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The Genetic Architecture and Evolution of Continuous Reaction Norms: Dissecting the Thermal Performance Curve

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Abstract

Genotypes can seldom achieve maximal performance across all environments they inhabit. Instead, increased performance in one environment often comes at a cost of lower performance in another. Such effects are the consequence of genotype-by-environment interactions, which are usually studied using reaction norms—functions describing the relationship between trait and environment. When the environmental variable is continuous rather than discrete, a continuous reaction norm is investigated, also known as function-valued or infinite dimensional traits. Through the study of continuous reaction norms, trade-offs that shape evolutionary trajectories can be identified. Whilst reaction norms have been studied extensively, we know little about their genetic basis, specifically their mutational inputs and the types of genes and genetic effects involved.

Thermal performance curves (TPC) are a specific, but common, class of continuous reaction norm mapping the response of a performance-associated trait to environmental temperature. TPCs are commonly used to explore the trade-offs involved in thermal adaption for ectothermic organisms. Three major 'modes' of variation have been identified; a 'faster-slower' axis representing overall increases or decreases in performance regardless of temperature, a 'hotter-colder' axis representing a trade-off between hot and cold temperatures, and a 'generalist-specialist' axis which explores a trade-off between the range of temperatures across which an organism can perform effectively and the maximal level of performance it can achieve. In this thesis, I have conducted three empirical studies using locomotor activity in two *Drosophila* species to investigate the genetic architecture of TPCs.

My first study investigated the contribution of new mutations to TPCs through a mutation accumulation assay followed by an eigendecomposition of the mutational variance-covariance matrix, **M**. Three independent axes of mutational variance were investigated that corresponded to the three major axes of TPC variation. In contrast to its near-absence from standing variation in this species, a 'faster-slower' axis, accounted for most mutational variance (75% in males and 66% in females) suggesting selection may easily fix or remove these types of mutations in outbred populations. Axes resembling the 'hotter-colder' and 'generalist-specialist' modes of variation contributed less mutational variance but nonetheless point to an appreciable input of new mutations to the two major trade-offs involved in thermal adaptation.

In my second study, I began to dissect the genotype-phenotype map for TPCs. A multivariate quantitative trait loci (QTL) analysis was performed on a panel of recombinant inbred lines (RILs) derived from an inter-population cross of *Drosophila serrata*. I found that vectors of QTL effects across temperatures were well-aligned with the major axes of genetic variance in the RIL population. Most QTL effects resembled either a 'faster-slower' or 'hotter-colder' axis whereas very few resembled 'generalist-specialist' like variation and those that did had small effects. Strong and directionally-biased transgressive segregation was also detected, consistent with weak selection between the two founder populations and a highly polygenic basis to TPC variation.

In the final study, I dissected the genetic architecture locomotor activity TPCs in a single population of *Drosophila melanogaster.* A genome-wide association study (GWAS) was conducted by assaying the TPC variation in 152 lines from the Drosophila Genetic Reference Panel (DGRP). The analysis was performed on four major components of the TPCs that were statistically extracted using a function-valued trait analysis (TMV) and on the first five principal component (PC) scores in an attempt to identify genomic elements underlying the biologically important axes of TPC variation. I identified polymorphisms associated with 'overall height', optimum temperature, 'generalist-specialist' and maximum performance TPC components of variation and the first five PC axes. Intriguingly, for components underlying thermal trade-offs, I observed strong skew in the direction in which the minor-frequency allele affected a TPC component and some of the PC axes, suggesting a role for natural selection in shaping standing variation. Although most significant variants were located within or near coding genes, they were located in areas more consistent with the regulation of gene expression than altering coding sequences, suggesting that changes in gene expression may be particularly important. Limited pleiotropic effects were detected between different TPC components possibly reflecting their highly optimised statistical partitioning along different trade-off axes. Gene ontology term enrichment analysis, revealed a degree of functional independence between the different modes of variation, which may translate into evolutionary independence between them. Where functional overlap did occur, it primarily involved neurological functioning and responses to stimuli.

My thesis places TPCs as highly complex traits, integrating a significant number of biological functions. It is clear that genetic variance is far from being equally distributed across the three major components of variation—likely a consequence of both contrasting

mutational inputs and differences in the efficacy of natural selection to shape standing variation. A role for selection in shaping standing variation is implicated in both species through either mismatches between patterns of mutational and standing variation or associations between allele frequency and the directions of additive effects on phenotype. Whilst this thesis has outlined the evolutionary genetic architecture of a single type of TPC, genomic-level investigation into these continuous reaction norms is only beginning.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

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Latimer, C. A. L., B. R. Foley, and S. F. Chenoweth. 2015. Connecting thermal performance curve variation to the genotype: a multivariate QTL approach. Journal of Evolutionary Biology 28:155-168.

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Contributor	Statement of contribution
Latimer, C. A. L. (Candidate)	Designed/implemented TPC assay (90%) Statistical Analysis (75%) Wrote/edited the paper (60%)
McGuigan, K.	Designed and created mutation accumulation

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Wilson, R. S	Wrote/edited the paper (5%)
Blows, M. W.	Designed and created mutation accumulation lines (20%) Wrote/edited the paper (5%)
Chenoweth, S. F.	Designed/implemented TPC assay (10%) Statistical Analysis (25%) Wrote/edited the paper (25%)

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Contributor	Statement of contribution
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Foley, B.	Designed and created the recombinant inbred lines used for assaying (100%) Wrote/edited the paper (5%)
Chenoweth, S. F.	Designed/implemented TPC assay (10%) Statistical Analysis (20%) Wrote/edited the paper (25%)

Contributions by others to the thesis

Conception and design of the project, critically revising: S. Chenoweth, R. Wilson

Analysis and interpretation of research data: S. Chenoweth

Creation of different panels of inbred lines: K. McGuigan/ W.M. Blows (Chapter 2), B. Foley (Chapter 3) and T. Mackay (Chapter 3)

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Keywords

thermal performance curves, trade-offs, evolutionary potential, genetic architecture.

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List of Abbreviations used in the text

Abbreviation	Un-abbreviated
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CRN	Continuous Reaction Norm
CVm	Mutational coefficient of variation
d	Multivariate vectors of divergence
DAM	Drosophila activity monitor
dCAPS	derived cleaved amplified polymorphic sequences
DGRP	Drosophila genetic reference panel
EST	Expressed sequenced tags
FDR	False discovery rate
G	Genetic variance-covariance matrix
GEI	Genotype-by-environment interaction
$\mathbf{G_f}$	Genetic variance-covariance matrix for females
$\mathbf{G_m}$	Genetic variance-covariance matrix for males
GO	Gene ontology
GOEAST	Gene ontology enrichment analysis

	software toolkit
GWA	Genome wide association
GWAS	Genome wide association study
h^2_m	Mutational heritability
M	Mutational variance-covariance matrix
m	Marker effect vectors
MA	Mutation accumulation
MANOVA	Multivariate analysis of variance
$\mathbf{M_f}$	Mutational variance-covariance matrix for females
$\mathbf{M_m}$	Mutational variance-covariance matrix for males
QTL	Quantitative trait loci
REML	Restricted maximum likelihood
RIL	Recombinant inbred line
RNAi	RNA interference
SNP	Single-nucleotide polymorphism
TMV	Template mode of variation
TPC	Thermal performance curve
V_E	Environmental variance
V_m	Mutational variance per generation

Chapter One: General Introduction

1.1 The Evolutionary Significance of Environmental variation

Understanding how the environment can influence organisms is of key importance to the study of many ecological and evolutionary processes (Schwenk et al. 2009; Angilletta and Sears 2011). External environmental conditions can influence multiple aspects of biological function, from biochemical and physiological processes (Hochachka and Somero 2002 ; Savage et al. 2004) to behaviour (Bennett 1990; Roff 2002) and ultimately fitness (Kingsolver and Watt 1983; Via and Lande 1985; Lynch and Gabriel 1987; Roff 2002). Well-adapted organisms are thought to have phenotypes well suited to the unique challenges of the environment within which they reside (Levins 1968). When an organism is poorly suited to its environment, stress may occur, resulting in consequences such as decreased fertility (Chakir et al. 2002; Marshall and Sinclair 2009; Ribeiro and Borghetti 2014) and survival (Lin et al. 1998; Hoffmann et al. 2003).

An additional complexity is that individuals seldom experience a single environmental condition within a lifetime and so must deal with multiple environmental conditions. Similarly, for a species to persist in the long term, it must be capable of coping with varying modes of environmental variation. Environments can vary spatially, differing between populations from different latitudes or altitudes (Angilletta 2009; Scheiner 2013). The environment also fluctuates over time, which may span timescales spanning thousands of generations such as the coming and going of ice-ages (Petit et al. 1999; Wolff et al. 2010; Scheiner 2013), or within a single generation over the course of seasons or within a single day (Higgins et al. 2007; Scheiner 2013).

The fundamental evolutionary and ecological consequence of a heterogeneous environment arises from the fact that individuals seldom have equal fitness in all environments they experience (Stearns 1989; Shaw et al. 1995; Fry et al. 1996). This inability of organisms to maintain fitness across all environments is commonly referred to as a trade-off, where high fitness in one environment is ‘traded’ for the lower fitness in another environment (Via 1991; Shaw et al. 1995; Lynch and Walsh 1998). In ecology, trade-offs may influence population dynamics. For example, high levels of environmental variation can negatively impact population growth rate and lead to a concomitant decline in population size (Kjaersgaard et al. 2012). Species distributions and invasions can be

affected by the ability of species to perform across a range of environments. For example, invasive species can often take advantage of a broad range of environments compared to indigenous species (Janion et al. 2010). Invasive plants can exploit a wide range of warm, moist and nutrient rich environments (Stohlgren et al. 2008) and invasive invertebrates are often small-bodied with high-growth rates, promoting their performance in novel environments (Lawton et al. 1986; Gaston et al. 2001).

In evolutionary biology, trade-offs are often assumed to constrain phenotypic evolution (Stearns 1992; Roff 2002; Roff and Fairbairn 2007). However, when genetic variation exists for a trade-off, they may also facilitate local adaptation through the evolution of specialisation to local conditions. Here, a specialist's performance can improve for a particular local environment but can decline in other environments via correlated responses to selection (Levins 1968; Huey and Hertz 1984; Angilletta et al. 2003). The empirical study of trade-offs has often focused on fitness components and has included a broad range of taxa, including plants (Chang and Shaw 2003), invertebrates (Fry et al. 1996) and bacteria (Knies et al. 2009). Life-history traits, such as growth rate and fecundity, vary across a range of environments, including different temperatures, humidities and nutrients (Fernandez and LopezFanjul 1997; Knies et al. 2006; Gutteling et al. 2007; Yamahira et al. 2007; Jordan et al. 2012). Behavioural traits, such as walking speed, have been measured as a response to temperature for multiple insect species (Gilchrist 1996; Latimer et al. 2011). Morphological traits, such a bristle numbers in *Drosophila* species, have been measured in response to temperature (Takahashi et al. 2012). While these studies and many others demonstrate the existence of trade-offs, we know comparatively little about how they evolve (Roff and Fairbairn 2007) and specifically, their genetic basis.

1.2 Trade offs and the Quantitative Genetics of Reaction Norms

In quantitative genetics, the response of a genotype to different environments is measured as a reaction norm (Schmalhausen 1949; Falconer and Mackay 1996; Lynch and Walsh 1998). In the simplest form, a reaction norm is a linear function describing the mean phenotypic values for a genotype in two discrete environments (Falconer and Mackay 1996). The slope of a reaction norm contains information about the degree of environmental sensitivity of a genotype. Environment-sensitive genotypes will have a

reaction norm with a non-zero slope (Fig. 1.1a), whereas insensitive genotypes have slopes that cannot be distinguished from zero (Fig. 1.1b).

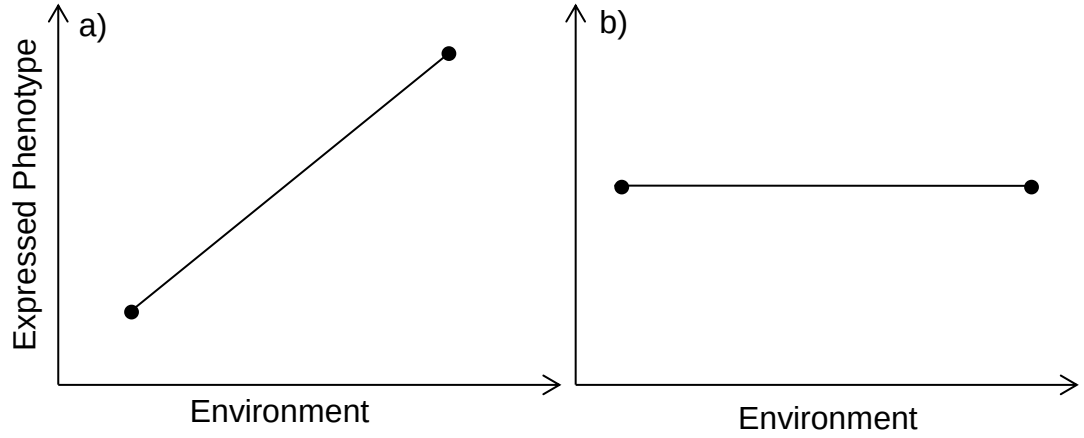

Figure 1.1: Simple reaction norms describing the response of genotypes to two discrete environments a) a sensitive genotype, and b) a non-sensitive genotype.

An opportunity for the evolution of trade-offs is apparent when multiple genotypes in a population differ in the slope of their reaction norms via differences in rank and/or scales across different environments (Lynch and Walsh 1998). A genotype-by-environment interaction (GEI) within a population can arise from quite different patterns of reaction norm variation. If no GEI is present, genotypes will maintain the same scale and rank of phenotype across the environments resulting in parallel reaction norms (Fig. 1.2a). When a GEI exists, non-parallel reaction norms between genotypes will be evident (Via 1987; Fry et al. 1996). Three different patterns can result in non-parallel reaction norms and indicate trade-offs across the environments. The first pattern has genotypes that vary in the scale of their phenotypes between environments (Fig. 1.2b). The third and fourth groups have patterns that represent crossing of reaction norms within populations. The crossing of reaction norms has been used to identify trade-offs between environments (Fry et al. 1996; Xu 2004) and can be due to either variation in rank (Fig. 1.2c) or variation in both rank and scale of the phenotypes (Fig. 1.2d).

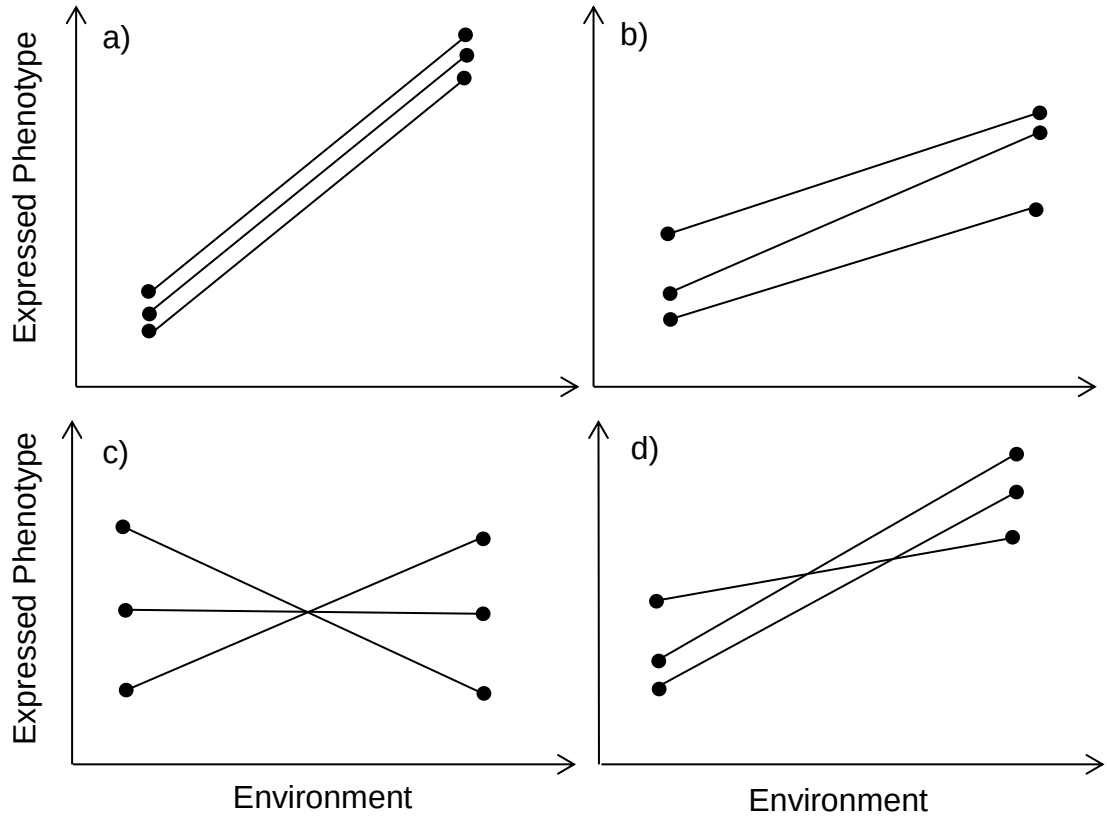

Figure 1.2: A schematic that represents the main patterns of variation in reaction norms for three genotypes, a) no variation in slope, rank or scale of reaction norms indicating no genotype-by-environment interaction or trade-off, b) variation in the scale of reaction norm, c) variation in the rank of reaction norms, and d) variation in the scale and rank of reaction norms. Adapted from Lynch and Walsh (1998).

Cross-environment genetic correlations are commonly used to explore trade-offs and genetic constraints between environments and are estimated from the genetic variance within each environment and the genetic covariance between the environments, whereby $r_{E1,E2} = \frac{Cov_{E1,E2}}{\sqrt{V_{E1} \times V_{E2}}}$ (Falconer and Mackay 1996; Lynch and Walsh 1998). A genetic constraint can arise, for example, when a trait is strongly selected in opposite directions between two environments, but the cross-environment correlation is strong and positive (Via and Lande 1985; Stinchcombe and Kirkpatrick 2012). In general, when a cross-environment correlation is low, the genetic variation is likely to be specific to one environment and selection in that environment will not affect performance in the other environment (Falconer and Mackay 1996; Lynch and Walsh 1998); therefore no trade-offs exists. However, if the trait is strongly genetically correlated between environments, then

selection in one environment will also be dependent upon selection in another environment (Via and Lande 1985; Andersson and Shaw 1994) and a trade-off exists.

However, there are different views on how to interpret genetic correlations to identify trade-offs, particularly regarding the sign of the correlation. A negative genetic correlation is posited in some studies to demonstrate a trade-off because it represents a negative association between the two environments (Lande 1982; Futuyma and Philippi 1987; Bell 1992; Roff and Fairbairn 2007). However, a genetic correlation less than one but different from zero, regardless of its sign, may also demonstrate a trade-off by illustrating that performance is not maximal across all environments (Fry et al. 1996). For example, studies often find positive, rather then negative, genetic correlations between environments (e.g. Van Noordwijk and de Jong 1986; Futuyma and Philippi 1987; Mackay and Lyman 1998; Baer et al. 2006; Ketola et al. 2012). The presence of a trade-off in a positive genetic correlation can be explained by Van Noordwijk & de Jong's (1986) acquisition-allocation model. This model posits that the allocation of resources can influence a negative trade-off, however when variability in the acquisition of resources exists, a positive correlation can potentially exist (Houle 1991; Roff and Fairbairn 2007).

