from zero by performing log likelihood ratio tests (Littell *et al.*, 1996; Simonsen & Stinchcombe, 2010).

Dimensions of G

Three complimentary approaches were used to estimate the number of dimensions of **G**, that is, to examine the distribution of genetic variance in multivariate space.

Eigen analysis of G – estimating $\mathbf{g_{max}}$

Eigen analysis of the unconstrained additive genetic variance covariance matrix, **G**, was first performed to determine how many genetically independent traits (eigenvectors) were represented by the original traits (phenotypes) actually measured, and how much genetic variance (eigenvalues) was associated with each independent set of eigenvectors. The eigenvector with the largest eigenvalue ($\mathbf{g}_{max}$, (Schluter, 1996) is the vector explaining most of the additive genetic variance in the **G** matrix.

Factor-analytic modelling of specific and common genetic variance

Factor-analytic modelling has also been used to determine how many dimensions of **G** contain significant amounts of genetic variance, the strength of genetic covariance among traits and sampling variance (Kirkpatrick & Meyer, 2004; Meyer & Kirkpatrick, 2005, 2008; Hine & Blows, 2006). The factor-analytic approach involves modelling a reduced covariance matrix ($\sum$) for the random effect representing the additive genetic variance (the sire-level term in a paternal half-sibling breeding design). The reduced-rank covariance matrix is given as

$$\sum = \Lambda\Lambda^T \tag{4}$$

where Λ is a $p \times m$ lower triangular matrix of constants representing factor loadings of the m latent factors. This model, which is analogous to a principal components analysis, explicitly assumes that all genetic variance is shared among traits, and that trait-specific variances are zero. As in any principal component analysis, the reduced-rank covariance matrix ($\sum$) can be represented by its eigenvalues and eigenvectors.

We were interested in estimating and interpreting trait-specific genetic variances. We therefore modelled a factor-analytic covariance structure that included trait-specific variances as well as the common variance–covariance matrix. Under this covariance structure, the reduced-rank additive genetic covariance matrix is given by

$$\sum = \Lambda\Lambda^T + \psi \tag{5}$$

where ψ is a $p \times p$ diagonal matrix of the specific variances for each trait. To be consistent with factor analysis terminology, we refer to the independent variances (ψ) as trait-specific variance, and the remaining genetic variance captured by the factors in $\Lambda\Lambda^T$ as the common or shared genetic variance (McGuigan & Blows, 2010). The total genetic variance is not directly estimated by **G** matrices of reduced rank, but rather corresponds to the genetic variance estimated when the covariance structure of **G** is modelled in an unconstrained manner. In this study, we focus on the trait-specific genetic variance, the proportion of the total (unconstrained) additive genetic variance that was trait specific (McGuigan & Blows, 2010) because we specifically wanted to ask whether male and female heat tolerance were free to respond to selection for increasing heat tolerance independently, or whether they might be constrained in their response to such selection by a shared genetic basis with each other or other traits.

In performing the factor analytic analyses, the variance at the dam level, δ_d, was modelled using an unstructured covariance matrix, whereas the variance at the sire level, δ_s, was modelled using an unstructured covariance matrix and the factor analytic covariance structures given in eqns (4) and (5). Since the factor analytic covariance structures were fit within a mixed model, individual elements (such as the trait-specific additive genetic variances) could be tested for significance using a series of nested log likelihood ratio tests (McGuigan & Blows, 2010). All analyses were implemented under the MIXED procedure in SAS (version 9.1; SAS Inc., Cary, NC, USA) using restricted maximum likelihood.

Following McGuigan & Blows (2010), we took two steps to analysing the data. First, we determined the rank of **G** (sire-level covariance matrix) under the hypotheses: (i) no specific variance, as in eqn (4) and (ii) specific variance, as in eqn (5). To fit the covariance structures corresponding to models (4) and (5), we used the TYPE = FA0(m) and TYPE = FA(m) statements at the sire level of (2). Log likelihood ratio tests were applied to determine which value of (m) best explained the data, that is, what the statistically supported number of dimensions of **G** were (Hine & Blows, 2006).