A large amount of research on trade-offs has focused on the response of a phenotype to discrete environments; this analysis is coined a 'character-state' approach (Via and Lande 1985; Walsh and Lynch 2014). However, many environmental factors are not naturally discrete, instead they vary along continuous scales, like temperature or salinity. In such cases, the character-state approach does not include information related to the ordering and spacing between the different levels of the variable (Walsh and Lynch 2014). Incorporation of this information into the study of trade-offs could provide a clearer understanding of the response of genotypes to the environment that underlie trade-offs (Kirkpatrick and Heckman 1989; Stinchcombe and Kirkpatrick 2012). A continuous reaction norm (CRN) measures the response of a trait across a continuous environmental factor, such as soil nutrition or population density (Fig. 1.3) (Kingsolver et al. 2004b). They are sometimes termed as function-valued (Pletcher and Geyer 1999; Meyer and Kirkpatrick 2005b) or infinite-dimensional traits (Kirkpatrick and Heckman 1989). CRNs feature heavily in agricultural quantitative genetics, the most common types are the growth curve, such as the change in body mass as an organism ages (Kirkpatrick et al. 1990; Kirkpatrick and Lofsvold 1992) and lactation curves—the amount of milk a cow lactates during reproduction (Shanks et al. 1981; Mellado et al. 2014).

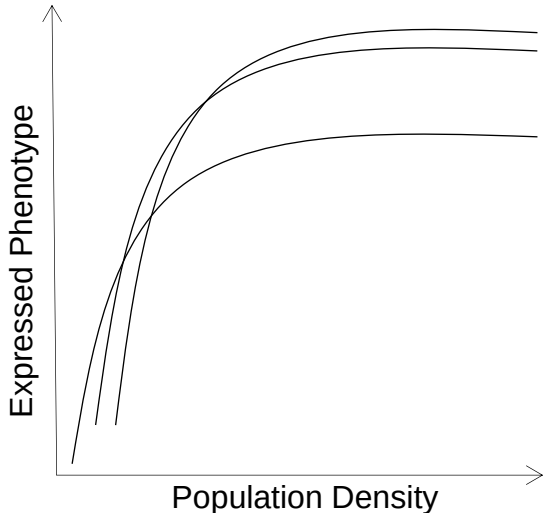

Figure 1.3: An example of a continuous reaction norm. The different lines indicate different genotypes.

1.3 Thermal Performance Curves

Temperature is an important environmental variable for many organisms and has been linked to species distributions (Kellermann et al. 2012), cellular (Clarke 2003) and metabolic (Galli and Richards 2012) processes, and behaviour (Gilchrist 1996). The thermal environment is particularly important for ectotherms as they have limited thermoregulation abilities (Gilchrist 1996) and as a result, their physiological processes are more likely to be affected by fluctuating environmental temperature (Gibbs 2002). Many phenotypes respond to temperature variation, for example, egg production (Berger et al. 2008), growth in bacteriophage (Knies et al. 2009), sprinting in lizards (Bennett 1980), and jumping distance in frogs (Wilson 2001). If traits do not adequately respond to the thermal environment, organism's can become stressed resulting in possible consequences for their physiological and behavioural state (Roberts et al. 2003), decreasing fitness, survival (Hoffmann et al. 2003), and ultimately leading to death.

Thermal adaptation is often investigated via the study of thermal performance curves (TPC). A TPC is a class of a continuous reaction norm that relates a temperature dependent trait, generally performance, to a range of acutely exposed temperatures (Kingsolver et al. 2001; Izem and Kingsolver 2005). TPCs exhibit a common shape, where trait values increase slowly, reaches a peak then decreases rapidly (Fig. 1.4). Despite a common shape, TPCs have been shown to vary between populations (Wilson 2001; Yamahira et al. 2007; Latimer et al. 2011), individuals (Kingsolver and Gomulkiewicz 2003;

Lachenicht et al. 2010), and genotypes (Gilchrist 1996; Izem and Kingsolver 2005; Knies et al. 2009). These changes occur in the components of a TPC; 'overall height', optimum temperature, width and maximum performance at optimum temperature (Fig. 1.4a). The width represents the range of temperatures across which an individual can operate; 'overall height' is the level of performance across all temperatures; and optimum temperature is the position of the temperature for maximum performance. Depending on the level of investigation (individual/genotype/population), differences in the shape of a curve can illustrate the outcomes of a plastic or genetically-based response to the environment (Izem and Kingsolver 2005).

Common theories of thermal adaption can be explored through investigation of genetic contributions to three modes or axes along which TPCs tend to vary; the three modes are the horizontal shift or 'faster-slower' axis, the vertical shift or 'hotter-colder' axis and the width shift or 'generalist-specialist' axis (Kingsolver et al. 2001; Izem and Kingsolver 2005). The 'faster-slower' axis represents variation for the overall performance across the TPC regardless of the temperature (Fig. 1.4b). The 'faster-slower' axis does not represent any trade-off between the environments unlike the 'generalist-specialist' and the 'hotter-colder' axes. The 'hotter-colder' axis incorporates variance in the optimum temperature and includes a trade-off for performance between hot and cold temperatures (Fig. 1.4c). For a population to have optimal performance at hotter temperatures, performance at colder temperatures will be lower (Kingsolver et al. 2001). Some evolutionary physiological models predict that the optimum temperature should match the most common environmental temperature (Lynch and Gabriel 1987; Gilchrist 1995).

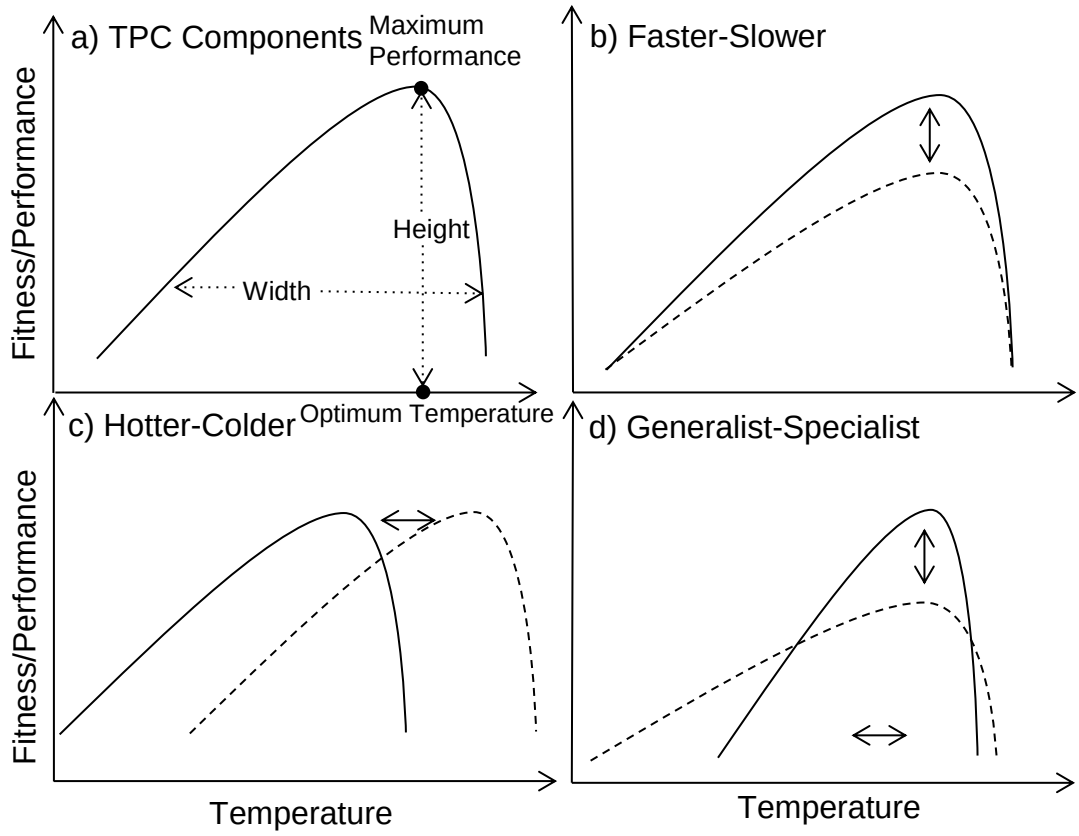

Figure 1.4: Schematic of a thermal performance curve, showing the general response of fitness/performance to temperature. a) Illustrates the four main components of the curve, height, width, optimum temperature and maximum performance, b) 'faster-slower'; a response of a by changing the overall height of the curve, c) 'hotter-colder'; a change in optimal temperature, and d) 'generalist-specialist'; a trade off between the width and height of a curve at its optimum. Modified from Izem & Kingsolver (2005).

The 'generalist-specialist' axis of variation represents variation in the width and maximum performance at optimum temperature components and is linked to the 'generalist-specialist' trade-off (Fig. 1.4d). The 'generalist-specialist', also known as the jack-of-all-trades or the master-of-none (Huey and Hertz 1984), has received a large amount of attention particularly in ecological studies. This trade-off underlies optimisation models that build upon Levins' (1968) Principle of Allocation, suggesting a trade-off, where a genotype's physio-chemical structures, reproductive constraints, and physiological/cellular mechanisms limit the range of temperatures it can perform across (Angilletta et al. 2003; Angilletta 2009). The trade-off posits that for an organism to be able to perform over a broad range of temperatures, a lower performance is required (the generalist) and that high performance can be achieved for only a small range of

temperatures (the specialist) (Huey and Hertz 1984). The two main optimisation models incorporating the 'generalist-specialist' trade-offs are Gilchrist's (1995), and Lynch and Gabriel's (1987) models. Gilchrist's (1995) model predicted that the breadth was a result of the amount of environmental variability populations experienced within and among generations. Lynch and Gabriel's (1987) model predicts that the range of temperatures experienced within an individual's lifetime determines their thermal breadth range. Both stipulate that specialists are thought to occur in a constant environment, and generalists when the environment is more temporally variable to enable the residents to have a higher fitness across a larger range of temperatures.

Dissection of the genetic basis of TPCs is needed to illuminate the mechanisms that may underlie the different axes of variation and determine how freely different axes can evolve in isolation of the others. Although physiologists have performed many studies on the trade-offs in TPCs, little is actually known about the genetic mechanisms and constraints that underlie these axes (Angilletta 2009). Input of genetic material to maintain sufficient genetic variation is required to generate a heritable response to selection for any trait (Barton 1990; Houle 1992; Barton 2010). Only a few experiments have been conducted to investigate the evolutionary potential of TPCs. Most experiments have detected genetic variation in one or more of the components, for example, growth rates of caterpillars (Izem and Kingsolver 2005) and bacteriophage (Knies et al. 2006; Knies et al. 2009), and the temperature for performance in fish (Yamahira et al. 2007). However, little is known about the mutational inputs that maintain the evolvability of these traits, with one study identifying specific nucleotide mutations in bacteriophage (Knies et al. 2006) and only one other that looked at mutation accumulation patterns in *Escherichia coli* (Cooper et al. 2001).

Furthermore, we also know very little of the types of molecular variants, pleiotropic or otherwise, that contribute to standing variance for TPCs and which may contribute to their adaptive evolution. No studies have directly attempted to estimate the magnitude and direction of allelic effects in TPC or their axes. Based on studies investigating local adaptation, including specialisation, genetic architectures might contain alleles with pleiotropic effects (Ostrowski et al. 2007; Anderson et al. 2013). Two main types of allele can exist that contribute to trade-offs and therefore may also be present in the 'hotter-colder' and 'generalist-specialist' axes of TPCs. First, antagonistic alleles that have opposing effects in different environments have been linked to trade-offs (Via 1991;

Cooper et al. 2001; Nikolin et al. 2012). Second, alleles have pleiotropic effects in the same direction across temperatures but differ in magnitude (Fry et al. 1996; Kawecki et al. 1997; Kawecki and Ebert 2004; Hall et al. 2010).

1.4 Study Species and traits

To investigate the genetic basis of TPCs, I have used two *Drosophila* species from the Melanogaster species group. *Drosophila serrata* is from the largest subgroup, *montium,* and is endemic to the Australasian region (Kellett et al. 2005). The geographic range for this species is from Papua New Guinea to Wollongong (Jenkins and Hoffmann 1999, 2001). Over this latitudinal gradient, populations experience a wide range of temperature fluctuations, possibly generating natural selection for different reaction norms. The different temperatures across the geographical range of *D. serrata* are known to affect life history traits (Ayala 1968; Sgro and Blows 2004) and stress related traits such as their ability to recover from cold temperatures (chill-coma recovery) (Jenkins and Hoffmann 1999, 2001; Hallas et al. 2002). The thermal sensitivity of locomotor activity in *D. serrata* has also been demonstrated to differ genetically between populations (Latimer et al. 2011).

The second species, *Drosophila melanogaster*, is well known*. D. melanogaster* is a cosmopolitan species that originated from Africa but is now found throughout the world (Bock 1980; Kellett et al. 2005). Its broad distribution covers a range of different environments with different thermal regimes; from cooler, dry, temporal environments to hot, humid, tropical climates. For this species to successfully colonise such a wide range of environments suggests that it may have the ability to adapt to different climates effectively. Vast numbers of studies have been conducted on *D. melanogaster,* including genomic studies, making it also a powerful genetic model for dissecting the genetic architecture of TPCs (Zou et al. 2000; Gopal et al. 2001; Noor et al. 2001; Blumenstiel et al. 2009; Deloger et al. 2009; Keightley et al. 2009; Angilletta and Sears 2011; Jordan et al. 2012; Mackay et al. 2012; Harbison et al. 2013).

To measure TPC for both these species, the model trait I used was locomotor performance. Locomotor performance is a complex trait, incorporating the fitness of an adult (Gilchrist et al. 1997; Long and Rice 2007), an individual's decision-making ability (Martin, 2003), and is influenced by temperature for a range of species (Gilchrist 1996; Lehmann 1999; Wilson et al. 2000; Chen et al. 2003; Martin 2003; Elnitsky and Claussen

2006). Locomotor performance changes dynamically in response to prior and current environmental conditions (Martin 2003). The influences of temperature on two aspects of locomotor performance, walking speed and locomotor activity, are widely studied (Crill et al. 1996; Gilchrist 1996; Roberts et al. 2003). For example, different thermal populations affect walking speed in *Drosophila* (Gibert et al. 2001). Reproductive successes, territorial defense, ability to avoid predators, and food detection (Gilchrist 1996; Roberts et al. 2003) have all been shown to vary with locomotor activity. Moreover, *Drosophila* species are often exposed to extreme temperatures, as both adults and larvae, at their preferred feeding and laying sites, rotten fruit (Feder 1997).

1.5 Thesis Approach and Aims

The overarching aim of my dissertation was to explore the genetic basis and evolutionary potential of the TPC. In Chapter 2, now published in the journal *Evolution* (Latimer et al. 2014), I investigated the evolutionary potential of locomotor TPCs by studying the supply of new genetic variation from spontaneous mutations. Employing a mutation accumulation experiment in *Drosophila serrata,* I estimated the contribution of mutational variation to the three major axes of TPC variation and compared their relative abundance to that commonly observed in standing genetic variation. My results provide an indication of the potential for mutation to generate variation in each of the major components of TPC variation, and establish a baseline against which to compare the distribution of standing genetic variation in different populations, which may shed light on how selection acts on TPC variation.

Chapters 3 and 4 focus on molecular dissections of the genetic architecture of two different aspects of TPC variation; inter-population divergence (Chapter 3) and standing variation within a single population (Chapter 4). In Chapter 3, I performed a QTL analysis in a cross between two populations of *D. serrata* spanning a latitudinal gradient along which TPCs are known to have genetically diverged. Using multivariate QTL analysis, I identified vectors of QTL effects across temperatures that were well-aligned with the major axes of genetic variance in this population and resembled the common axes of TPC variation.

In Chapter 4, I dissected the genetic architecture of TPC standing variation in a single population of *D. melanogaster* using a panel of 152 fully-sequenced wild-derived isogenic lines from the Drosophila Genetic Reference Panel (DGRP) (Mackay et al. 2012;

Huang et al. 2014). I first performed two different approaches to dissect the TPC; I first performed a character-state eigendecomposition approach and then a template mode of variation analysis (TMV), a functional trait analysis that partitions among line variance among the three major axes of TPC variation components associated with the TPC shape. I then performed a genome-wide association (GWA) analysis to provide unprecedented insight into the allelic nature of quantitative genetic variation for thermal performance curves. I identified polymorphisms associated with the axes of TPCs that had a strong skew in allelic effects suggesting a role for natural selection in shaping standing variation. I also found that the regulation of gene expression may be heavily involved in TPC shape variation.

Chapter Two:
The contribution of spontaneous mutations to thermal sensitivity curve variation in *Drosophila serrata*

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2.1 Abstract

Many traits studied in ecology and evolutionary biology change their expression in response to a continuously varying environmental factor. One well-studied example are thermal performance curves (TPCs); continuous reaction norms that describe the relationship between organismal performance and temperature and are useful for understanding the trade-offs involved in thermal adaptation. I characterised curves describing the thermal sensitivity of voluntary locomotor activity in a set of 66 spontaneous mutation accumulation lines in the fly *Drosophila serrata.* Factor-analytic modeling of the mutational variance-covariance matrix, **M**, revealed support for three axes of mutational variation in males and two in females. These independent axes of mutational variance corresponded well to the major axes of TPC variation required for different types of thermal adaptation; 'faster-slower' representing changes in performance largely independent of temperature, and the 'hotter-colder' and 'generalist-specialist' axes, representing trade-offs. In contrast to its near-absence from standing variance in this species, a 'faster-slower' axis, accounted for most mutational variance (75% in males and 66% in females) suggesting selection may easily fix or remove these types of mutations in outbred populations. Axes resembling the 'hotter-colder' and 'generalist-specialist' modes of variation contributed less mutational variance but nonetheless point to an appreciable input of new mutations that may contribute to thermal adaptation.

2.2 Introduction

Trade-offs are central to many concepts in ecology and evolutionary biology such as life-history evolution (Stearns 1992) and local adaptation (Kawecki 1995; Kawecki et al. 1997) and have been studied deeply at biochemical (Gillooly et al. 2001; Gillooly et al. 2002; Savage et al. 2004), physiological (Huey and Kingsolver 1993; Gilchrist 1995; Angilletta 2009; Angilletta et al. 2010), and population levels (Gebhardt and Stearns 1988; Stearns 1989, 1992, 2000). Fundamental to the concept of any trade-off is that fitness, or one of its components, differs between the environments that an organism may inhabit (Levins 1968; Via 1991; Shaw et al. 1995; Fry et al. 1996). For trade-offs to evolve, fitness differences between environments must have a genetic basis, and more specifically, involve a genotype-by-environment interaction (GEI).

Cross-environment genetic correlations have been the primary tool for inferring the GEIs that underlie trade-offs (Fry et al. 1996). GEIs are indicated when a genetic correlation is less than one (Lynch and Walsh 1998). Two classes of pleiotropic mutation can lower a cross environment genetic correlation; those that decrease fitness in one environment but increase it in the other, and those sharing direction of effect but differing in magnitude (Lande 1982; Futuyma and Philippi 1987; Houle 1991; Arnold 1992; Bell 1992; Roff and Fairbairn 2007). Because cross-environment genetic correlations can change when allele frequencies change through drift or selection (Falconer and Mackay 1996; Lynch and Walsh 1998), they can sometimes provide a misleading picture of the relative contributions of these different types of pleiotropic mutation to the genetic architectire of a trait. By estimating cross-environment correlations within a set of mutation accumulation lines, it becomes possible to obtain a clearer view of the contribution of different classes of pleiotropic mutation to the genetic covariance between environments before selection acts (Houle 1991).

With few exceptions (see Fernandez and LopezFanjul 1997), studies taking a mutation accumulation approach report positive cross-environment genetic correlations that are less than one. For example, cross-environment genetic correlations for fitness in *Drosophila melanogaster* and *Caenorhabditis elegans* fall in the ranges of 0.29-0.98 and 0.38-1 respectively (Fry et al. 1996; Fernandez and LopezFanjul 1997; Vassilieva et al. 2000; Baer et al. 2006; Baer 2008), while estimates for bristle number in *D. melanogaster* range from 0.5 to 0.98 (Mackay and Lyman 1998). These results suggest that perhaps the

most common form of GEIs are those due to deleterious mutations that vary in effect size, but not sign, across environments. It is therefore possible that the evolution of specialisation is often due to such mutations rather those that increase fitness in one environment while lowering it another.

Most mutation accumulation studies conducted to date have focused on discrete reaction norms that describe the relationship between genotype and discrete environments with a GEI indicated when reaction norm slopes are non-parallel (Stearns 1989; Falconer and Mackay 1996; Fry et al. 1996; Lynch and Walsh 1998). While these environments may be naturally categorical factors, such as different food types (Fry et al. 1996), the vast majority of traits studied in ecology and evolutionary biology are expressed at different levels of a continuously-varying environmental factor such as temperature, time or salinity (Stinchcombe and Kirkpatrick 2012). Such traits are termed function-valued (Pletcher and Geyer 1999; Meyer and Kirkpatrick 2005a) or infinite-dimensional (Kirkpatrick and Heckman 1989; Kirkpatrick et al. 1990). Typically, mutational studies have measured only a small number of environments (Mackay and Lyman 1998; Wayne and Mackay 1998; Vassilieva et al. 2000; Chang and Shaw 2003; Kavanaugh and Shaw 2005; Baer et al. 2006) or explored a range of unrelated environments (Kondrashov and Houle 1994; Fry et al. 1996; Fernandez and LopezFanjul 1997; Xu 2004). Little is known of the mutational inputs to continuous reaction norms.

Thermal performance curves (TPC) are a class of continuous reaction norm useful for understanding the trade-offs predicted by thermal adaptation theory (Levins 1968; Huey and Kingsolver 1989). A TPC describes the relationship between organismal performance and temperature (Huey and Stevenson 1979; Izem and Kingsolver 2005), a factor which has been shown to be important for ectotherms through its effects on physiological processes mediating locomotion (e.g. Bennett 1980; Weinstein 1998; Lyon et al. 2008), growth (e.g. Kingsolver 2000; Yamahira et al. 2007), resource acquisition (e.g. Greenwald 1974; Ayers and Shine 1997), and survival (e.g. Domenici and Blake 1993; Ahnesjo and Forsman 2006). TPCs have a characteristic shape; performance gradually increases with temperature, reaches a maximum and then falls sharply (Huey and Stevenson 1979; Huey and Kingsolver 1989; Hallas et al. 2002).

Variation in TPCs among individuals is often summarised along axes that reflect the different ways in which thermal adaptation may occur. These axes differ in the extent to

which they require the contribution of alleles with negative pleiotropic effects between environments (Kingsolver et al. 2001). The first axis represents overall increases or decreases in performance and is known as the ‘faster-slower’ axis Fig. 2.1a). Genetic variation along this axis could be due to mutations that affect performance in similar directions and equally across temperatures but also mutations that share sign-of-effect but differ in magnitude. The two remaining axes require the existence of at least some mutations with negative pleiotropic effects. The ‘hotter-colder’ axis represents variation in the temperature at which performance is maximised, where increased performance at the hotter temperatures trade-off for decreased performance at colder temperatures (Fig. 2.1b) (Kingsolver et al. 2001). The ‘generalist-specialist’ axis represents a trade-off between the width of a curve and the maximum level of performance achieved; a generalist has a wider curve but a lower maximal level of performance, while a specialist has a narrower curve but a higher maximal level of performance (Fig. 2.1c).

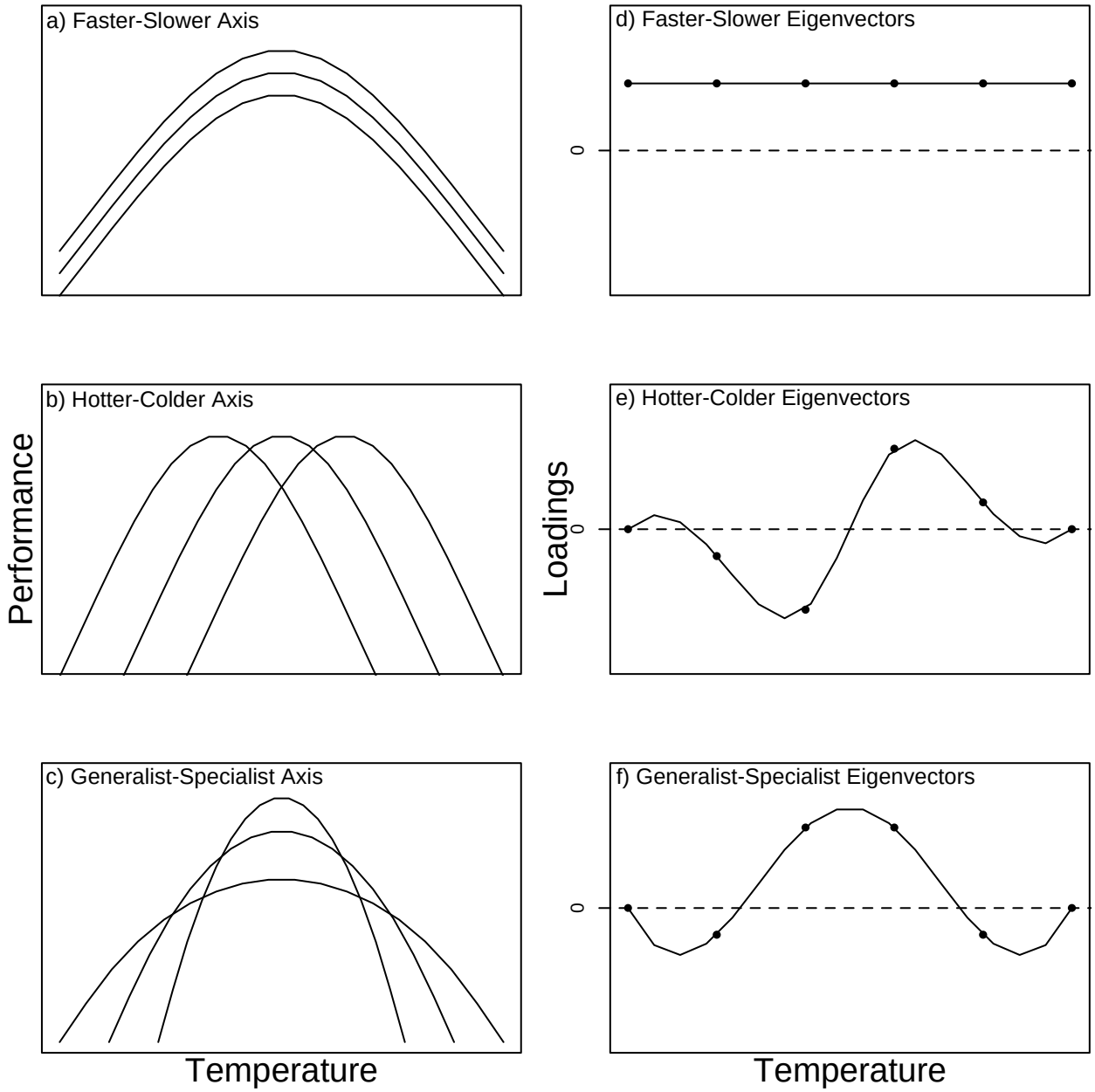

Figure 2.1: Schematic diagram of the patterns of the three axes of TPC shape variation a) ‘faster-slower’ axis b) ‘hotter-colder’ axis and c) ‘generalist-specialist’ axis. Shown in the right column are example eigenvector loadings typical of each of the three modes of variation; d) ‘faster-slower’ e) ‘hotter-colder’ and f) ‘generalist-specialist’. For the patterns in e) and f) multiple eigenvectors can exist in the variance covariance matrix among test temperatures; refer to Izem and Kingsolver (2005) for further details.

To my knowledge, the mutational contributions to TPC shape variation remain unknown for any multicellular organism, the only studies to investigate the contribution of new mutations to thermal dependence have been restricted to microbial systems (Cooper

et al. 2001; Knies et al. 2006). Measuring the mutational contribution to these traits through estimation of the mutational analogue of the additive genetic variance-covariance matrix **G** (Lande 1979), the mutational variance-covariance matrix, **M** (Lande 1975), can provide insights into their ultimate evolutionary potential. **M** summarises the effects new mutations have on individual trait variances and also their pleiotropic effects across traits (or environments), which influence genetic covariances and can ultimately bias evolutionary trajectories (Lande 1979, 1980; Camara et al. 2000; Phillips and McGuigan 2006; Chenoweth et al. 2010).

In this study, I estimated the mutational variance for the thermal sensitivity of locomotor activity in the fruit fly *Drosophila serrata.* Quantitative genetic experiments conducted under common-garden conditions have established that TPCs are genetically variable and have diverged between populations inhabiting different thermal environments in this species (Latimer et al. 2011); a result consistent with the observation of TPC evolution during experimental adaptation to different thermal environments in *D. melanogaster* (Gilchrist et al. 1997). Within natural populations, the majority of the standing genetic variance was distributed along the 'generalist-specialist' axis with little standing genetic variance for the 'faster-slower' axis and, surprisingly no evidence for the 'hotter-colder' axis (Latimer et al. 2011). I focus on locomotor activity because it has been associated with fitness in other *Drosophila* species through its links with reproductive success, dispersal, predator avoidance and foraging (Partridge et al. 1987; Gilchrist 1996; Roberts et al. 2003; Long and Rice 2007).

I used a spontaneous mutation accumulation assay to estimate the mutational variance-covariance matrix, **M**, for male and female activity at different temperatures. My main focus was to ask whether the major axes of mutational variance reflect the three classic modes of variation thought critical for adaptive evolution of TPCs, the 'faster-slower', 'hotter-colder' and 'generalist-specialist' axes. I also compare the relative contribution of these different axes of variation from new mutations to that observed in standing variance in this species and in other ectotherms.

2.3 Methods

Mutation Accumulation Assay

I used a set of spontaneous mutation accumulation (MA) lines that were created to test the role of sexual selection in purging deleterious mutations (McGuigan et al. 2011). Initially, 200 MA lines were founded from a single inbred line established from a lab-adapted stock from Forster, Australia (32.12^{o}S, 152.32^{o}E). These 200 lines were randomly allocated to two mating treatments. One that represented a classic mutation accumulation protocol of maintenance via single pair full-sib mating, hereafter referred to as “standard” and a second that manipulated the opportunity for pre-copulatory sexual selection, hereafter referred to as “sexual-selection”. The treatment involved a female choosing between approximately four brothers as mates with no opportunity for multiple mating thereby equalising the effective population size of the two treatments. Comprehensive detail of the creation of these lines has been outlined in McGuigan et al. (2011).

The existence of an evolutionary stable control is an important issue in mutation accumulation studies especially for the inferring magnitude of mutational effects that can be overestimated (Lynch et al. 1999; Halligan and Keightley 2009). In flies, it is not possible to freeze the founding line as it is in other systems such as *C. elegans* or bacteria. Instead, I maintained the founding inbred Forster line at a large effective population size to reduce its accumulation of new mutations while the MA lines were being established. It is nonetheless likely that some mutations accumulated in the founding line during the study period. I note that the estimation of mutational (co)variances—the main focus of this paper—is not greatly affected by the stability of the control (Lynch et al. 1999; Halligan and Keightley 2009).

The locomotor activity assay was performed after 32 generations of spontaneous mutation accumulation. After generation 25, the sexual selection treatment ceased and all sexual-selection lines continued to be maintained by the mutation accumulation protocol that was applied to the standard lines. I assayed a total of 66 MA lines (30 standard and 36 sexual-selection lines) as well as the inbred Forster founder line. I estimated individual level activity curves for 12 males and 12 females per MA line and used 60 males and 60 females for the Forster inbred founder line. For each MA line three single mating pairs were each randomly allocated to a vial for egg laying. Adults were discarded after 48hrs. Upon emergence, I sexed 4 males and 4 females from each replicate vial and held them

singly in vials until the time of the assay. Phenotyping was conducted on five to nine day-old virgins. During both mutation accumulation and phenotypic assays phases of the study, flies were maintained in the lab on a yeast-sucrose agar medium at 25 ± 0.5^{o}C in a 12:12 hr light cycle.

Assay of the Thermal Dependence of Locomotor Activity

Individual TPCs were estimated by measuring locomotor activity when exposed briefly to six temperatures in the order of 30, 25, 33, 36, 35, and 38 ± 0.3^{o}C (Latimer et al. 2011). I measured the highest temperature last to prevent any detrimental effects interfering with subsequent measurements (e.g. Bennett 1980; Weinstein 1998; Lyon et al. 2008). Between each temperature exposure, flies were held at 25 ± 0.5^{o}C for 40 minutes to prevent acclimation to the temperature exposure. All measurements were conducted in the same constant temperature cabinet. Due to logistical constraints imposed by the availability of a limited number of locomotor activity testing devices, assays were conducted in 12 completely randomised blocks performed over six consecutive days. Two blocks were processed per day, each containing one male and one female from each of the 66 lines and five individuals per sex from the founding Forster line, ensuring that all the lines and founder was represented in all blocks.

Locomotor activity was measured using Drosophila Activity Monitors (DAM, TriKinetics, Waltham, Massachusetts, USA) in a method similar to Latimer et al. (2011). A DAM comprises 32 x 5 mm holding tubes that are bisected by an infrared beam. When a fly crosses the beam, a connected computer records the number of intersections. Recordings were taken over a period of twenty minutes to prevent acclimation to the temperature treatment. I measured activity between 4.15am and 1.30pm to match with the diurnal activity patterns shown in a preliminary study I conducted (flies were maintained at a 12-hour light-cycle, 4am:4pm). Flies were placed in the 5 mm measurement tubes the afternoon prior to allow the flies to adjust to the new physical environment. Each 5 mm vial contained 1.5 cm of a three-day-old agar-sucrose medium that was caped and was stoppered with 0.2 mm of foam on the opposing end.

My measure of locomotor activity differs from some of the previous measures that have been used as proxies for thermal performance in flies such as walking speed, which usually involves measurement after reaction to a stimulus such as flicking flies to the base of a vial (Crill et al. 1996; Gilchrist et al. 1997). Although all activity metrics include both

physiological and motivational aspects of physical movement, it could be argued that my metric includes more motivational variation (i.e. the willingness to move) than other measures (e.g. sprint speed) used in previous studies (Gilchrist et al. 1997; Lachenicht et al. 2010). Nonetheless, basal activity is positively correlated with walking speed in *D. melanogaster* (Burnet et al. 1988). Perhaps more critical, however, is the extent to which my measure is associated with fitness. This is the case in *D. melanogaster*, where voluntary activity co-varies with both male and female fitness (Long and Rice 2007).

Statistical Analyses

Univariate analyses

I first tested for an effect of experimental mating treatment on both the mean and among-line variance of activity using the following mixed effects model:

$$a = \mu + b + t + b \times t + l(t) + v(l(t)) + \varepsilon \,, \qquad [2.1]$$

where, a is a vector of locomotor activity scores taken at the six test temperatures, μ is the mean, b is a block effect reflecting the twelve experimental runs, t is the mating treatment, $l(t)$ is the random MA line effect nested within mating treatment and $v(l(t))$ is the effect of replicate rearing vial nested within line. Males and Females were analysed separately. Models were fitted using restricted maximum likelihood (REML) using PROC MIXED in SAS (ver. 9.2; SAS Institute, Cary NC). Treatment and block were modeled as fixed effects, whilst all others were considered random effects. In all analyses, locomotor activity was square root transformed to normality.

I was unable to detect an effect of the sexual selection treatment on locomotor activity. Locomotor activity did not significantly differ in either mean or genetic variance between the treatments in either sex at any temperature (Appendix 2.2, see also Appendix 2.1). I therefore performed all further analyses by pooling the MA lines across treatments.

Four measures of mutational variability were calculated from the variance component and mean estimates from the linear model:

$$a = \mu + b + l + v(l) + \varepsilon \,, \qquad [2.2]$$

where *a* is the activity score, *μ* is the population mean, *b* is a fixed block effect reflecting the twelve experimental runs, *l* is the random line effect and *v* is the effect of vial nested within line. The mutational variance per generation, V_m, was calculated by dividing the among-line variance component for each temperature by 2*t*, where *t* is the number of generations, a value of 32 in my case (Lynch and Walsh 1998; Halligan and Keightley 2009). The mutational heritability (h^2_m) was estimated as the mutational variance per generation divided by the environmental variance (V_m/V_E) (Lande 1975). The mutational coefficient of variation was estimated as, $CV_m = 100 \times \sqrt{V_m}/\overline{X}$, where $\overline{X}$ is the trait mean (Houle et al. 1996). To test for directional bias in the effect of new mutations on any trait (locomotor activity at each temperature), the activity change per generation (analogous to Mukai's ΔM for fitness effects) was standardised by the trait mean: $\Delta M/ M_C = (M_{MA} - M_C)/M_C$ (Keightley and Ohnishi 1998), where M_C is the mean of the founding Forster line and M_{MA} is the mean for all the MA lines. Confidence intervals for all four measures were calculated by bootstrapping at the level of MA line 1000 times with replacement.

Multivariate analyses

The statistical analysis of function-valued traits such as TPCs is complex as a result of their nonlinear nature (Izem and Kingsolver 2005; Griswold et al. 2008). There are two broad approaches available by which function-valued traits can be analysed; functional (Griswold et al. 2008) and multivariate (Kirkpatrick and Heckman 1989). Functional analyses fit a continuous function to the vector of observations spanning an environmental axis for each individual. Differences between populations or genotypes are then revealed as differences in the means or variances of the underlying function coefficients. Although functional approaches have good statistical power when the number of phenotypic measurements per individual is large (owing to the relatively modest number of parameters that need to be estimated from the data (Griswold et al. 2008)), they assume that all individuals share a common underlying function (Izem and Kingsolver 2005). In situations where the heterogeneity in curve shape is large, this assumption may be violated, thereby weakening the approach. For example, different orders of polynomial may fit some genotypes better than others. In such instances, the multivariate approach, which treats all temperatures as discrete traits, has greater flexibility, because there is no *a priori* assumption of underlying curve shape. My mutational data set revealed heterogeneity in curve shapes that contrasted to what I had previously observed in the pattern of standing genetic variance in *D. serrata* (Latimer et al. 2011). Not all lines

exhibited a drop in activity at the highest test temperatures. Consequently, nonlinear functional approaches were unable to effectively model such heterogeneity in curve shape and for this reason I rely on the multivariate approach.

Multivariate approaches treat all measurements as discrete traits and fit a classic MANOVA model to the data. For analyses involving randomly sampled genotypes, the variance-covariance matrix at genotype level is analogous to the genetic variance covariance matrix, **G** (Lande 1979), of evolutionary quantitative genetics or in my case because I was assaying mutation accumulation lines, the mutational variance-covariance matrix, **M** (Lande 1975). The major axes of TPC genetic variance are revealed through an eigenanalysis of the estimated variance covariance matrix (Kirkpatrick et al. 1990; Kirkpatrick and Lofsvold 1992; Gilchrist 1996; Kingsolver et al. 2001). The 'faster-slower' axis is represented by eigenvectors with all positive (or negative) temperature loadings and is indicated when performance is positively genetically correlated between all pairs of temperatures (Fig. 2.1a, d). Variance for the 'hotter-colder' axis can be detected by an eigenvector with negative loadings at the lower temperatures and positive ones at the hotter temperatures (or vice versa), because this trade-off involves negative associations between performance at the lower temperatures and performance at the hotter temperatures (Fig. 2.1b, e). The 'generalist-specialist' axis is represented by eigenvectors where the loadings for temperatures in the center of the curve oppose those at either end, because intermediate temperatures are negatively genetically correlated with temperatures at the extreme (Fig. 2.1c, f).