We then used the Akaike information criterion (AIC) to identify the best overall fit from the FA(m) and FA0(m) models. This comparison specifically tests whether modelling specific variances improved model fit over a model in which specific variances were assumed to be zero, and thus whether specific additive genetic variance

accounted for significant variation in the suite of traits (McGuigan & Blows, 2010). We complimented this test of specific variances with log likelihood ratio tests of significance of individual trait-specific variances using the PARMS statement in Proc MIXED for the best FA(m) model to hold the specific variance to zero, testing one trait at a time. The AIC comparison of model fit between FA(m) and FA0(m) models provides a more sensitive test of specific variances in the traits measured because it tests the hypothesis that there is specific variance across all traits simultaneously (McGuigan & Blows, 2010).

Kirpatrick's dimensionality approach

Finally, to further explore the dimensions of the **G** matrix for the traits considered, we used the approach outlined by Kirkpatrick (2009), which strictly considers the geometry of **G** without regard to the direction of selection and the predicted response. This method determines the effective number of dimensions, n_D, in a **G** matrix by measuring whether there is an even distribution of genetic variation explained by all the eigenvalues of the **G** matrix estimated from the unconstrained model. If most of the genetic variation occurs in the first one or two dimensions, that matrix is ill conditioned and will permit evolution in only a few dimensions. Kirkpatrick suggests measuring n_D as the sum of the eigenvalues of **G** divided by the largest eigenvalue. If n_D is close to 1, most of the genetic variation in **G** is explained by the first and largest eigenvalue, and the matrix has an effective dimension of 1.

Additive genetic correlations between all traits

To complement the multivariate methods described above, we also estimated the additive genetic correlation between all six traits examined using the relationship

$$r_{S(1,2)} = \frac{\mathrm{Cov}_{S(1,2)}}{\sqrt{\sigma^2_{S1} \times \sigma^2_{S2}}} \quad (6)$$

where $\mathrm{Cov}_{s(1,2)}$ is the sire-level additive genetic covariance between traits 1 and 2, and σ^2_{S1} and σ^2_{S2} are the sire-level variance components for traits 1 and 2. Log likelihood ratio tests were used to test whether any of the additive genetic correlations were significantly from both zero and one.

Results

Means and phenotypic variances for all traits are displayed in Table S1. We observed significant sexual dimorphism in heat tolerance. An unpaired equal variances t-test showed that mean heat knockdown time was significantly higher in males ($t = -30.397$, $df = 1836$, $P < 0.0001$) than in females. On average, males took 9 min 38 s longer to be knocked down by the heat stress than females (Table S1).

Genetic variation and covariation in heat tolerance and fitness traits

Significant levels of additive genetic variance were detected for all six traits examined (Table 1). Additive genetic covariances between female and male heat, male heat and ovariole number and male wing size and accessory gland size were positive and significantly different from zero (Table 1).

Four of the pairwise additive genetic correlations were significantly different from zero (Table 1). Significant, positive genetic correlations were found between male and female heat tolerance, male heat tolerance and ovariole number, male wing size and testis size, and male wing size and accessory gland size (Table 1). All four of these genetic correlations were significantly different from zero and one (Table 1), implying that both correlated and independent evolutionary responses to selection pressures would be possible.

Eigen analysis of G – estimating $\mathbf{g}_{max}$

The eigen analysis revealed an uneven distribution of genetic variance in **G**. The first three eigenvectors of **G** accounted for 92.22% of the total additive genetic variance of **G** (Table 2). The leading eigenvector ($\mathbf{g}_{\max}$) accounted for 54.83% of the variance in **G**. Female and male heat tolerance and ovariole number loaded positively to $\mathbf{g}_{\max}$, with negative contributions from the remaining three traits. Female heat tolerance made the largest contribution to this vector (Table 2). All traits, except for female heat, loaded positively onto the second eigenvector of **G** ($\mathbf{g}_2$), with the largest contribution coming from male wing size and ovariole number. Ovariole number made the largest (positive) contribution to the third eigenvector ($\mathbf{g}_3$) (Table 2).

Factor-analytic modelling of specific and common genetic variance

Since additive genetic variation was detected for all six traits examined (Table 1), we had sufficient statistical power to test hypotheses about the partitioning of additive genetic variation into specific vs. common genetic factors. Analyses determined that the FA(2) and FA0(5) models best described the additive genetic variance in all traits examined (Table 3). The AIC supported FA(2) as the model that best described the data (Table 3) indicating support for significant trait-specific additive genetic variation in the traits measured. All of the six traits were associated with nonzero estimates of specific additive genetic variation, although only three of these, ovariole number, testes size and accessory gland size, had specific variances that were significantly different from zero (Table 4). The proportion of the total genetic variance that was trait-specific (autonomous genetic variation, Hansen & Houle, 2008) in each of the traits

Table 1 Additive genetic variance and covariance matrix (**G**) estimated from the model with unconstrained sire-level variances and covariances, and additive genetic correlations. Additive genetic variances on the diagonal, additive genetic covariances above the diagonal, additive genetic correlations below the diagonal.

	Female heat	Male heat	Ovariole number	Testis size	Accessory gland size	Male wing size
Female heat	46.004*	13.7508*	6.3828	−1.278	−1.4584	−4.9884
Male heat	0.6780***††	9.2872*	5.4352**	0.1679	0.2172	−1.5336
Ovariole number	0.2330	0.4330***††	16.9388*	−0.9188	1.9444	3.3516
Testis size	−0.1588	0.0454	−0.1846	1.4663*	−0.1674	1.3356
Accessory gland size	−0.1276	0.0415	0.2751	−0.0808	2.9516*	2.7084*
Male wing size	−0.1718	0.1154	0.1888	0.2531***††	0.3613†,††	19.0328*

*$P < 0.05$; **$P < 0.07$ for log likelihood ratio test of significant difference from zero.
***$P < 0.05$; †$P = 0.053$ log likelihood ratio test of significant difference from zero.
††$P < 0.05$ log likelihood ratio test of significant difference from one.

Table 2 Eigen analysis of genetic variation for all traits examined. Trait loadings on eigenvectors of the unconstrained sire-level additive genetic variance covariance matrix (**G**), the additive genetic variance, V_A, (eigenvalue) associated with each eigenvector and the percentage of the total additive genetic (% V_A) variance explained by each eigenvector.

	$\mathbf{g}_{max}$	$\mathbf{g}_2$	$\mathbf{g}_3$	$\mathbf{g}_4$	$\mathbf{g}_5$	$\mathbf{g}_6$
V_A	51.51	22.33	12.81	3.96	2.38	0.97
% V_A total	54.82	23.76	13.63	4.21	2.53	1.03
Female heat	0.9109	−0.0489	−0.3104	−0.2485	0.0434	0.0889
Male heat	0.3278	0.1066	0.1558	0.8875	−0.0314	−0.2610
Ovariole number	0.2058	0.5915	0.7207	−0.2479	−0.1279	−0.1027
Testis size	−0.0295	0.0262	−0.0913	0.2849	−0.2551	0.9186
Accessory gland size	−0.0252	0.1728	0.0304	0.0859	0.9518	0.2349
Male wing size	−0.1375	0.7783	−0.5922	0.0225	−0.0985	−0.1198

ranged from 9% to 92% (Table 4). Male heat tolerance had the lowest level of trait-specific additive genetic variance (9%, Table 4), followed by female heat tolerance and male wing size (Table 4). Overall, the specific variances accounted for 43.26% of the total additive genetic variation in the traits examined.

The first two eigenvectors of **G** from the FA(2) and the FA0(5) models were very similar (Table 5; vector correlation between $\mathbf{g}_{S1}$ and $\mathbf{g}_{N1}$, and between $\mathbf{g}_{S2}$ and $\mathbf{g}_{N2}$ was 0.89). The leading eigenvector accounted for 39% [FA(2)] or 55% [FA0(5)] of the additive genetic variance and was determined both by contributions from traits with low trait-specific variance, namely female and male heat tolerance and male wing size, and high trait-specific variance (ovariole number, testes size and accessory gland size; Tables 4 and 5), but with the largest contributions from female and male heat tolerance.