I estimated **M** for each sex using the multivariate mixed effects model:

$$\boldsymbol{a = \mu + b + l + v(l) + \varepsilon}\ , \qquad [2.3]$$

which is the multivariate equivalent of eqn. 2.2 where $\boldsymbol{a}$ is a vector of locomotor activity scores taken at the six test temperatures fit separately for males and females, $\boldsymbol{\mu}$ is the mean, $\boldsymbol{b}$ is a fixed block effect reflecting the twelve experimental runs, $\boldsymbol{l}$ is the random line effect and $\boldsymbol{v}$ is the effect of vial nested within line. Significance of line terms was assessed using likelihood ratio tests, comparing a model without a line effect to the full model [2.3]. Here, estimation was based on an unstructured variance-covariance matrix (type=UN) using PROC MIXED in SAS 9.2 (SAS Institute, Cary NC).

To visualise the major axes of mutational covariance, I conducted an eigenanalysis on each matrix and plotted the loadings of each eigenvector. To ensure that the eigenanalysis results were not influenced by differences in means across temperatures, I first mean standardised the among-line variance covariance matrix through element-wise division by $[\overline{\mathbf{X}}][\overline{\mathbf{X}}]^{-1}$ where $\overline{\mathbf{X}}$ refers to the six element mean vectors for each sex (Hansen and Houle 2008). I report values for **M** on a per generation scale, dividing all variance and covariance estimates by 2*t*, where *t* is the number of generations (*t* = 32) (Lynch and Walsh 1998; Halligan and Keightley 2009). To compare males and females, the vector correlations and angles between the sexes for each significant eigenvector and the trace of $\mathbf{M}_m$ and $\mathbf{M}_f$ were calculated. The vector correlations were calculated by the sum product of the normalised eigenvectors and the angle calculated by $\cos^{-1}\left(\frac{m^T f}{\|m\|\|f\|}\right)$ (Schluter 1996; Walsh and Blows 2009).

I also investigated the dimensionality of **M** using a factor analytic modeling approach in SAS (Hine and Blows 2006; McGuigan and Blows 2007). A series of likelihood ratio tests were performed to compare the fit of models differing in the number of independent of dimensions of among MA line (co)variance. I specified a factor analytic variance-covariance structure at line level (Type =FA0(*n*)) and adjusted *n* from one to six reflecting the specific number of dimensions to be tested.

2.4 Results

Thermal Performance Curve Shape

When investigating the shape of the TPC, I found a general increase in locomotor activity with temperature (Fig. 2.2). However, for both sexes, the peak associated with the typical shape of TPCs was not evident for all lines. Instead for some lines, the highest mean performance was observed at the highest test temperature.

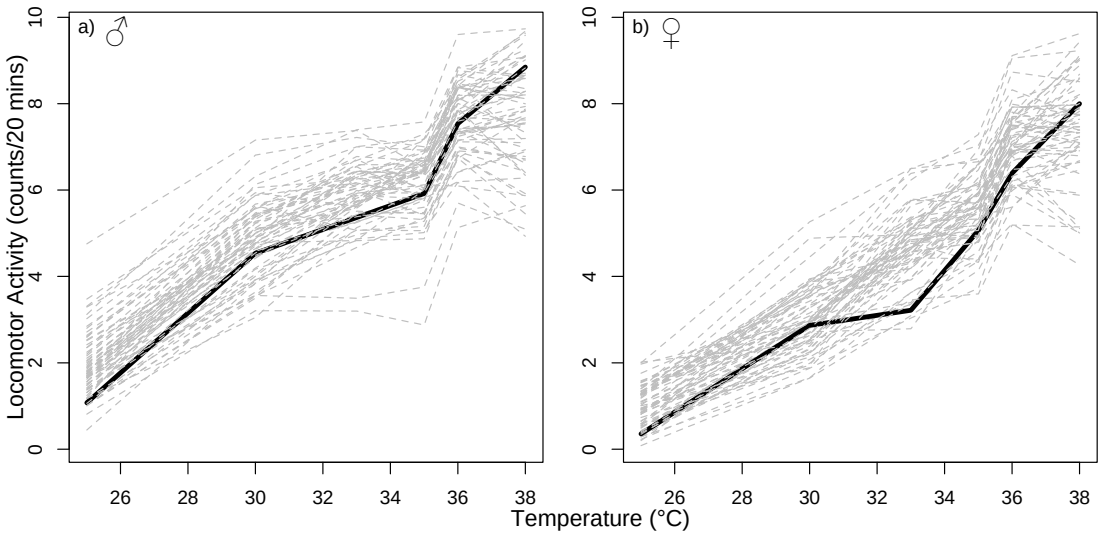

Figure 2.2: The mean locomotor activity TPCs for a) females and b) males of each mutation accumulation line. Locomotor activity was measured as counts per twenty-minutes and has been square root transformed. The founder line is indicated by the solid black curve while individual MA lines appear in grey. As no difference between the treatments in the mutation accumulation experiment was found, the two treatments groups were not shown in this graph. See Appendix 2.1 for a plot highlighting both treatment groups.

Univariate Mutational Variability

The mutational heritability of locomotor activity at each temperature ranged between 2.618×10^{-3} and 5.329×10^{-3} for males, and 0.538×10^{-3} and 4.722×10^{-3} for females (Table 2.1). These values fall within the range of estimates reported for life-history traits (2×10^{-3} to 30×10^{-3}: Lynch et al. 1999) except for females at 25^{o}C with a value of 0.583×10^{-3} being lower than this range. For both sexes, the mutational heritability was generally larger at higher temperatures; the difference in mutational heritability between 25^{o}C and 38^{o}C was two-fold for males and eight-fold for females. I found that the mutational coefficient of variation, CV_m ranged between 1.1% and 3.59% for all temperatures, within the range of estimates for other life-history traits (0.5 - 4.0%: Houle et al. 1994). In contrast to heritability, CV_m was maximal at 25 ^{o}C (Table 2.1).

I observed significant asymmetry in the direction of mutational effects across all temperatures with the single exception of 36^{o}C in males (Table 2.1). In both sexes, the values of $\Delta M/ M_C$ were positive for all temperatures below 38^{o}C in females and below 36^{o}C in males (Table 2.1, Figure 2.2). By contrast $\Delta M/M_C$ was negative for 38^{o}C in both

sexes, indicating that most new mutations affecting locomotor activity at this temperature decreased it.

Table 2.1: The estimates for mutational variance (V_m), mutational heritability (h^2_m), the coefficient of mutational variance (CV_m) and the activity change per generation ($\mathbf{\Delta M/ M_C}$) for males and females at each temperature. Bootstrap 95% confidence intervals are presented in parentheses.

Temp (°C)	**V_m x 10^{-3}**	**h^2_m x 10^{-3}**	**CV_m**	**100 × ΔM/ M_C**
Males				
25	4.784	2.618	3.589	97.015
	(0.271,8.861)	(0.141,5.33)	(0.711,4.733)	(92.476, 101.646)
30	7.134	3.131	1.736	11.776
	(0.166,11.45)	(0.079,5.36)	(0.266,2.186)	(10.644, 13.531)
33	6.091	3.598	1.326	9.468
	(0.545,10.38)	(0.317,6.866)	(0.403,1.785)	(8.446, 10.655)
35	6.819	5.160	1.334	3.973
	(1.149,12.24)	(1.029,9.686)	(0.543,1.827)	(2.866, 4.769)
36	7.883	4.846	1.161	0.814
	(3.376,12.67)	(2.141,9.396)	(0.769,1.482)	(-0.433, 1.261)
38	15.995	5.329	1.636	-12.847
	(6.586,26.19)	(2.043,9.081)	(1.037,2.118)	(-14.360, -12.281)
Females				
25	0.852	0.583	3.001	179.885
	(0,1.873)	(0,1.602)	(0,4.748)	(169.669, 188.863)
30	4.311	1.744	2.194	9.083
	($2.234x10^{-17}$,8.733)	($9.35x10^{-18}$,3.771)	($5.16x10^{-9}$,3.13)	(6.273, 10.500)
33	6.433	2.392	1.692	42.613
	(0.200,10.88)	(0.084,4.514)	(0.287,2.230)	(41.211, 44.840)
35	6.134	2.766	1.405	9.380
	(1.828,10.14)	(0.841,5.143)	(0.776,1.826)	(7.859, 10.218)
36	6.606	2.582	1.146	10.214
	(1.304,11.65)	(0.456,5.098)	(0.519,1.528)	(8.906, 10.833)
38	15.738	4.722	1.688	-8.008
	(4.402,25.51)	(1.266,8.176)	(0.899,2.208)	(-9.291, -7.076)

Multivariate Mutational Variance

To test for the presence of mutational variance and covariance for locomotor activity among all the six temperatures, I tested the significance of the MA line term in linear model [2.3]. Removal of the line term resulted in a significant drop in likelihood in males (χ^2=137, d.f. = 21, p< 0.0001) and females (χ^2=87.9, d.f. = 21, p< 0.0001) indicating that I had captured significant mutational variance in both sexes. Further factor analytic modeling of the (co)variance among lines supported a maximum of three independent axes of mutational variation in males, and two in females (Table 2.2). For both male and female **M** matrices, the covariances between all pairs of temperatures were positive (with one exception: 25C vs. 38C in females) and the mutational correlations tended to weaken as the differences between the temperatures increased (Table 2.3).

Table 2.2: Likelihood ratio tests and information criteria for reduced rank analyses testing the dimensionality of the mutational variance-covariance matrix, **M**, for locomotor activity in male and female *D. serrata.* The best model (based on AIC) is shown in bold for each sex.

Factor	Parameter	-2LogLikelihood	AIC	χ^2	d.f.	p-value
Males						
6	63	14089.1	14209.1			
5	62	14089.1	14209.1	0	1	1.00
4	60	14090.2	14206.2	1.1	2	0.577
3	**57**	**14092.4**	**14202.4**	**2.2**	**3**	**0.532**
2	53	14116.8	14218.8	24.4	4	6.64×10^{-5}
1	48	14162.0	14258.0	45.2	5	1.32×10^{-7}
Females						
6	63	15457.1	15577.1			1.00
5	62	15457.1	15577.1	0	1	1.00
4	60	15457.2	15573.2	0.1	2	0.951
3	57	15459.7	15569.7	2.5	3	0.475
2	**53**	**15465.0**	**15567.0**	**5.3**	**4**	**0.258**
1	48	15481.7	15575.7	16.7	5	0.0051

Table 2.3: The mutational variance-covariance matrices for locomotor activity in males, $\mathbf{M_m}$ (left panel), and females, $\mathbf{M_f}$ (right panel) across the six test temperatures (°C). Variances are on the diagonal in bold, the covariances below the diagonal and the correlation between temperatures above the diagonal. Estimates are mean-standardised. Variances and covariances estimates are reported as × 1000.

	Males						**Females**					
	25	**30**	**33**	**35**	**36**	**38**	**25**	**30**	**33**	**35**	**36**	**38**
25	**1.243**	0.911	0.718	0.484	0.438	0.154	**0.820**	0.768	0.871	0.569	0.311	-0.048
30	0.557	**0.301**	0.839	0.642	0.519	0.341	0.473	**0.462**	0.676	0.685	0.605	0.333
33	0.344	0.198	**0.185**	0.874	0.680	0.549	0.403	0.235	**0.261**	0.741	0.678	0.270
35	0.226	0.148	0.157	**0.175**	0.836	0.860	0.230	0.208	0.169	**0.199**	0.953	0.696
36	0.178	0.104	0.107	0.128	**0.133**	0.940	0.104	0.151	0.127	0.156	**0.135**	0.890
38	0.088	0.097	0.122	0.186	0.177	**0.266**	-0.023	0.121	0.074	0.165	0.174	**0.284**

The loadings for the first three individual eigenvectors of **M**, which contribute more then 95% of the variance, was consistent with the observed strong covariance between temperatures (Fig. 2.3). All three eigenvectors show similar patterns across the sexes. The first eigenvector accounted for 75.6% and 69.7% of the total mutational variance for males and females respectively. The eigenvector loadings shared the same sign across all temperatures and the eigenvector most closely resembles the 'faster-slower' axis of TPC variation. In both sexes, loadings tended to weaken towards higher temperatures (Fig. 2.3; Appendix 2.3). The second eigenvector explained 20.3% and 22.7% of the total mutational variance for males and females respectively. The loadings indicated negative mutational covariance between activity expressed at cooler and hotter temperatures. Such a change in direction in loadings is consistent with a 'hotter-colder' axis of TPC variation. Although the existence of a third dimension was not statistically-supported for females, the third eigenvector, explained 2.75% and 6.08% of the total mutational variance for males and females respectively. A trade-off between the intermediate and extreme temperatures was displayed in the loadings. This pattern of negative correlation between intermediate and the most extreme temperatures is consistent with a 'generalist-specialist' axis of TPC variation.

Figure 2.3 suggests mutations affected males and females similarly. The trace (the sum of the diagonal elements) of **M** supports this, being only slightly larger in males than the females ($\mathbf{M}_{\boldsymbol{m}}$: 2.303; $\mathbf{M}_{\boldsymbol{f}}$: 2.161) (Table 2.3). Vector correlations between the males and females for both the first and second eigenvectors were strong, indicating the male and female loadings were similar, ($\lambda_{1:}$ r = 0.977, angle = 12.07^{o} $\lambda_{2:}$ r = 0.966, angle = 15.05^{o}). However, for the third eigenvector the loadings for males and females were quite different, with a low vector correlation of 0.031 corresponding to an angle of 88.22^{o}.

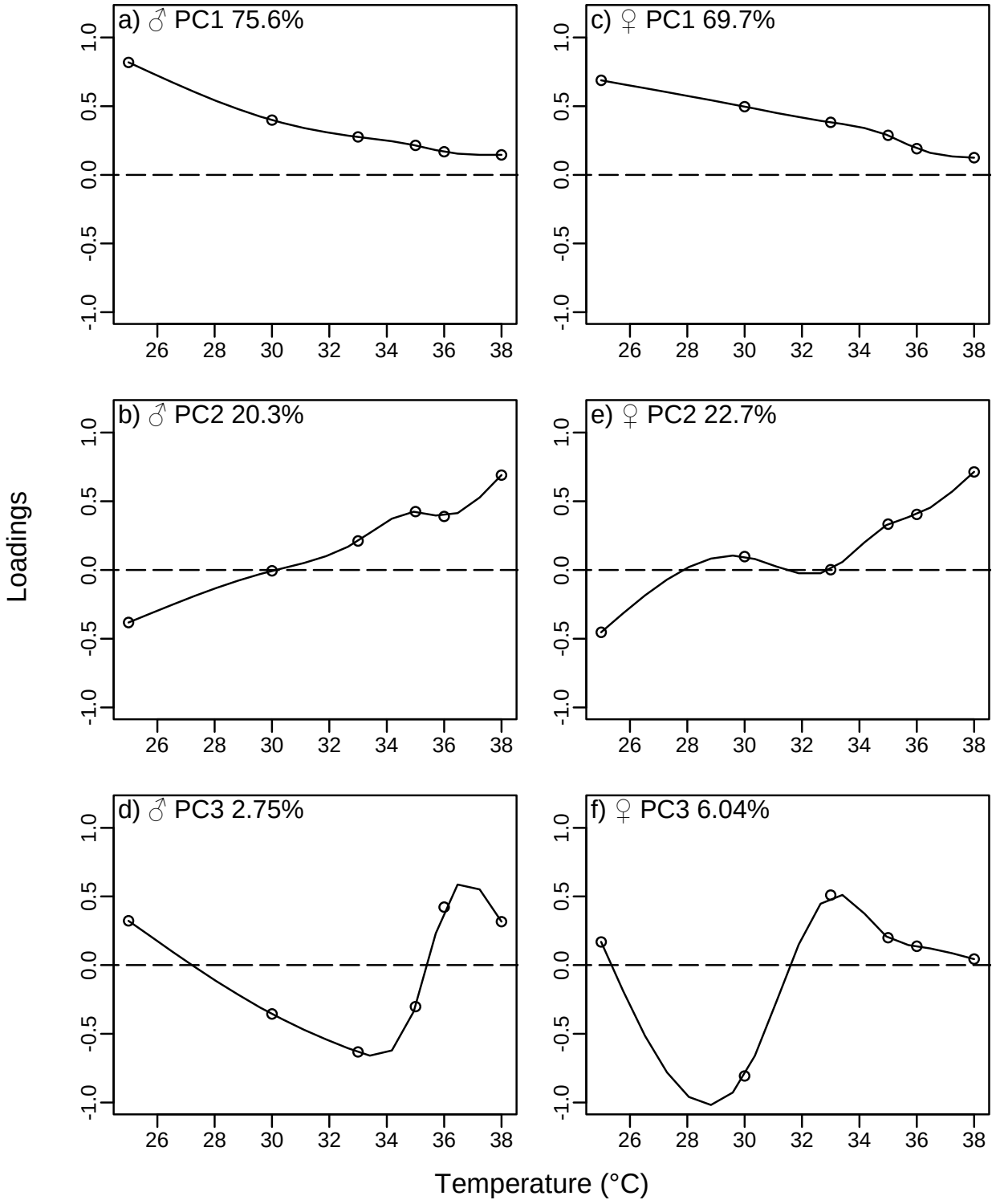

Figure 2.3: Eigenvector loadings for the male and females mutational variance-covariance matrices, $\mathbf{M_m}$ and $\mathbf{M_f}$. **M** was mean standardised (sex-specific means) before eigenanalyses were conducted. The first three eigenvectors are shown for each sex (males: a-c, females e-f) and all are normalised to unit length.

2.5 Discussion

Mutation and the Components of Thermal Performance Curve Variation

I detected significant mutational (co)variance for the TPC of *D. serrata.* For both males and females, more than 95% of the mutational variance occurred along the first three eigenvectors of **M**. Each of these independent axes shared broad similarities with one of the three commonly documented axes for thermal performance curve variation; 'faster-slower, 'hotter-colder' and 'generalist-specialist'. At first glance these results suggest that a variety of different pleiotropic mutations make up the genetic variance for thermal adaptation trade-offs (Gilchrist 1996; Izem and Kingsolver 2005; Knies et al. 2006; Yamahira et al. 2007; Angilletta 2009; Knies et al. 2009; Latimer et al. 2011). However their relative contribution to mutational variation was in many cases different to what has commonly been observed in the pattern of standing variation for these traits.

The first eigenvector of **M**, which described the largest axis of mutational variance, had loadings for all temperatures that were positive, which corresponds to positive correlations of mutational effects across all temperatures. In terms of thermal adaptation, all positive loadings resemble a 'faster-slower' axis of variation (Fig. 2.1a, d; Kingsolver et al. 2001; Izem 2004; Izem and Kingsolver 2005). Having the mutational variance dominated by this form of variation is particularly interesting because the 'faster-slower' axis often contributes the smallest amount of standing genetic variance in natural populations (Gilchrist 1996; Izem and Kingsolver 2005; Knies et al. 2006; Yamahira et al. 2007). Further, a quantitative genetic study of TPC in natural populations of *D. serrata* failed to detect significant standing variance for a 'faster-slower' axis of variation, despite detecting 'generalist-specialist' and 'hotter-colder' variation (Latimer et al. 2011), which suggests low 'faster-slower' variance in outbred populations.

It is possible that the majority of the mutational variance for the 'faster-slower' axis is maladaptive which would explain why it is observed infrequently in nature but remains observable in a mutation accumulation study where natural selection is greatly reduced. In general, correlations estimated from standing genetic variance tend to be considerably lower than estimates from mutational variance (Houle 1994). Estes and Phillips (2006) also found that mutational covariances were positive and larger before selection occurred.

In my case, the general increase in activity in the MA lines suggests that higher activity may be maladaptive (see section below).

An interesting aspect to the first eigenvector of **M** was that although all loadings were positive, they tended to decrease as temperature increased. General mean-variance scaling effects cannot explain this pattern because my eigenanalysis was performed on mean-standardised values. The pattern may be a true reflection of the genotype-phenotype relationship where the allelic effects on locomotor activity are larger at cooler rather than warmer temperatures. Given that activity was quite low at cooler temperatures, there may be a greater scope to increase activity from these low levels as opposed to the higher temperatures, where activity levels are already high. An alternative explanation is that simply more locations in the genome affect activity at lower temperatures than higher ones.