The second eigenvector accounted for 16% [FA(2)] or 24% [FA0(5)] of the additive genetic variance and was once again determined by contributions from traits with low trait-specific variance (male wing size) and high trait-specific variance (ovariole number; Tables 4 and 5), but with the largest contribution from male wing size. The third eigenvector from the FA0(5) model was associated with large trait-specific variance; $\mathbf{g}_{N3}$ was dominated by ovariole number, with opposing effects of male wing size (Tables 4 and 5).

Table 3 Genetic model fit for all traits examined. Model-fit information (number of parameters estimated, −2 log likelihood score, and the Akaike Information criterion (AIC) score) from REML mixed models of genetic variation in six thermotolerance and fitness-related traits when specific variances for each trait were explicitly estimated [FA(*m*)] or zero trait-specific variances were assumed [FA0(m)]. The difference in −2 log likelihood scores gives a statistic with a χ^2 distribution; degrees of freedom are equal to the difference in the number of parameters estimated by each model. The genetic variance explained by each model is given as the percentage of the total additive genetic variance (V_A), which was determined from a model with unconstrained sire-level variances. Best AIC model fit is shown in bold.

Model	Parameters	−2 Log likelihood	AIC	χ^2 statistic	% V_A
No sire variance†	6	33618.5	33630.5		
FA(1)	18	33230.3	33264.3	388.21*	95.74
FA(2)	23	33217.3	**33263.3**	13.00*	99.48
FA(3)	27	33213.1	33265.1	4.20	99.90
FA(4)	30	33213.0	33273.0	0.10	100
FA0(1)	12	33430.0	33452.0	188.50*	21.80
FA0(2)	17	33314.9	33348.9	115.10*	74.74
FA0(3)	22	33257.0	33299.0	57.90*	82.36
FA0(4)	24	33225.1	33275.1	31.90*	88.67
FA0(5)	26	33216.2	33266.2	8.90*	95.53
FA0(6)	27	33213.0	33267.2	3.2	99.76

*$P < 0.05$ for log likelihood ratio test.
†This model does not contain a sire-variance covariance matrix and is the lowest level in the hierarchical comparison of log likelihood statistics for both the FA(1) and FA0(1) models.

Dimensionality of G

Using Kirkpatrick's (Kirkpatrick, 2009) method, we estimated $n_D = 1.82$. This value n_D implies that most of the genetic variation in **G** is explained by the first and

Table 4 Common (shared) and trait-specific additive genetic variances (V_A) for heat tolerance and fitness traits. The additive genetic variance that is explained by trait-specific variance is given as the percentage of total trait-specific additive genetic variance for each trait, determined from a model with unconstrained sire-level variances.

Trait	Common V_A†	Trait-specific V_A†	% Trait-specific V_A
Female heat knockdown	26.979	17.102	37.175
Male heat knockdown	8.234	0.874	9.41
Ovariole number	4.210	12.642*	74.63
Testis size	0.103	1.351*	92.34
Accessory gland size	0.630	2.311**	78.29
Male wing size	9.192	7.114	37.38

*$P < 0.05$; **$P = 0.0607$.
†Estimated from the best-fit model in Table 2.

Table 5 Eigen analysis of genetic variation for all traits examined. Trait loadings on eigenvectors of each **G**, the additive genetic variance (eigenvalue) associated with each eigenvector and the percentage of the total additive genetic variance (from the unconstrained model) explained by each eigenvector.