The second eigenvector of **M** showed patterns consistent with the 'hotter-colder' axis. In both males and females, the loadings were of opposing sign between 25^{o}C and the warmer temperatures suggesting a 'hotter-colder' trade-off and therefore a relatively high propensity for pleiotropic mutations that differ in their direction-of-effect between temperatures. Studies of standing variance typically see the 'hotter-colder' trade-off (Izem and Kingsolver 2005; Knies et al. 2006; Latimer et al. 2011) and may owe in part the contribution of pleiotropic mutations seen in my study.

The third eigenvector of **M** was consistent with the 'generalist- specialist' axis for which standing genetic variation is typically detected (but see Yamahira et al. 2007). The 'generalist-specialist' axis was the dominant mode of variation for standing genetic variance in my earlier study of *D. serrata* (Latimer et al. 2011). However, it contributes relatively little to mutational variation (3 and 6% in males and females), which again suggests natural selection may have altered the frequency of alleles with different classes of pleiotropic effects. Selection on 'faster-slower' variation might increase the frequency of 'generalist-specialist' alleles, which are likely to affect fitness only under fluctuating selection. Whether this mechanism might also apply to TPCs in other species will require further study.

In more general terms, my analyses suggest that alleles with quite varied pleiotropic effects across temperatures play an important role in thermal adaptation. In addition to

variation in the direction of effect across temperatures, my results suggest the existence of alleles that differ in their effect size across environments. Differences in allelic effect sizes suggest that some specialisation on the 'faster-slower' axis of variation might be possible even under consistent directional selection (Fry et al. 1996). Indeed, the fitness effects of new mutations in different environments are often deleterious in each environment but to differing degrees (Kondrashov and Houle 1994; Fry et al. 1996; Fernandez and LopezFanjul 1997; Mackay and Lyman 1998; Wayne and Mackay 1998; Xu 2004).

An unusual feature of my data that could affect interpretation of the distribution of mutational variance across the three major axes of TPC variation was that some lines, including the founder, lacked an activity peak within the range of tested temperatures. Although an inability to measure activity at sufficiently high temperatures is a common issue in these types of assays (Wilson 2001; Angilletta 2009), my previous study of natural populations used an identical range of test temperatures and revealled activity peaks between 35 and 37^{o}C (Latimer et al. 2011). It appears that the founding genome may differ genetically in its temperature of maximal activity. One key point of difference between the founder genome and the samples used in prior work was the population of origin. The founder genome was sourced from Forster, approximately 700kms away from the closest population assayed by Latimer et al. (2011). Genetically based divergence in the temperature of maximal activity was indeed observed among three northern populations, indicating genotypic variability for this trait (Latimer et al. 2011).

Perhaps more important than understanding its origin is understanding how the lack of a peak could have influenced my findings. As my data essentially contained a mixture of genotypes, those exhibiting an activity peak and those still monotonically increasing, I reanalysed the male and female data excluding all MA lines lacking an activity peak. The results were remarkably similar to the previous analyses in Figure 2.3, both in terms of the distribution of mutational variance across the first three eigenvectors of $\mathbf{M_m}$ and $\mathbf{M_f}$, and also, in terms of correspondence between vector loadings and the biological axes of TPC variation (Appendix 2.4). The loadings for the first eigenvector remained all positive loadings in both sexes and the fall in magnitude with increasing temperature was also preserved. The second eigenvector continued to resemble a 'faster-slower' axis with loadings changing sign between hotter and cooler temperatures. The third eigenvector again resembled a 'generalist-specialist' axis with loadings changing sign between

intermediate and extreme temperatures. Thus it does not appear that my biological conclusions have been greatly affected by not capturing optimal temperature values for all lines.

The Direction of Mutational Bias Varies with Temperature

Mutational bias occurs when the average effect of new mutations on a trait mean deviates from zero. I observed a high degree of mutational bias in my analysis of locomotor activity in *D. serrata.* With the single exception of 36°C in males, the values of $\Delta M/M_C$ were significantly different from zero. For all but the highest temperatures, the direction of bias was consistent in increasing the level of locomotor activity. However, the pattern flipped at the highest temperature in my assay of 38°C, with most MA lines having lower levels of activity than the control. Beyond the mutational bias well-known presence for fitness itself (Keightley and Lynch 2003), the frequency with which it has been detected for phenotypes tends to be quite variable. Unsurprisingly, life history traits thought to be closely associated with fitness often display bias for mutagen-induced (Lyman et al. 1996; Keightley and Ohnishi 1998; Yang et al. 2001) and spontaneous (Santiago et al. 1992) mutations. The pattern is arguably less common for morphological traits such as abdominal (but not sternoplural) bristle number in *D. melanogaster* (Mackay et al. 1992) and wing shape (Houle and Fierst 2012; McGuigan and Blows 2013). A notable exception is the strong bias for body size in nematodes (Ostrow et al. 2007).

I might have reasonably expected that locomotor activity would decrease rather than increase, as I saw for most temperatures, under mutation accumulation. The evolutionary significance of mutational bias depends upon the nature of selection acting on a trait (Waxman and Peck 2003). For traits under directional selection, like fitness, a downward bias is expected (Iwasa and Pomiankowski 1991; Pomiankowski et al. 1991). An analysis of adult fitness in these MA lines has indicated that both males and females indeed suffer reduced fitness relative to the founder line at 25°C (S. Allen, K. McGuigan, M. Blows, S, Chenoweth, manuscript in prep) and reductions in fitness components such as male mating success are also consistent with a reduction in fitness during mutation accumulation (McGuigan et al. 2011; McGuigan and Blows 2013). However, we do not yet know to what extent locomotor activity is a direct target of selection or exhibits these mutational bias effects through pleiotropic association with fitness-reducing alleles. Mutational data for performance and life history traits combined are scarce as both are

rarely measured in the same study or on the same sets of lines. A notable exception is the *D. melanogaster* study of Huey et al. (2003), who reported that the mutational correlations between multiple performance and multiple life history traits were not consistently positive and are indeed highly variable. For the specific case of the largely-upward mutational bias for locomotor activity I have observed here, one intriguing possibility that may help to explain it is that the trait may experience sexually antagonistic selection. For example, in *D. melanogaster,* voluntary activity is under positive directional selection in males but negative directional selection in females (Long and Rice 2007); perhaps the fitness reduction via increased locomotor activity occurs on females, with any apparent benefit to males being offset through the fitness reduction in females.

Conclusion

Significant mutational variance exists for the thermal dependence of locomotor activity in *D. serrata* and it is distributed across at least three independent axes of variation. The majority of mutational variance appears to be along the 'faster-slower' axis where overall activity can increase or decrease across all temperatures. However, underlying this axis there appears to be differences in the strength of allelic effects between temperatures, suggesting that a degree of thermal specialisation remains feasible through this type of genetic variation. Two other axes of variation consistent with trade-offs central to thermal adaptation theory, the 'generalist-specialist' and 'hotter-colder' axes, were also evident. Opposing patterns of mutational and standing variance suggest selection affects the cross-temperature covariance structure of these traits in outbred populations. Association studies that can estimate both the frequency and pleiotropic additive effects of DNA sequence polymorphisms across different temperatures will be useful in furthering our understanding of the evolutionary dynamics of thermal performance curves.

Chapter Three:
Connecting thermal performance curve variation to the genotype: a multivariate QTL approach

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3.1 Abstract

Thermal performance curves (TPCs) are continuous reaction norms that describe the relationship between organismal performance and temperature and are useful for understanding trade-offs involved in thermal adaptation. While thermal trade-offs such as those between generalists and specialists or between hot- and cold-adapted phenotypes are known to be genetically variable and evolve during thermal adaptation, little is known of the genetic basis to TPCs–specifically, the loci involved and the directionality of their effects across different temperatures. To address this, I took a multivariate approach, mapping QTL for locomotor activity TPCs in the fly, *Drosophila serrata,* using a panel of 76 recombinant inbred lines. The distribution of additive genetic (co)variance in the mapping population was remarkably similar to the distribution of mutational (co)variance for these traits. I detected 11 TPC-QTLs in females and 4 in males. Multivariate QTL effects were closely aligned with the major axes genetic (co)variation between temperatures; most QTL effects corresponded to variation for either overall increases or decreases in activity, with a smaller number indicating possible trade-offs between activity at high and low temperatures. QTLs representing changes in curve shape such as the 'generalist-specialist' trade-off, thought key to thermal adaptation, were poorly represented in the data. I discuss these results in light of genetic constraints on thermal adaptation.

3.2 Introduction

Temperature is an important environmental factor for ectotherms, owing to their limited ability to thermoregulate, which in turn influences physiological (Hochachka and Somero 2002 ; Roberts et al. 2003; Kingsolver 2009) and behavioural (Bennett 1980; Gilchrist 1996) processes that link to growth and survival (Hoffmann et al. 2003; Angilletta 2009). Therefore, ectotherms face a distinct challenge due to the fact that environmental temperatures vary in space and time (Gibbs 2002). If temperature fluctuates too widely or too rapidly, organismal performance can decline as a result, for example, through reduced reproductive success (Hoffmann et al. 2003; Berger et al. 2008) or increased predation risk due to suboptimal locomotion (Roberts et al. 2003; Lyon et al. 2008).

Thermal performance curves (TPCs) provide a framework for understanding how changes in environmental temperature affect organismal performance (Huey and Stevenson 1979; Izem and Kingsolver 2005). TPCs are a non-linear continuous reaction norm that link values of a performance-related trait of an organism acutely exposed to a range of environmental temperatures. TPCs of ectotherms exhibit a characteristic shape, where performance gradually increases with temperature, reaching a maximum before decreasing sharply (Fig 1a) (Huey and Stevenson 1979; Huey and Kingsolver 1989; Angilletta et al. 2002b; Angilletta 2009). These functions have been used to understand the various trade-offs thought central to thermal adaptation (Huey and Kingsolver 1989; Kingsolver 2009). Specifically, three major components of TPC variation have been identified (Kingsolver et al. 2001; Izem and Kingsolver 2005). First, the 'hotter-colder' axis represents variation in the temperature where performance is maximised. Along this axis a trade-off is implied when increased performance at a high temperature involves decreased performance at a cold temperature or vice versa. Second, the 'generalist-specialist' axis represents a trade-off due to a negative correlation between the width of a performance curve and the maximum level of performance achieved; generalists have wider curves but achieve a lower maximal level of performance, whereas specialists have narrower curves but can attain a higher maximal level of performance (Huey and Slatkin 1976; Huey and Kingsolver 1989; Kingsolver et al. 2001). Third, the 'faster-slower' axis represents variation for an overall increase or decrease in performance independent of temperature and therefore does not involve any trade-offs for performance at different temperatures.

Phenotypic variation in TPCs can arise through plasticity in response to environmental factors. For example, changes in TPC elevation and shape have been observed following exposure to different constant temperature treatments in both vertebrate and invertebrate species (Deere and Chown 2006; Condon et al. 2010). A genetic basis to TPC variation has also been established through a variety of approaches including mutation accumulation (Cooper et al. 2001; Knies et al. 2006; Chapter 2), quantitative genetic experiments (Gilchrist 1996; Izem and Kingsolver 2005; Berger et al. 2013), and common-garden studies (Yamahira and Conover 2002; Van Doorslaer and Stoks 2005). Moreover TPCs diverge genetically between natural and experimental populations from different thermal environments suggesting local adaptation (Knies et al. 2006; Yamahira et al. 2007; Latimer et al. 2011). Although it is clear that TPCs are genetically variable and evolve during thermal adaptation, we know little of their genetic architecture (Angilletta 2009). Quantitative genetic studies can indicate the overall distribution of genetic variance across TPCs, but the number, genomic distribution and pleiotropic effects of variants that underlie TPC variation may set bounds on their ultimate evolutionary potential. For example, we do not know whether the different axes of TPC variation are due to specific classes of pleiotropic variants or whether these axes are more ordinarily made up of multiple variants that affect different parts of a curve independently.

A practical first step towards dissecting the genetic architecture of TPC variation would be to study their underlying Quantitative Trait Loci (QTL). Although QTL studies estimating the environmental sensitivity of quantitative traits are by no means new (Fry et al. 1998; Gurganus et al. 1998; Gutteling et al. 2007; Bergland et al. 2008), none have estimated QTLs for TPCs specifically. Mapping QTLs for TPC variation is a problem best suited to multivariate QTL mapping methods. There are a large number of multivariate approaches available (e.g. Jiang and Zeng 1995; Mangin et al. 1998; Knott and Haley 2000; Banerjee and Yi 2012), and typically approaches emphasise one of two areas: statistical demonstration of pleiotropy for pairs of traits, or the estimation of effects across many traits simultaneously. For mapping TPC QTLs the latter feature is perhaps most important, owing to the often-large number of temperatures assayed and the complex covariance structure between them.

In this study I have applied a multivariate regression based mapping approach that permits estimation of QTL effects across all test temperatures simultaneously while

accounting for the covariance between temperatures. This approach yields an additive 'effects vector'—the loadings in each vector supplying information about the magnitude and direction of effect on performance at each temperature. A desirable property of this approach is that it provides estimates in the same 'trait space' as evolutionary quantitative genetic parameters of interest, such as the additive genetic variance covariance matrix across temperatures, **G** (Lande 1979), its mutational variance counterpart, **M** (Lande 1975), and multivariate vectors of divergence between populations, ***d*** (Schluter 1996). Further, the approach allows one to determine how well QTL effects match the three major axes of TPC variation. For example, a 'faster-slower' QTL would have effects for all temperatures in the same direction; a 'hotter-colder' QTL would have similar direction of effects at one end of the temperature range but in the opposite direction at the other; and 'generalist-specialist' QTL would have similar effects at mid-range temperatures but opposing effects at extreme temperatures (Fig. 3.1b-d).

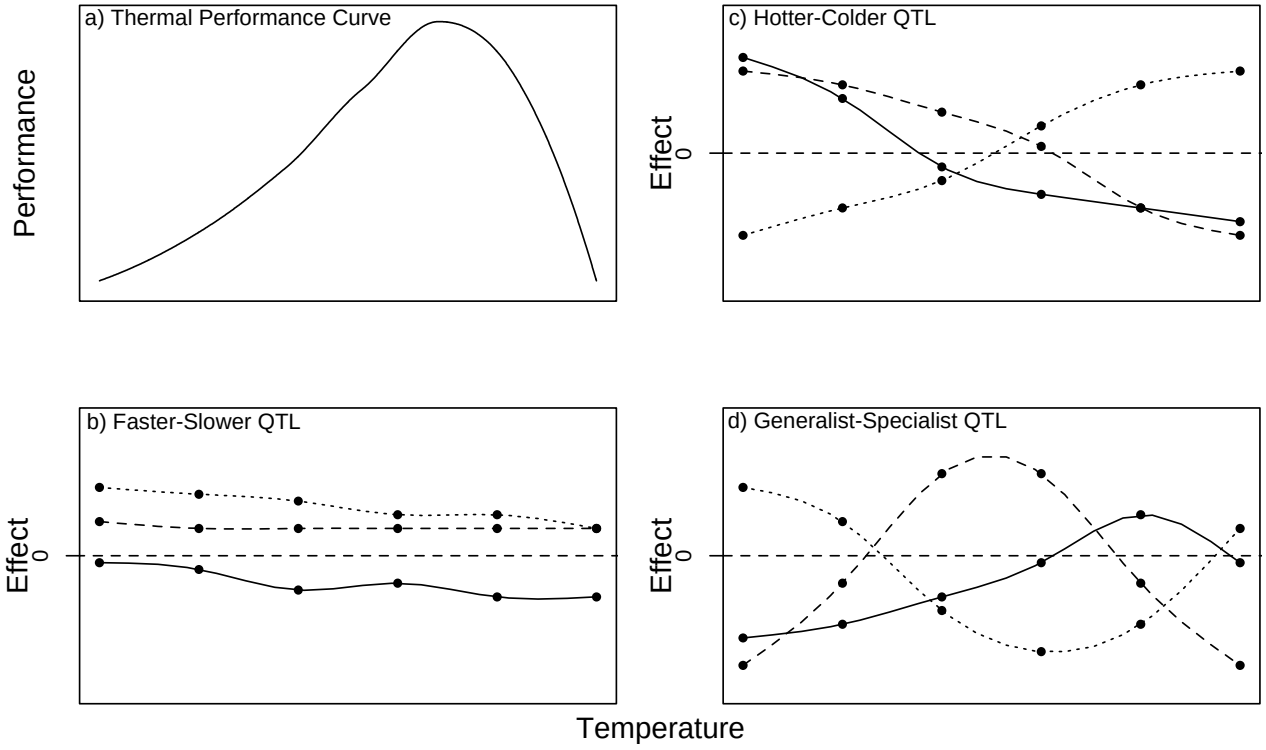


Figure 3.1: Schematic diagram of a) a thermal performance curve illustrating the common shape, and b-d) possible multivariate QTL effects resembling the three major axes of TPC variation: b) faster-slower QTL c) hotter-colder QTL and d) generalist-specialist QTL.

Here, I have implemented the multivariate QTL mapping framework for locomotor activity TPCs in the fruit fly *Drosophila serrata* using a panel of recombinant inbred lines

(RILs) that were established from a cross between two natural populations. Locomotor activity was used due to its known thermal sensitivity in ectotherms and association with fitness in *Drosophila* species where it has links with reproductive success, dispersal, predator avoidance and foraging (Gilchrist et al. 1997; Roberts et al. 2003; Long and Rice 2007). In *D. serrata*, both mutational genetic variance (Chapter 2) and standing genetic variance for TPCs has been characterised (Latimer et al. 2011) as has their genetic divergence along a latitudinal gradient (Latimer et al. 2011). I discuss my results in light of genetic constraints on thermal adaptation.

3.3 Methods

Recombinant Inbred Lines

I used a panel of 76 Recombinant Inbred Lines (RIL) of *Drosophila serrata* founded from a divergent line cross (Foley 2008). Founder lines were sampled from two natural populations (Eungella, QLD, 21.17° S, 148.50° E; Forster, NSW, 32.22° S, 152.53 E) spanning 11.05 degrees of latitude that represent opposing ends of the eastern Australian distribution of *D. serrata*. Founder lines were inbred for ten generations post collection. The founder lines were then checked for major inversions by examining polytene chromosomes to ensure that they were homosequential prior to crossing (Stocker et al. 2004). The F2 offspring of the cross were then inbred via full-sib mating for a minimum of a further 17 generations to isogenise them. All lines were maintained at 25^{O}C in a 12:12 dark-light cycle on a yeast-sucrose agar medium.

Thermal Performance Curve Assay

Individual TPCs were estimated by measuring locomotor activity when exposed briefly (20 minutes) to each of six temperatures in the order of 30, 25, 33, 36, 35, and 38 ± 0.3^{o}C (similar to Latimer et al. 2011; Chapter 2). Temperature was measured in this order to minimise differences between runs and prevent overly sudden changes in temperature. I measured the highest temperature last to prevent any detrimental effects interfering with any subsequent measurements (Gilchrist 1996). Single fixed, as opposed to multiple random, testing orders are often used when estimating TPCs (Gilchrist 1996; Wilson 2001; Wilson et al. 2001; Angilletta et al. 2002a; Ben-Ezra et al. 2008). In an earlier study, my colleagues and I conducted a pilot experiment to determine the influence testing order had

on the curves (Latimer et al. 2011). The average correlation between TPCs from 6 randomly selected testing orders was very high in both sexes (females: r = 0.9216; males: r = 0.9346), and therefore I opted for higher throughput with a fixed testing order over the use of random testing orders which would have dramatically reduced the number of samples we could process. All measurements were conducted in one single temperature cabinet and flies were rested at 25 ± 0.5^{o}C for 40 minutes between each temperature treatment to prevent stress and acclimation. TPC assays were conducted in 12 completely randomised runs performed over six consecutive days. Two runs were processed per day; each run contained one male and one female per RIL and four males and four females per founder line. I estimated individual-level TPCs for 12 males and 12 females per RIL and for 48 males and 48 females for each of the two founder lines. Flies were sexed across three density controlled replicate vials for the RILs and founder lines and held singly in vials until the time of the assay. Phenotyping was performed on five to seven day-old virgin flies.

Locomotor activity was measured using Drosophila Activity Monitors (DAM, TriKinetics, Waltham, Massachusetts, USA) in line with the method used by Latimer et. al (2011; 2014). A DAM comprises 32 holding tubes that are bisected by an infrared beam. Activity is recorded as a count of intersections across the beam made by the fly over a period of 20 minutes, thus preventing acclimation to the temperature treatment. Assays were run between 04:15 and 13:30 each day. Flies were placed in the 5 mm x 65mm holding tubes and held at a constant temperature of 25^{o}C the prior afternoon to allow the flies to adjust to the new physical environment. Each 5 mm holding tube contained, at one end, 1.5 cm of a three- day-old agar-sucrose medium to impede desiccation.

The traits used to measure TPCs are ideally an aspect of an organism's overall capacity to function (Angilletta 2009). All activity metrics involve both physiological and voluntary aspects of movement; however, it could be argued that activity captured by the DAMs, potentially involves a greater voluntary component than other performance metrics, such as sprint speed (Angilletta et al. 2002a; Lachenicht et al. 2010). Previous studies using the DAM based measure have successfully recovered expected TPC shapes and detected the expected trade-offs (Latimer et al. 2011), as have similar activity-based assays (Gilchrist 1996; but see Angilletta 2009).

Molecular Markers

A total of 49 molecular markers were used to genotype the RILs (Appendix 3.2). This panel of markers consisted of 16 expressed sequenced tag (EST) derived single-nucleotide polymorphisms (SNPs) genotyped using a SEQUENOM Assay (Stocker et al. 2012), 7 derived Cleaved Amplified Polymorphic Sequences (dCAPS) markers genotyped using Agarose gels and 23 microsatellites. Microsatellites were derived chiefly from a *D. serrata* EST collection (Frentiu et al. 2009) with the exception of *Dser6* (marker 25), which was developed earlier (Magiafoglou et al. 2002). Because of the strong conservation of chromosome arm-level gene content between *D. serrata* and *D. melanogaster* genomes (Stocker et al. 2012), I was able to use BLAST (Altschul et al. 1997) to assign markers to chromosomal arms. Additionally, the 16 SNPs genotyped using SEQUENOM have been placed on the *D. serrata* linkage map (Stocker et al. 2012). Although it was possible to assign almost all the markers to chromosomal arms, it was not possible to create a linkage map for this set of markers because of low linkage disequilibrium between markers on the same chromosome. For this reason, I performed all QTL analyses using a single-marker approach as opposed to interval mapping. Although some markers exhibited segregation distortion, those with minor allele frequencies greater than 20% were retained for QTL mapping because single marker analysis is not particularly sensitive to segregation distortion (Xu 2008).

Statistical Analysis

Quantitative Genetic Analysis

I first tested for differences in mean locomotor activity between the two Eungella and Forster founder lines. For all analyses, locomotor activity was square root transformed to improve normality. Sexes were analysed separately, due to locomotor activity across temperatures being sexually dimorphic in *D. serrata* (Latimer et al. 2011). The mixed effects model was:

$$a = \mu + f + t + b + f \times t + f \times \mathrm{b} + t \times b + f \times t \times \mathrm{b} + v(f) + \varepsilon \,, \qquad [3.1]$$

where, $\boldsymbol{a}$ is locomotor activity taken at the six test temperatures for each sex, μ is the mean, f is founder line, t is temperature (here modelled as a categorical factor), b is block, reflecting the twelve experimental runs and v(f) is vial nested within founder line. I

modelled t, f, b and all interactions as fixed effects and the vial term, v(f), as random. I treated block as a fixed effect because the blocks were temporal instead of a random sample of times, where they were twelve successive runs conducted over the 6 test days (Quinn and Keough 2002). Analyses were performed using the MIXED procedure in SAS (ver. 9.3; SAS Institute, Cary NC).

I also estimated the multi-temperature vector of divergence between the two founder lines, ***d***, from:

$$d = \left[\overline{\mathbf{X}}_{Eungella} - \overline{\mathbf{X}}_{Forster}\right]\left[\left(\overline{\mathbf{X}}_{Eungella} - \overline{\mathbf{X}}_{Forster}\right)'\left(\overline{\mathbf{X}}_{Eungella} - \overline{\mathbf{X}}_{Forster}\right)\right]^{-1/2} \quad [3.2]$$

where, $\overline{\mathbf{X}}$ refers to the six element mean vectors for each founder (Schluter 1996). Before divergence was calculated, the data were mean standardised using the overall population mean of RILs (founders included) to permit comparison with the QTL effects that were also estimated on a mean standardised scale.

To test for genetic variance among the RILs, I fitted a multivariate mixed effects model:

$$\boldsymbol{a} = \boldsymbol{\mu} + \boldsymbol{b} + \boldsymbol{l} + \boldsymbol{v(l)} + \boldsymbol{\varepsilon}\,, \quad [3.3]$$

where, ***a*** is a vector of locomotor activity scores (mean standardised) taken at the six test temperatures, ***μ*** is the vector of means, ***b*** is the block effect reflecting the twelve experimental runs, ***l*** is the line (RIL) effect and ***v(l)*** is the effect of vial nested within line. Block was modeled as a fixed effect (for the same reasons explained above), whereas all other terms were treated as random. I estimated the genetic variance-covariance matrix, **G** (Lande 1979), from the (co)variance component estimates corresponding to the line term. An unstructured covariance matrix (Type=UN; SAS MIXED procedure) was fitted at this level. Significance of the line term was tested using likelihood ratio tests against the null model where the line term was removed. To identify the major axes of genetic (co)variance among the RILs, I performed an eigenanalysis on the **G** matrix from [3] for each sex.

Multivariate QTL Mapping Analysis

A multivariate QTL analysis was performed to find markers associated to locomotor activity across all temperatures. The QTL mapping model was:

$$\mathbf{A} = \boldsymbol{X_i B} + \mathbf{E}\ , \qquad [3.4]$$

where, **A** is a n = 76 x 6 element matrix of means for the 76 RILs at six temperatures, **X** is an 76 x 2 element design matrix with 1 in the first column and the coding variables for the marker genotype *i* (0,1) in the second column, **B** is a 2 x 6 element matrix containing the six intercepts in the first row and six slope estimates in the second row that correspond to the additive 'effects vector' of the marker at each temperature. The residual error, **E**, here equates to the among RIL variance-covariance matrix. This model was performed for each of the 49 markers separately. I fitted this model using the MIXED procedure in SAS Version 9.3 (SAS Institute Inc. Cary NC) and assumed an unstructured variance covariance matrix for **E.** The approach is essentially a multivariate regression and is similar to that based on canonical correlation used for QTL mapping by Leamy et al. (1999) and Wolf et al. (2005). More recently, this approach has also been adopted for use in association mapping (Ferreira and Purcell 2009).

All QTL mapping was conducted at the level of RIL means. I performed a permutation test to check that the F-ratio approximated p-values for the QTL model were sensible. Briefly, I randomly shuffled the 6 element RIL mean activity vectors against the marker data 1000 times and estimated the linear model [4] retaining the F-ratio and p-value at each iteration. I found that the permutation-based p-values were always extremely close to the F approximation and no systematic bias was apparent (Appendix 3.1). To correct for multiple testing, a false discovery rate (FDR) analysis was performed taking the approach of Storey and Tibshirani (2003) implemented in the qvalue package (Dabney et al. 2004) in R (R Core team version R 3.1.0).

To provide a preliminary representation of the types of QTLs detected, I categorised QTL effects into three major groupings according to the consistency of the direction of QTL effects across temperatures. For the first class all the effects had to be in the same direction (i.e. all positive or all negative; Fig. 1b) but they could vary in magnitude, making this a broad definition of the 'faster-slower' axis. For second class, one switch in direction had to occur across the curve resulting in the vectors splitting into two groups with opposing direction (e.g., +++---, ++++-- or +++++-; Fig. 1c). For the final group two switches in direction had to occur, where the effects for intermediate temperatures were in the opposite direction to those at the extremes (e.g. ++--++, +----+, ++-+++; Fig. 1d). Because these groupings were initially based on the point estimates of QTL effects, I also

examined the nominal significance of effects at the individual testing temperatures, testing for a significant deviation from zero.

I was also interested in the alignment of marker effects with the divergence vector between the founder lines, ***d***. To explore this association I calculated angles between marker effects vectors, ***m***, and the vector of divergence, ***d***. I calculated the angles between $\boldsymbol{d}_i$ and ***m*** from $\theta = \cos^{-1}\left(\frac{\boldsymbol{di}^T\boldsymbol{m}}{\|\boldsymbol{di}\|\|\boldsymbol{m}\|}\right)$ (Schluter 1996; Blows and Walsh 2009). The angles between the marker effects, ***m,*** and the eigenvectors of **G**, $\boldsymbol{\lambda}_i$, were also calculated in the same way. This allowed me to assess the orientation of QTL effects with the major axes of the genetic variance created in this synthetic recombinant population. Angles were calculated for all significant QTLs. The lengths of the effects vectors were taken as a measure of overall QTL effect size.

3.4 Results

Thermal Performance Curve Shape

Locomotor activity was positively correlated with temperature, with males generally having a higher level of locomotor activity than females (Fig. 3.2). A gradual increase in activity to a peak followed by a reduction was pronounced in most lines (in 59 and 63 out of 76 RILs for males and females respectively). The reduction in activity after the peak did not fall back all the way to zero at 38^{o}C, as seen in the idealised thermal performance curve shape (Fig. 3.1), suggesting that higher testing temperatures may have been required to observe further reductions in activity. A small number of lines had highest mean performance at the hottest temperature of 38^{o}C.

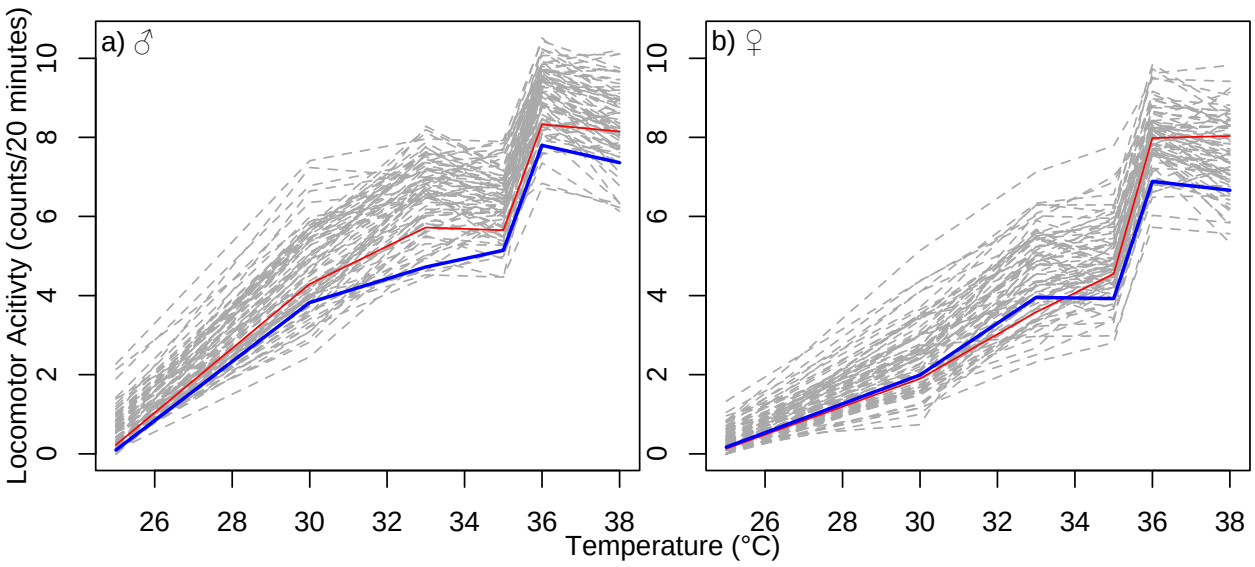


Figure 3.2: The mean locomotor activity TPCs for each recombinant inbred line for a) males, b) females. Locomotor activity was measured as counts over a twenty-minute period and square root transformed (see methods). The Eungella parent is in red, the Forster parent in blue, and the recombinant inbred lines means are broken grey lines.

Quantitative Genetic Analysis

My analysis of the founder lines detected significant divergence between the northern and southern genotypes. For males, there was a significant founder line effect (Table 3.1: $F_{1,426}$=33.42, p<0.0001), with Eungella males having consistently higher locomotor activity than Forster males. However, the interaction term was marginally non-significant (Table 3.1: $F_{5,426}$=1.88, p=0.0960), suggesting that males differed in overall locomotor activity across all six temperatures but to a lesser degree in the shape of the curve. For females, the overall founder line effect was not significant (Table 3.1: $F_{1,402}$=0.36, p=0.5482) but the interaction was highly significant (Table 3.1: $F_{5,402}$=4.37, p=0.0023). This suggests that female founder genotypes differed to a greater degree in the shape of the performance curve, as can be observed by the crossing of founder TPCs in Figure 3.2b. Eungella line females had higher activity than Forster line females at 25^{o}C, 30^{o}C and 33^{o}C whereas Forster had higher activity at temperatures of 35^{o}C and above. When comparing the RIL means to the founder lines for all temperatures, the RILs often spanned a far wider range of activity levels than the founders (Figs. 3.2&3.3). This pattern suggests extensive transgressive segregation for locomotor activity across the six temperatures (See Discussion).

Table 3.1: Mixed effects linear model testing for differences in *D. serrata* locomotor activity between the Eungella and Forster founder lines.

Effects	**Test Statistic**	**p-value**
Males		
Temperature	$F_{5,426} = 353.86$	<0.0001
Founder Line	$F_{1,426} = 33.42$	<0.0001
Block	$F_{11,426} = 18.43$	<0.0001
Founder Line x Temperature	$F_{5,426} = 1.88$	0.0960
Founder Line x Block	$F_{11,426} = 1.73$	0.0650
Temperature x Block	$F_{55,426} = 4.99$	<0.0001
Founder Line x Temperature x Block	$F_{55,426} = 0.97$	0.5463
Vial(Founder Line)	$\chi^2_1 = 93.8$	<0.0001
Females		
Temperature	$F_{5,402} = 275.67$	<0.0001
Founder Line	$F_{1,402} = 0.36$	0.5482
Block	$F_{11,402} = 11.00$	<0.0001
Founder Line x Temperature	$F_{5,402} = 4.37$	0.0007
Founder Line x Block	$F_{8,402} = 2.55$	0.0102
Temperature x Block	$F_{55,402} = 4.71$	<0.0001
Founder Line x Temperature x Block	$F_{40,402} = 0.94$	0.5719
Vial(Founder Line)	$\chi^2_1 = 0.0$	1.000

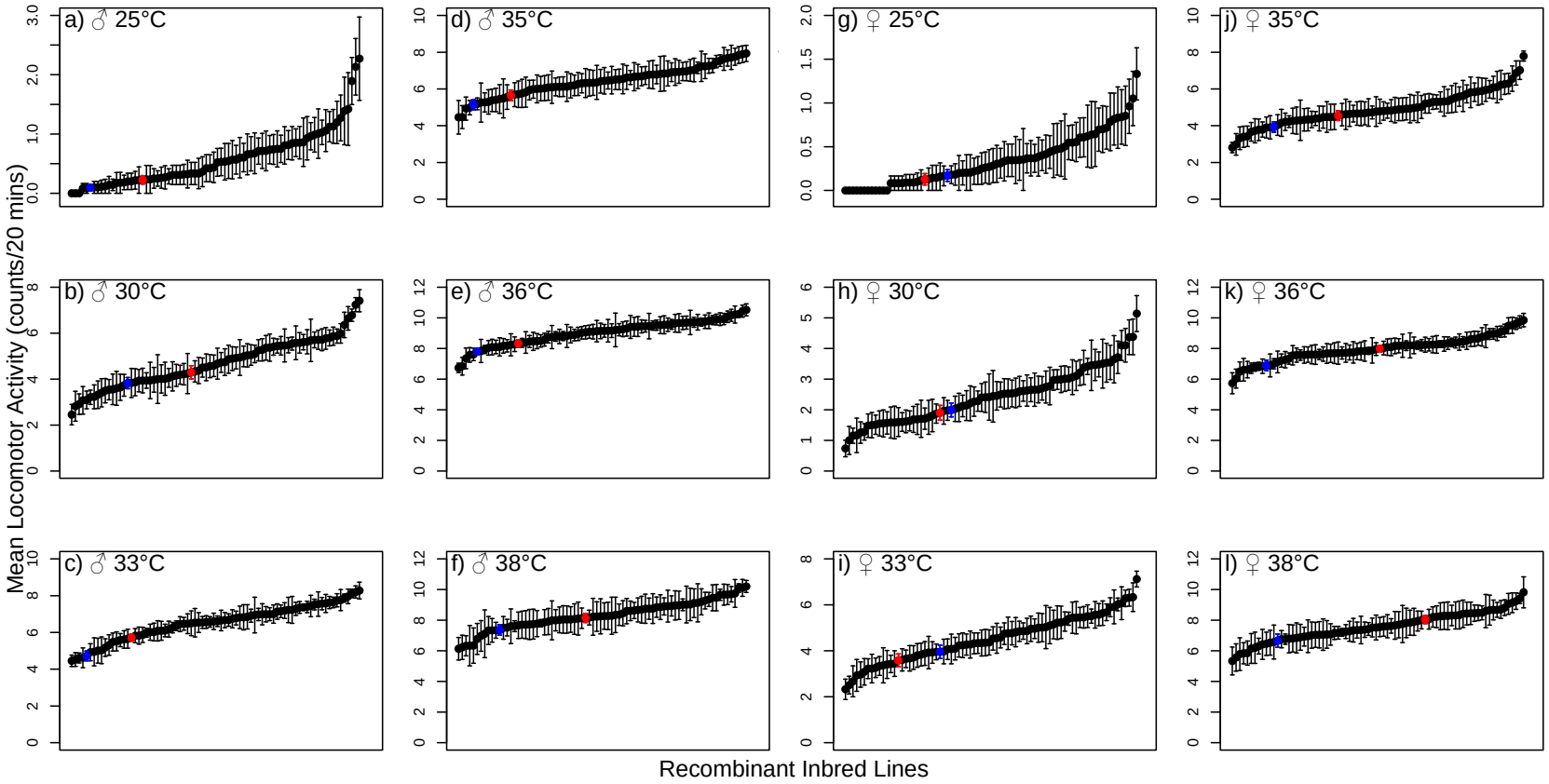


Figure 3.3: Mean locomotor activity (square-root transformed) ± s.e. for each of the recombinant inbred lines and parents at each of the six temperatures in *Drosophila serrata*. The recombinant inbred lines are sorted in ascending order. Eungella parent is in red and Forster parent is in blue.

My analyses of genetic variance across the six temperatures in the RILs detected significant genetic (co)variance for both males (LRT χ^2=153.4, p<0.0001) and females (LRT χ^2=128.6, p<0.0001). For males and females, the genetic covariances between temperature pairs were positive except for the most extreme pair of temperatures, 25^{o}C:38^{o}C. Furthermore, the genetic correlations between the temperatures tended to be strongest for more similar temperatures (Table 3.2).

Table 3.2: The among RIL genetic variance covariance matrix (**G**) for *D. serrata* locomotor activity across the six temperatures. Variances are along the diagonal in bold, covariances below the diagonal, and the genetic correlations above the diagonal. Also indicated on the far right are the multi-temperature divergence vectors, ***d***, between founder lines and the first three eigenvectors of **G** and the percentage of variance they explain.