	FA(2)		FA0(5)		
	$\mathbf{g}_{S1}$	$\mathbf{g}_{S2}$	$\mathbf{g}_{N1}$	$\mathbf{g}_{N2}$	$\mathbf{g}_{N3}$
V_A	9.159	3.859	12.831	5.557	3.150
% V_A total	38.99%	16.43%	54.63%	23.66%	13.80%
Female heat	0.8553	−0.1075	0.9077	0.2065	0.1186
Male heat	0.4404	0.2702	0.3016	0.2381	−0.0469
Ovariole number	0.2379	0.3719	−0.2313	0.5104	0.8145
Testis size	−0.0355	0.0607	−0.0237	0.0223	−0.1194
Accessory gland size	−0.0229	0.1989	−0.0473	0.1633	0.0361
Male wing size	−0.1270	0.8566	−0.1698	0.7829	−0.5521

largest eigenvector, with a smaller contribution in this case from the second eigenvector. Thus, **G** has an effective dimension of 1 or 2. This is consistent with the eigen analysis of the unconstrained **G** matrix (Table 2) that indicates that the leading eigenvector ($\mathbf{g}_{max}$) accounts for more than half of all genetic variation in **G**.

Discussion

The genetic basis of traits, and in particular the genetic covariation among traits, is expected to constrain the direction and rate of phenotypic evolution (Lande, 1979; Lande & Arnold, 1983; Falconer & Mackay, 1996; Lynch & Walsh, 1998). Understanding the extent to which traits are genetically independent is therefore central to understanding and identifying evolutionary constraints (Schluter, 1996; Hansen & Houle, 2008; Agrawal & Stinchcombe, 2009; Kirkpatrick, 2009; Walsh & Blows, 2009). Previous studies that have looked at the potential for the evolution of increased heat tolerance in response to a warming climate have taken a univariate or bivariate approach and studied either one sex only or pooled both sexes together in their analyses. Moreover, few studies have examined whether negative genetic covariances between traits important in thermal responses and those important in reproductive success could constrain an adaptive response to changing climates, within or across the sexes.

Thus, the motivation of our study was to take a multivariate approach to determine the extent to which a response to thermal selection might be constrained by additive genetic variances for, and covariances between, heat tolerance and key life-history traits within and between the sexes. We first showed that significant additive genetic variance is present for all six traits examined. We then determined that the additive genetic variance–covariance matrix, **G**, is of reduced rank, with three eigenvectors explaining more than 90% of the total additive genetic variance in **G**, with $\mathbf{g}_{max}$ and $\mathbf{g}_2$ accounting for 78%. Female heat tolerance, followed by male heat tolerance and ovariole number, made the largest contribution to the leading eigenvector of **G** ($\mathbf{g}_{max}$), suggesting that selection for increased heat tolerance (in the direction of $\mathbf{g}_{max}$) should result in an evolutionary increase in heat tolerance in both sexes. The observation of reduced dimensionality of **G** is consistent with Kirkpatrick's (2009) study, where most estimates of n_D were close to one. Thus, the genetic variance in **G** is distributed unevenly, enabling responses to selection for a limited number of trait combinations.

Most genetic correlations between homologous traits in males and females are expected to be large and positive because the sexes essentially share the same genome, and thus, selection on either of the sexes should result in closely correlated responses in the other sex (Lynch & Walsh, 1998; Poissant *et al.*, 2010). It has been argued that such correlated responses to selection in males and females should constrain the evolution of sexual dimorphism (Lynch & Walsh, 1998). Although we found a large difference between male and female mean heat knockdown time, we also showed that the genetic correlation between male and female heat tolerance was significantly less than one. This suggests that both shared and trait-specific additive genetic variances contribute to female and male heat tolerance. In addition, all of the significant additive genetic correlations were significantly less than one, implying both a shared and a trait-specific component to the underlying additive genetic variance for the traits examined.

To examine this in more detail, we then applied the analytical approach developed by McGuigan & Blows (2010) to partition genetic variance in thermal tolerance and fitness-related traits to independent (trait-specific) vs. nonindependent (common) variance. We implemented factor analytical modelling within the hypothesis-testing framework outlined by McGuigan & Blows (2010) to explicitly address questions about the effect of a shared

genetic basis on the potential for independent phenotypic evolution in the six traits considered.