Males	**25**	**30**	**33**	**35**	**36**	**38**	**d**	λ_1 (77.79%)	λ_2 (16.29%)	λ_3 (4.92%)
25	0.105	0.751	0.581	0.117	0.131	-0.139	0.476	0.864	-0.356	0.331
30	0.046	0.035	0.746	0.471	0.321	0.137	0.371	0.443	0.350	-0.796
33	0.022	0.016	0.013	0.892	0.676	0.611	0.600	0.222	0.457	0.193
35	0.003	0.008	0.009	0.008	0.814	0.835	0.305	0.071	0.508	0.140
36	0.003	0.004	0.006	0.005	0.005	0.993	0.231	0.048	0.343	0.318
38	-0.003	0.002	0.005	0.006	0.005	0.006	0.360	-0.002	0.407	0.314
Females								(75.52%)	(19.00%)	(5.33%)
25	0.064	0.766	0.662	0.340	0.002	-0.061	-0.200	0.589	-0.595	0.462
30	0.049	0.063	0.800	0.692	0.385	0.249	-0.118	0.636	0.040	-0.764
33	0.029	0.035	0.030	1.009	0.763	0.547	-0.286	0.418	0.354	0.381
35	0.011	0.022	0.022	0.016	0.950	0.789	0.438	0.246	0.471	0.133
36	0.000	0.008	0.011	0.010	0.007	0.974	0.506	0.094	0.400	0.132
38	-0.001	0.005	0.008	0.008	0.007	0.007	0.645	0.065	0.371	0.150

My eigendecomposition of **G** from [3.3] found that the first three eigenvectors explained 99% of the variance for both males and females, with similar vector loadings for both sexes (Fig. 3.4). The first eigenvector explained the majority of the variance of **G**, 77.79% and 75.52% for males and females respectively, and the majority of the loadings were positive. Therefore this vector most closely resembled a “faster-slower” mode of TPC variation for females. However for males, loadings were all positive with the exception of 38^{o}C, for which a very small negative loading was observed (Fig 3.4a,d). The loadings for

the first eigenvector also tended to weaken towards higher temperatures in both sexes. The second eigenvector accounted for 16.29% and 19.00% of the variance for male and female **G** respectively (Fig. 3.4b,e). Loadings indicated a change in sign between the lowest temperature of 25^{o}C and all other temperatures. Although a classic interpretation of this vector would most closely fit the “hotter-colder” mode of variation, because our RIL performance curves were largely monotonically increasing, this axis also contains variation for thermal sensitivity or slope variation. The third eigenvector explained only a small amount of genetic variance in each sex; 4.92% and 5.39% in males and females respectively (Fig. 3.4c,f). The loadings indicated two changes of sign along the curve resulting in a contrast between intermediate (30^{o}C) and high and low temperatures. This axis corresponds to changes in curvature, which may underlie trade-offs, consistent with the “generalist-specialist” axis of variation.

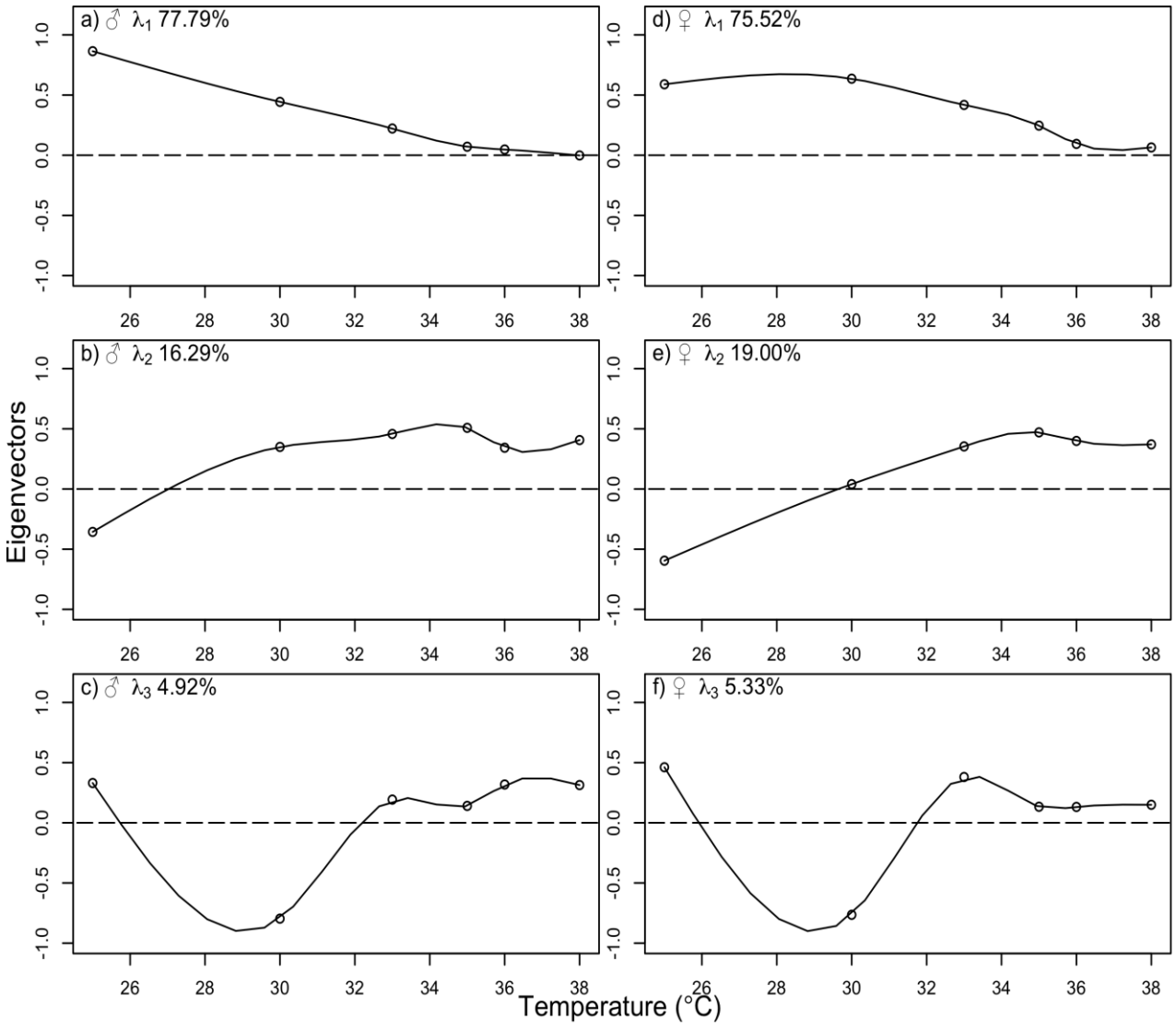


Figure 3.4: Loadings across temperatures for the first three eigenvectors of the male (a-c) and female (d-f) among-RIL **G** matrices for locomotor performance in *D. serrata*. Circles indicate vector loadings for each testing temperature and lines were fit using a lowess smoother. Each **G** matrix was mean standardised before the eigenanalysis was conducted.

Multivariate QTL Analysis

Of the 49 markers assayed, two (4 and 23) had severe segregation distortion with minor allele frequencies less than 20% and so were not considered further. In general, any distortion tended to be towards the Forster parent. As the general goal of my study was the broad exploration of possible QTL effects across the different temperatures rather than a fine-scale dissection of specific QTLs, I used a false discovery rate of 0.10. At this threshold we found eleven (female) and four (male) markers significantly associated with locomotor activity across the six test temperatures (Table 3.3).

Table 3.3: Significant markers from the multivariate QTL analysis model ordered by effect size (length of effects vector). Positive values for the temperature-specific QTL effects indicate that the Forster allele increases activity. The F-value and p-value, with the q-value in parenthesis, the marker vector length, pattern of sign change, the angles (***θ***) between the marker vectors and the divergence vector (***d***), and the first three eigenvalues (**λ**) of six are displayed. Note that the variation in error degrees of freedom for the QTL F-values is due to variation among the RILs in missing genotype data. QTL effects also appear visually in Figure 5.

Marker	**Chr. Arm**	**F-value**	**P (qvalue)**	**QTL Effect (s.e.)**						**Effect size (length)**	**Sign change pattern**	**θ**			
				25°C	**30°C**	**33°C**	**35°C**	**36°C**	**38°C**			**D**	**λ_1**	**λ_2**	**λ_3**
Males															
11	2R	$F_{6,53}$=3.230	0.009 (0.094)	0.339 (0.119)	0.195 (0.056)*	0.125 (0.037)*	0.104 (0.036)*	0.050 (0.025)*	0.065 (0.031)*	0.431	++++++	29.5	15.0	76.4	85.7
7	3R	$F_{6,71}$=4.111	0.001 (0.057)	0.331 (0.098)*	0.203 (0.046)*	0.113 (0.031)*	0.059 (0.028)*	0.018 (0.021)	0.009 (0.026)	0.409	++++++	37.1	7.0	83.7	88.1
35	3R	$F_{6,72}$=3.267	0.007 (0.094)	-0.139 (0.099)	-0.157 (0.046)	-0.019 (0.033)*	-0.005 (0.028)	-0.002 (0.021)	0.012 (0.025)	0.211	+++++-	129.1	22.8	86.8	68.3
44	3R	$F_{6,72}$=3.413	0.005 (0.094)	0.033 (0109)	-0.086 (0.053)	-0.044 (0.036)	-0.018 (0.031)	-0.051 (0.022)*	0.009 (0.028)	0.116	+----+	119.3	78.6	42.7	62.0
Females															
25	N/A	$F_{6,67}$=5.330	0.0002 (0.005)	0.107 (0.098)	0.273 (0.074)*	0.126 (0.054)*	0.162 (0.043)*	0.030 (0.025)	0.039 (0.031)	0.362	++++++	86.6	22.2	74.8	77.2
35	3R	$F_{6,72}$=4.405	0.001 (0.012)	-0.097 (0.086)	-0.270 (0.064)*	-0.152 (0.045)*	-0.104 (0.039)*	-0.024 (0.024)	-0.019 (0.028)	0.342	------	85.8	20.1	77.8	75.7
40	X	$F_{6,72}$=2.620	0.024 (0.096)	0.263 (0.078)*	0.160 (0.068)*	0.091 (0.047)	0.021 (0.041)	0.013 (0.024)	0.003 (0.028)	0.322	++++++	104.3	20.6	71.6	83.0
7	3R	$F_{6,71}$=3.283	0.007 (0.054)	0.222 (0.085)*	0.142 (0.072)*	0.139 (0.047)*	0.099 (0.040)*	0.048 (0.024)*	-0.007 (0.029)	0.318	+++++-	96.9	14.5	87.4	78.1

6	N/A	$F_{6,66}$=3.434	0.005 (0.054)	-0.094 (0.090)	-0.235 (0.067)*	-0.106 (0.049)*	-0.066 (0.043)	0.001 (0.026)	-0.010 (0.029)	0.282	----+-	81.5	19.5	84.8	72.5
5	3R	$F_{6,66}$=2.490	0.031 (0.096)	-0.080 (0.092)	-0.218 (0.071)*	-0.104 (0.048)*	-0.090 (0.041)*	-0.014 (0.025)	-0.027 (0.029)	0.272	------	88.3	20.4	78.1	74.6
49	2R	$F_{6,70}$=2.548	0.027 (0.096)	0.008 (0.090)	0.177 (0.071)*	0.094 (0.050)	0.103 (0.042)*	0.036 (0.024)	0.067 (0.029)*	0.238	++++++	76.1	37.3	58.7	73.5
41	3L	$F_{6,70}$=2.464	0.032 (0.096)	-0.092 (0.088)	-0.168 (0.070)*	-0.068 (0.049)	-0.068 (0.042)	0.011 (0.025)	0.009 (0.029)	0.214	----++	79.4	17.8	90.0	75.5
26	3L	$F_{6,73}$=2.687	0.021 (0.096)	-0.068 (0.085)	-0.170 (0.066)*	-0.046 (0.047)	-0.042 (0.040)	0.021 (0.023)	0.018 (0.027)	0.195	----++	75.0	26.7	86.4	65.5
12	2R	$F_{6,72}$=2.723	0.019 (0.096)	0.092 (0.097)	-0.012 (0.079)	-0.127 (0.051)*	-0.072 (0045)	-0.055 (0.026)*	-0.028 (0.031)	0.184	+-----	108.6	80.4	24.9	84.5
46	2L	$F_{6,73}$=2.520	0.028 (0.096)	-0.007 (0.093)	0.049 (0.076)	-0.035 (0.052)	-0.096 (0.043)*	-0.043 (0.025)	-0.035 (0.030)	0.126	-+----	129.8	81.9	50.0	51.9

* $p < 0.05$

For females, five QTL (Table 3.3: markers 5,25,35,40,49) had effects with a common direction across all temperatures indicating genetic variation for overall increases or decreases in activity (i.e. 'faster-slower' variation). Follow up testing suggested that at most 5, and as few as 2 temperatures, were nominally significant, likely reflecting the fact that, although directionality was shared, effect sizes differed greatly between temperatures. Four QTLs (Table 3.3: markers 7,12,26,41) had a single sign change along the curve, representing a switch in direction of effect between high and low temperatures. However, for only one of these (marker 41) was the change in sign significant (i.e. a significantly negative and a significantly positive value). For the other three QTL all significant effects had a common direction, suggesting that many of these QTL probably contribute more to variation for overall activity than to trade-offs between temperatures. For two QTL in females (Table 3.3: markers 6 and 46), I observed two sign changes in the point estimates, however, in no case were both sign changes supported by individual significance testing. As above, only effects with a consistent direction were significant at individual level for these QTL, suggesting that curve shape changes or 'generalist-specialist' effects were poorly supported by the data (Fig 3.5d-f).

For males, two QTL (Table 3.3: markers 7 and 11) were detected with consistent directions of effect across temperatures, indicating overall increases or decreases in activity. One QTL (marker 35) had a single significant sign change along the curve between 36^{o}C and 38^{o}C. The remaining QTL (marker 44) had two sign changes in the point estimates suggesting a change in curvature, but these sign changes were not supported in follow up testing (Fig 5a-c). For both sexes, QTLs contributing to variation in overall activity, with either all positive or all negative loadings, tended to have larger effect sizes than those involving any changes in sign (Table 3.3). The emergent pattern from the multivariate QTL analysis was one where most QTL contributed to variation for overall activity and had larger effect sizes than the smaller number of QTLs involving changes in sign along the curve.

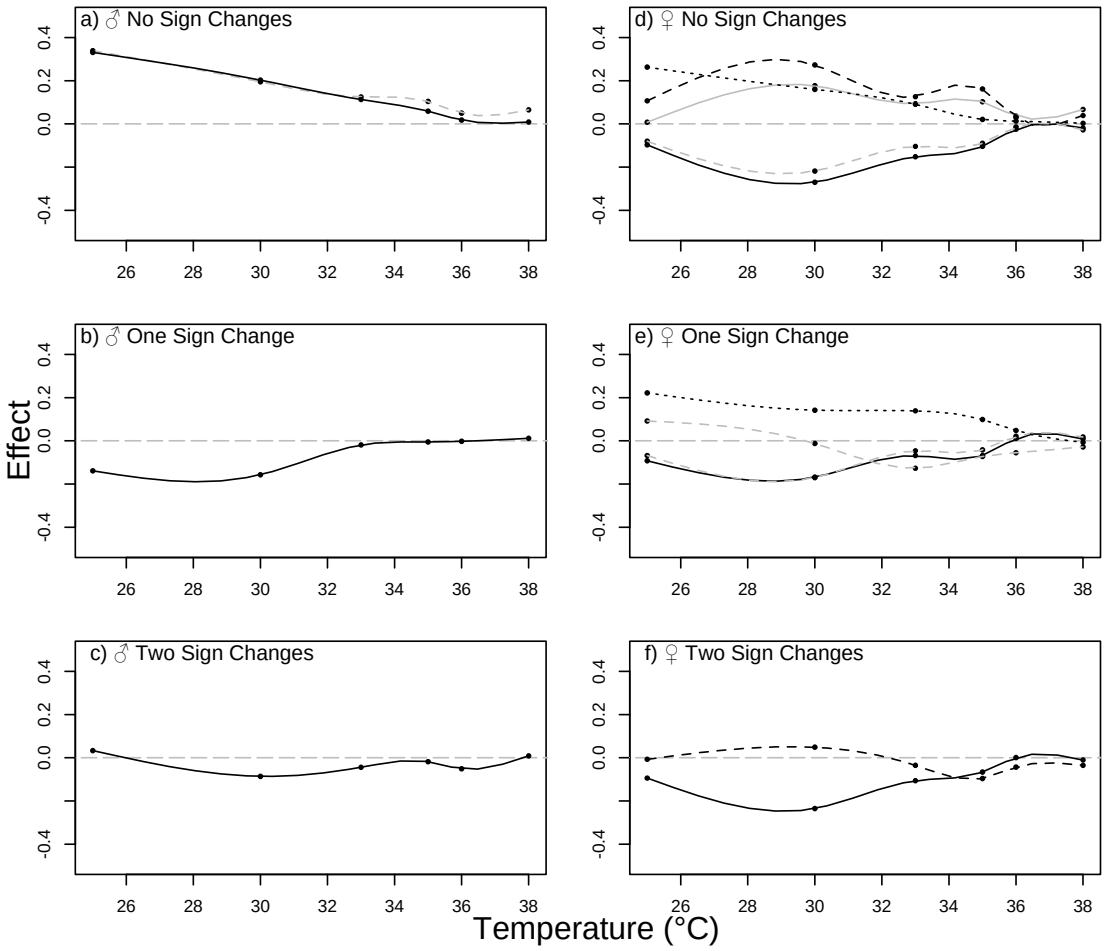


Figure 3.5: The effects vector loadings for the significant QTLs across the six temperatures for mean standardised locomotor activity TPCs in D. serrata males (a-c) and females (d-f). Black dots indicate the effects on each testing temperature. Lines were fit using a lowess smoother. QTLs were classified according to the number of sign changes between temperatures moving along the curve from left to right. Positive loadings indicate that the Forster allele increases the trait.

Alignment of QTL effects with population divergence and axes of genetic variance

I calculated the angles between multivariate QTL effects and the multivariate divergence vector, **d**, between the two founder lines; a divergence angle close to 0^o would indicate a marker more associated with Eungella while angles near 180^o indicate alignment with Forster. No QTLs were a particularly good match to the pattern of divergence between Eungella and Forster lines (Table 3.3). Divergence angles ranged between 75^o and 130^o for females, and between 29^o and 129^o for males. I also compared QTL effects with first three eigenvectors of **G**. The vast majority of QTLs were best aligned

with the first eigenvector of **G** (males: 3/4, females: 9/11) with angles ranging between 7^{o} and 23^{o} in males, and between 15^{o} and 38^{o} in females. Remaining QTLs were best aligned with the second eigenvector of **G** (females: 2/11, 15^{o} and 38^{o}; males: 1/4; 43^{o}), and none aligned well with the third eigenvector.

3.5 Discussion

Thermal adaptation and the genetic architecture of thermal performance curve variation

A major goal of this study was to begin to understand the different types of allelic effects that constitute genetic variance for TPCs and the modes of variation involved in thermal adaptation. As the cross is between different populations, the genetic variances and covariances among temperatures contained within the **G** matrix can be considered 'synthetic. Furthermore, because allele frequencies are close to symmetrical, additive genetic variance is likely to be maximised (Falconer and Mackay 1996) and relatively unaffected by natural selection, which is not always the case when examining standing variation within a population. *D. serrata* locomotor activity TPCs are unique among other TPCs traits in that estimates of mutational (co)variance are available for an identical assay set up as used in the present study (Latimer et al. 2014). To determine the extent to which the genetic variance captured in our mapping population represented the mutational variation in these traits, I examined the orientation between the first three eigenvectors of **G** and the first three eigenvectors of **M**. Alignments were remarkably tight, for the first two eigenvectors (Males: $\boldsymbol{\lambda_1}$ r = 0.97 or 14.5 degrees; $\boldsymbol{\lambda_2}$ r = 0.86 or 30 degrees and Females: $\boldsymbol{\lambda_1}$ r = 0.98 or 12.2 degrees, $\boldsymbol{\lambda_2}$ r = 0.86 or 30 degrees). The association was still strong in females for the third eigenvector in females ($\boldsymbol{\lambda_3}$ r = 0.94 or 19.930 degrees) but weakened for males ($\boldsymbol{\lambda_3}$ r = 0.46 or 62.6 degrees). These observations suggest that although synthetic, the cross has captured biologically relevant variation such that the estimated QTL effects should be informative of the TPC genotype-phenotype map, thereby allowing us to determine how allelic effects generate genetic variance in these curves and how they align with the major trade-offs involved in thermal adaptation.