Across all of the traits examined, 43% of the total additive genetic variation in the thermal tolerance and fitness-related traits was observed to be trait specific, although only three of these traits were associated with significant levels of unique (trait specific) additive genetic variation. Our analyses indicate that trait-specific additive genetic variance contributes to male and female heat tolerance. This suggests that independent evolution of heat tolerance in males and females should be possible. Indeed, the significant sexual dimorphism in heat tolerance that we observed indicates that independent evolution of this trait in the population examined has taken place in the past. Taken together, our analyses of heat tolerance in males and females suggest that both independent, and correlated, responses to thermal selection are possible, at least within the population we examined here.

Our results contradict the expectation that, because the sexes essentially share the same genome, selection on either of the sexes should result in closely correlated responses in the other sex (Lynch & Walsh, 1998) and hence constrain the evolution of sexual dimorphism. In a recent review of 114 studies of bivariate cross-sex genetic correlations, Poissant *et al.* (2010) found that cross-sex genetic correlations were usually large and positive. However, such an analysis precludes insight into the role that trait variances and covariances play on phenotypic evolution that comes from multivariate analyses. In our study, whereas female and male heat tolerance were indeed positively genetically correlated to each other, male heat tolerance was also positively correlated with ovariole number, and all three traits loaded positively to $\mathbf{g}_{max}$, implying that direct selection for heat tolerance (in the direction of $\mathbf{g}_{max}$) could result in similar evolutionary trajectories in all three traits.

However, we should also note that a limitation of analyses that are based on half-sibling breeding designs is that they cannot explicitly partition out the variance attributable to the X chromosome (Falconer & Mackay, 1996; Lynch & Walsh, 1998). Furthermore, sons do not receive a copy of the X chromosome from the sire in a half-sibling design, whereas daughters do. Any additive genetic effects caused by alleles on the X chromosome will thus be included in the additive genetic variance component estimates for daughters, but not sons, thereby underestimating the additive variance estimates in males. This technical discrepancy is likely to be significant in *Drosophila* species given that the X chromosome represents about one-third of the haploid *D. melanogaster* genome (Celniker & Rubin, 2003), and therefore, the contribution of the X chromosome to the genetic variance of any given trait is likely to be substantial. Indeed, genetic studies of quantitative trait loci (QTLs) have indicated that the percentage of phenotypic variation in male heat knockdown time explained by genes that occur on the X chromosome is between 15% and 20% (Norry *et al.*, 2004, 2007). Nonetheless, given that sons did not receive a copy of the X from their fathers, this then suggests that the large and significant positive additive genetic correlation between female and male heat knockdown, revealed in our study, largely reflects shared autosomal genetic variance for this trait in this population. Furthermore, cross-sex genetic correlations estimated from paternal half-sibling data are in fact likely to be downwardly biased due to the inclusion of autosomal-only variance in the male–female covariance [numerator (6)], autosomal-only variance in the male variance component but autosomal plus X-linked variance in the female variance component [denominator (6)].

To directly test the extent to which males and females share the autosomal vs. X-linked additive genetic variance for heat tolerance, we compared the intersex genetic correlation that explicitly includes X-linked additive genetic variance (the total additive genetic correlation) with the genetic correlation that only includes autosomal additive genetic variance (autosomal additive genetic correlation) following Cowley & Atchley (1988) and Chenoweth & Blows (2003) (Appendix S1). We found that both genetic correlations were similarly large and positive. This tells us that males and females share much of the autosomal and X-linked additive genetic variance for heat tolerance (Chenoweth & Blows, 2003). Thus, our results are unlikely to be confounded by the paternal half-sibling breeding design we used.

Two scenarios might then explain the presence of the observed sexual dimorphism in heat tolerance. Firstly, our results are consistent with the idea that males have been under stronger directional selection for heat tolerance, historically, driving a greater tolerance in males, but also depleting the genetic variance for this trait in males below that of females. Secondly, a history of strong directional selection on female fecundity in this population might have driven increases in male heat tolerance beyond those of female tolerance, given the strong positive intersexual correlation between ovariole number in females and heat tolerance in males. Clinal patterns in ovariole number in *D. melanogaster* suggest selection on female fecundity in nature (Azevedo *et al.*, 1996). It is also possible that fecundity selection may have resulted during laboratory adaptation (Sgrò & Partridge, 2000), although we performed these experiments at generation eight of laboratory culture in an effort to minimize this occurring. An empirical test of this argument, however, requires both an estimate of the additive genetic variance–covariance matrix (**G**) and the vector of directional selection gradient, β, for all traits. Whereas we have estimated the former, we do not have direct estimates of β for any of the traits examined. We can only infer the role of natural selection from clinal studies of *D. melanogaster* from eastern Australia that demonstrate clines in heat tolerance (Hoffmann *et al.*, 2002; Sgrò *et al.*, 2010), ovariole number (Azevedo *et al.*, 1996) and body (wing)

size (James *et al.*, 1995; van Heerwaarden & Sgrò, 2011) that have been shown to result from selection rather than genetic drift.

We have sampled just one population along a latitudinal cline where we know that the main trait of interest (heat tolerance) differs in expression along the cline, at least in females. Although we know the clinal patterns in expression of this trait for females, what we do not know is how much phenotypic expression in this trait changes in males along the cline (although sexual dimorphism in heat tolerance is present in the cline end populations of Cairns and Melbourne, C. M. Sgrò unpublished), nor do we have empirical measures of the vector of directional selection in nature. Such knowledge would provide us with a clear indication of the strength of directional thermal selection on males relative to females along the cline. Furthermore, although we found positive genetic correlations between thermal traits across sexes, and between female fecundity and male thermal tolerance in the mid-latitudinal population that we sampled, these genetic correlations might erode or differ in magnitude or sign in other populations along the cline, and this will be an avenue for further research.

Finally, we found no evidence to suggest that covariances between heat tolerance and key life-history traits will constrain the evolution of higher levels of heat tolerance. The expectation of ongoing selection for increased heat tolerance under climate change has renewed interest in the extent to which adaptation to thermal stress will result in trade-offs in performance or fitness at different temperatures. Previous studies have found mixed evidence for such trade-offs. Laboratory selection experiments in *Drosophila* have provided evidence that some measures of performance at extreme temperatures are correlated with shifts in performance at moderate temperatures (Huey & Kingsolver, 1993; Gilchrist *et al.*, 1997; Hoffmann *et al.*, 2003). Studies using laboratory experimental evolution in microbes have observed trade-offs between performance across different environments and thermal tolerance in some instances (Cooper *et al.*, 2001; Knies *et al.*, 2006), but not others (Bennett & Lenski, 1993; Knies *et al.*, 2009). Finally, recent studies utilizing the intertidal marine copepod *Tigriopus californicus* suggest that thermal adaptation might indeed be constrained by fitness trade-offs (Willett, 2010) or limited genetic variation in the direction of selection (Kelly *et al.*, 2012). Yet, none of these studies have examined the importance of constraint to thermal adaptation in a multivariate context. This current study is the first to do so, albeit in a single population of *D. melanogaster* in a single environment.

In conclusion, using a combination of multivariate and bivariate approaches, we found no evidence to suggest that the evolution of heat tolerance will be constrained in response to selection for increasing heat tolerance expected to occur under climate warming in the population of *D. melanogaster* examined. Further multivariate studies of the importance of multivariate constraints on the evolution of thermal tolerance, which not only examine populations sampled from along the species distribution, but also explicitly consider multiple environments, are needed.

Acknowledgments

Research was supported by Australian Research Council funding to CMS, and funds from Monash University to CMS and DKD. The authors thank Richard Lee, Fiona Cockerell and Winston Yee for their assistance with the heat tolerance assay, and Katrina McGuigan and Steve Chenoweth for discussions on analytical approaches.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Testing for X-linked contributions to the male heat tolerance.

Table S1 Mean (± standard error) and total phenotypic variance (V_P) for all traits measured.

Table S2 Design matrix of causal variance components.

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Received 28 February 2012; accepted 6 April 2012