The first eigenvector of **G** had positive loadings across all temperatures, with an exception of a very small negative value for the highest temperature in males and most likely reflects an availability of genetic variance for overall activity variation. This axis most closely resembles the faster-slower axis of TPC variation. However, although all

temperature loadings were positive, there was a noticeable decline in their strength towards the hotter temperatures. As my analyses were performed on mean standardised trait values, this association between temperature and loading strength is not a simple consequence of mean-variance scaling effects; instead it is likely a true reflection of a decrease in genetic variance at hotter temperatures. This pattern was also reflected in the multivariate QTL effects; the overwhelming majority of QTLs were best aligned with the first eigenvector of **G** and had a characteristic pattern of lower additive effects at high temperatures (see Table 3 & Figure 5). The tight correspondence between axes of mutational and genetic variance and multivariate QTL effects suggests that a class of pleiotropic variants that increase (or decrease) overall activity, but at the same time have ever shrinking effects towards high temperatures, are a dominant feature of the genetic architecture of the thermal sensitivity of locomotor activity in *D. serrata*.

In some studies of TPC variation, larger, not smaller, genetic variances have been observed at the highest temperatures (Gilchrist 1996; Kingsolver et al. 2004b; Knies et al. 2009). It is difficult to make direct comparisons between these studies because they do not report mean standardised estimates of genetic variance. However, one possibility is that because I did not observe large falls in activity at the highest testing temperatures, greater genetic variance could have been observed if higher testing temperatures were used. Genotypes may exhibit greater variation in the temperature at which activity declines rapidly than they do close to the activity peak.

Smaller genetic variances at hotter rather than cooler temperatures, as we have observed, have also been reported for growth rate TPCs in natural populations of *Sepsid* flies (Berger et al. 2013). As their study examined standing, as opposed to mutational variance, the authors interpreted the lower genetic variances at high temperatures in terms of a mutation-selection balance, arguing that perhaps some genetic variance has been depleted at high temperatures owing to stronger selection in this part of the curve. My study suggests that depletion of genetic variance may not be required to explain this pattern and that it could be a genuine feature of the genotype-phenotype map for TPCs rather than a consequence of allele frequency changes. This then begs the question of why allelic effects might be smaller at hotter rather than cooler temperatures? The asymmetric shape of TPCs implies that the fitness costs of increasing activity at the highest temperatures are far more severe than at lower temperatures (Martin and Huey

2008). One possibility is that a history of stronger selection at hotter temperatures has favoured the evolution of smaller mutational effects in this part of the curve. Alternatively, there may be physiological limits to increasing activity level when it is already high. Under this scenario, the smaller mutational effects would not have occurred from a history of selection but rather are a reflection of constrained physiology or biomechanics at higher temperatures.

The QTLs with a consistent direction of effect across all temperatures best resemble the 'faster-slower' axis of TPC variation and would generate positive genetic covariance between temperatures. However, because none had effects that were equal across all temperatures, a degree of thermal specialisation could nonetheless evolve through frequency changes of such variants (Fry et al. 1996). The large shifts in QTL effect sizes between temperatures makes thermal adaptation along the 'hotter-colder' axis also possible through these types of QTL effects. There were remarkably few QTLs showing significant sign changes between high and low temperatures. Such QTLs effects would suggest antagonistic pleiotropy between temperatures and may be classic indicators of the 'hotter-colder' trade-off. One possible reason for limited 'hotter-colder' variation could be due to our lowest testing temperature being 25^{o}C. Temperatures lower than 25^{o}C occur during the year in both parental (Eungella and Forster) populations. It may be the case that different physiological mechanisms, and therefore other loci, influence activity levels at lower temperatures. Assaying flies at lower temperatures may therefore reveal greater variation along a hotter-colder axis.

Experimental evolution studies have reported genetic constraints on the 'generalist-specialist' axis and have suggested that it may be the least evolvable of all axes (e.g. Berger et al. 2014). In this study, the 'generalist-specialist' axis was very poorly represented in the distribution of genetic variance; the third eigenvector of **G**, which accounted for less than 5% of the genetic variance possibly, contained an element of this type of variation. However, there were no convincing QTLs with 'generalist-specialist' like effects. The very few QTLs, showing two sign changes along the curve, had small overall effect sizes, and the sign changes were not supported by follow up testing. My results suggest that there may be fewer regions of the genome that, when variable, contribute 'generalist-specialist' genetic variance. Owing to the relatively large level of linkage disequilibrium generated in a QTL mapping cross, it remains possible that the effects

estimated here are due to multiple causal loci within the single QTL (Whitlock 1996); and these genomic regions could harbour variants with different types of pleiotropic effects across temperatures, perhaps masking ‘generalist-specialist’ like effects. Mapping studies conducted in populations with much lower levels of linkage disequilibrium, like outbred *Drosophila* populations, which tend to have very short range linkage disequilibrium, may be a useful next step in understanding the genetic architecture of TPCs.

Population differentiation and transgressive segregation

At all assay temperatures, the population of RILs spanned a much wider range of activity levels than the founder lines. This is the hallmark of transgressive segregation, which occurs when phenotypes with larger or smaller trait values than the founders arise from interactions among, or recombination of founder alleles, that increase or decrease the trait. In the simplest form, alleles associated in repulsion phase in parents (i.e. AAbb and aaBB parents with selection of A and B) results in offspring with either higher (AABB) or lower (aabb) trait values than the parents through recombination (Rieseberg et al. 1999; Rieseberg et al. 2003; Johansen-Morris and Latta 2006). The extensive nature of the transgressive segregation seen here suggests that *D. serrata* TPCs are highly polygenic. Although epistasis is not required to explain transgressive segregation, it can exacerbate it, sometimes resulting in a pattern of directional bias where more segregants have phenotypes above (below) than below (above) the parental values (Peiffer et al. 2013). For *D. serrata* there was a strong directional bias towards higher activity levels at all temperatures. Epistasis therefore may be a feature of the genetic architecture of *D. serrata* TPCs, at least at the scale of among-population differentiation examined here. This directional bias cannot necessarily be accounted for by segregation distortion (e.g. elimination of deleterious recessives, which might be expected to confer overall lower levels of activity). Although distortion was present for some markers, it was most often biased towards the southern Forster allele, which for all but the three lowest temperatures in females had lower activity levels than Eungella. To some extent, the transgressive segregation seen here is not surprising because the founder lines were not originally selected to be highly divergent, but rather were simply from different populations along the *D. serrata* eastern Australian distribution.

Some QTL-based tests for local adaptation rely on detecting a bias in the direction of QTL effects between founder lines derived from different populations. A significant

excess of QTLs with effects in one direction suggests a history of directional selection, while a random mixture of QTL effect directions is consistent with divergence via neutral processes such as genetic drift (Orr 1998; Rice and Townsend 2012). There was limited directional skew in the QTL effects across the six assay temperatures with more or less even numbers of positive and negative QTL effects in either sex, with the single exception of all positive effects for the 4 male QTLs at 38°C. Extending to a multivariate view, under strong directional selection we may expect to observe a close alignment between QTL effects vectors and the vector of divergence between populations. However, QTL effects were poorly aligned with the divergence between populations with angles generally quite large. If the different founder lines do indeed represent their original donor populations, divergent natural selection between them, in either univariate or multivariate form, may have been quite weak.

Conclusion

Studies of TPC divergence have revealed differences in the nature of thermal adaptation between populations; in some cases changes in reaction norm shape occur (Kingsolver et al. 2007; Berger et al. 2013), but sometimes divergence occurs mainly in overall performance with little change in shape (Klepsatel et al. 2013). Divergence can also occur via different modes across different parts of a species range (Berger et al. 2013). These observations can lead to quite different views on the importance of genetic constraints on thermal adaptation, and it has been suggested that the evolvability of TPCs might be taxon or trait specific (Angilletta et al. 2002b; Angilletta 2009). My study suggests that the overwhelming majority of QTL effects tended to involve changes in overall performance. Other axes, better aligned with changes in temperature of maximal performance or reaction norm shape, contributed far less genetic variance and were supported by fewer QTLs with generally smaller effects. It is also apparent that a degree of thermal specialisation remains possible through frequency change at loci affecting overall performance but with differing strength between temperatures. My results do not support a view of complete evolutionary independence between variation for overall performance, changes in thermal sensitivity, and changes in reaction norm shape.

Chapter Four:
Genome-wide dissection of natural variation in thermal performance curves for locomotor activity in *Drosophila melanogaster*

4.1 Abstract

Theories of thermal adaptation seek to explain how organisms adapt to their local thermal environments and in particular identify two major trade-offs, the 'generalist-specialist' and 'hotter-colder' trade-offs. Thermal adaptation is commonly explored through thermal performance curves (TPCs), reaction norms that characterise the relationship between organismal performance and temperature. Little is known about the genetic basis of TPCs, especially on a fine genomic scale. To address this, I performed a genome-wide association study (GWAS) of locomotor activity TPCs in 152 lines from the Drosophila Genetic Reference Panel (DGRP). Two types of analysis were performed to partition TPC variation. A character-state analysis detected five axes (principal components) of variation that described more than 95% of the genetic variance in both overall activity and TPC shape. The number of significant associations detected in the GWA analysis for each principal component (1 – 5) was 66, 29, 43, 12, and 39 respectively for females and 87, 53, 12, 16, and 23 variants respectively for males. A function-valued trait analysis showed the numbers of associated variants for each trait corresponded closely to their relative contributions to standing variation with 222 and 135 variants for 'hotter-colder', 66 and 17 for 'generalist-specialist', 56 and 18 for maximum performance, and only 20 and 5 for 'overall height' for females and males respectively. Annotation of associated polymorphisms suggested that gene expression is likely an important process shaping TPC variation compared with protein coding changes. I found strong skew in the direction in which the minor-frequency alleles affected TPC traits, suggesting a role for natural selection in shaping standing variation. Alleles producing specialised TPCs tended to occur at higher frequencies than alleles that produced generalists. An enrichment analysis on gene ontology terms showed that neurological functioning and responses to stimuli were key functional processes of the genes involved. This study provides insights into the

underlying genetic architecture of trade-offs that can have implications for thermal adaptation.

4.2 Introduction

Environmental temperature has a major influence on the performance of ectotherms through its influences on growth (Knies et al. 2009; Nilsson-Ortman et al. 2012), reproduction (Berger et al. 2008), and behaviour (Bennett 1980; Gilchrist 1996; Lyon et al. 2008), which in turn affects survival (Hoffmann et al. 2003; Ahnesjo and Forsman 2006) and fitness (Gilchrist et al. 1997). However, adaptation to specific temperatures is challenging because temperature varies greatly in both time and space (Gilchrist 1995; Angilletta 2009). Theories of thermal adaptation seek to explain how organisms adapt to their local thermal environments, and in particular, to identify the performance trade-offs that may exist between different temperatures.

Fitness usually cannot be maintained at a high level across all temperatures that an organism encounters (Levins 1968; Via and Lande 1987; Fry et al. 1996); instead, fitness trade-offs often occur between temperatures. These trade-offs are based on the performance of a genotype at one temperature being constrained by performance at an alternative temperature (Levins 1968; Palaima 2007). Two major trade-offs have been identified in thermal adaptation theory. First, a 'generalist-specialist' trade-off describes essentially a negative association between maximal fitness and the breadth of temperatures across which an organism can operate (Huey and Hertz 1984; Lynch and Gabriel 1987; Gilchrist 1995). Second, a 'hotter-colder' trade-off describes how individuals performing better in a hotter environment tend to have lower fitness in a cooler environment (Kingsolver et al. 2001).

These thermal trade-offs can be summarised by variation in thermal performance curves (TPC), a common class of continuous reaction norm characterising the relationship between organismal performance and temperature (Huey and Stevenson 1979; Izem and Kingsolver 2005). TPCs tend to exhibit a common shape across species; performance slowly increases with temperature, reaches a peak before falling suddenly at high temperatures (Huey and Stevenson 1979; Huey and Kingsolver 1989; Angilletta 2009). Generally, four components of the curves are identified; (Fig. 1a): 'overall height', indicating the performance across all the temperatures (Izem and Kingsolver 2005), curve

width, which describes the range of temperatures across which an individual can perform (Izem and Kingsolver 2005), maximum performance (z_{max}) at the peak, and optimum temperature (T_{opt}), indicating the temperature where performance is maximized (Izem and Kingsolver 2005).

It has been a challenge to develop statistical methods that can define these components for multiple individuals or genotypes in a consistent manner. For example, TPCs for some individuals may best resemble a third order polynomial function whereas for others, in the same data set, a fourth order or more complex function may be more suitable (Krenek et al. 2011). One method that attempts to deal with these shortcomings is the Template Mode of Variation (TMV) approach, created by Izem and Kingsolver (2005). Based on Lawton's (1972) shape invariant model, the approach fits a common 'template curve' to all individuals and then scores individual-level deviations from the common template in specific directions of biological interest. The attractive feature of TMV is the decomposition of phenotypic and genetic variation into three modes that describe the relevant trade-offs and thermal adaptation, the 'faster-slower', the 'hotter-colder', and the 'generalist-specialist' axis (Fig. 4.1) (Huey and Kingsolver 1989; Kingsolver et al. 2001; Izem and Kingsolver 2005). The 'faster-slower' axis explores the variation in 'overall height' of the curve, and represents performance across all temperatures. The 'faster-slower' axis is independent of temperature values but the other two shifts involve trade-offs, as they capture temperature-dependent variation (Fig. 4.1b). The 'hotter-colder' axis represents variation in the T_{opt} with TPCs shifting right or left along the temperature axis. Performance decreases at temperatures opposite to the direction of curve shift (Fig. 4.1c). For example, a hotter T_{opt} will result in loss of performance at the lower range of temperatures. Last, the 'generalist-specialist' axis represents variation in the width and z_{max} and incorporates a 'generalist-specialist' trade-off by restraining the area under the curve so that a wider curve has a lower maximal performance (Fig. 4.1c).

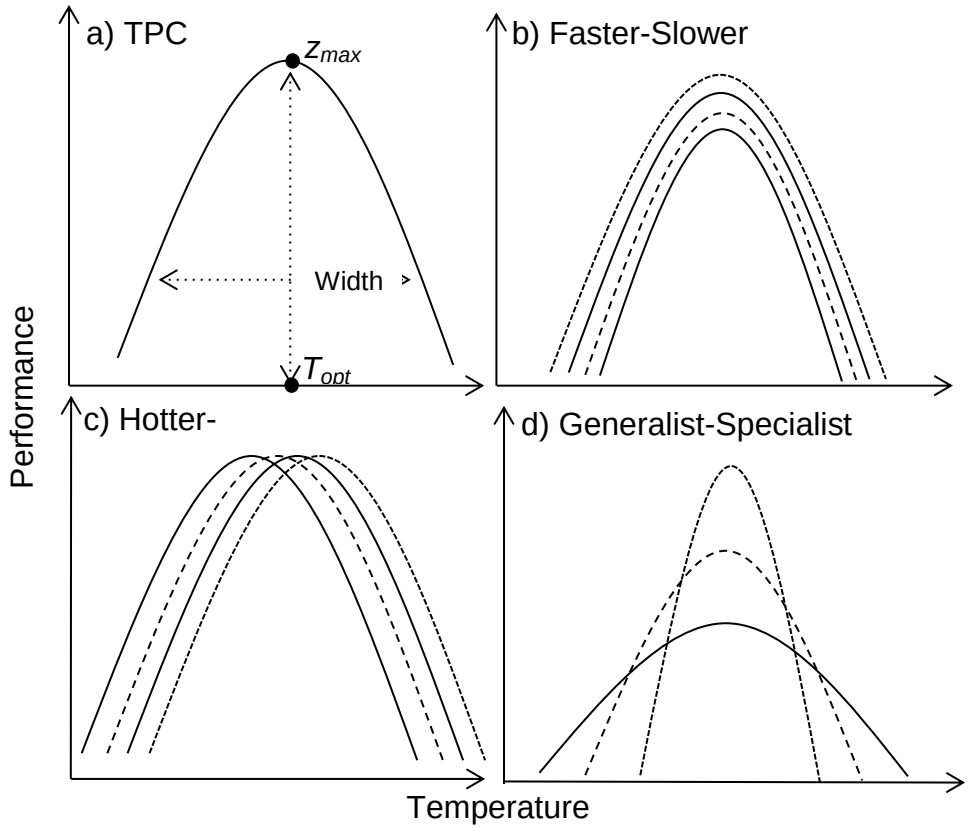


Figure 4.1: Schematic of thermal performance curves and axes of variation. a) A thermal performance curve illustrating the width, z_{max} and T_{opt} components, b) the 'faster-slower' axis, c) the 'hotter-colder' axis and d) the 'generalist-specialist' axis. Adapted from Izem and Kingsolver (2005).

The shape of TPCs and the trade-offs inherent in them, can arise from at least three physiological processes and the underlying genetic architecture (Angilletta 2009). First, the functions of enzymes across temperatures can pose thermal constraints; as temperatures increase the catalytic reaction time must decrease to maintain conformity for substrate binding (Somero 1995; Fields 2001; Hochachka and Somero 2002 ; Yang et al. 2013). Second, temperature impacts the movement and conformation of cellular membranes; high temperatures increase movement, disrupting the laminar structure, whereas cold temperatures decrease movement (Hazel and Eugene Williams 1990). Third, oxidation-limitation theory posits that thermal limits are set where aerobic respiration fails to meet energetic requirements. At higher temperatures, the energy required for performance is restricted due to the limited oxygen supply from respiration and circulatory processes, while at low temperatures the mitochondria cannot produce enough ATP for performance to be maintained (Pörtner et al. 2000). From a genetic perspective, trade-offs between

different environments arise as a consequence of the pleiotropic effects of different segregating variants (Fry et al. 1996). Broadly, two classes of pleiotropic effects could underlie a trade-off: antagonistic pleiotropic effects that increase performance at one temperature but decrease it at another, or pleiotropic effects that differ in magnitude but in the same direction across temperatures (Fry et al. 1996). The 'hotter-colder' and 'generalist-specialist' are perhaps likely to consist of antagonistic pleiotropic effects (Berger et al. 2014, but see Chapter 3). In addition to these classes of pleiotropic variant, there may be multiple variants that each affect only one TPC component and may therefore permit independent evolution of shape components (Anderson et al. 2013).

A genome-wide-association study (GWAS) in a natural population can capture natural variation and allow the identification of common variants affecting phenotypes (Iles 2008), such as TPC components. In contrast to the synthetic variation created by the crossing of pre-selected parents in my previous QTL chapter, a GWAS enables the mapping of standing variation. The analysis of a GWAS facilitates a more accurate estimation of individual allelic effects due to a finer mapping resolution. Whilst many studies have associated genes with responses to temperature (Nielsen et al. 2006; Sørensen et al. 2007; Jensen et al. 2008; Colinet et al. 2010; Svetec et al. 2011; Carreira et al. 2013), to my knowledge none have associated genes with TPCs. When applied in a well-annotated model species, the GWAS approach may be particularly powerful for linking genetic variance with the key physiological processes, such as enzyme function, cellular structure and oxidation-limitation, predicted to play a part in thermal trade-offs.

In this Chapter, I performed a GWAS on the major components of TPC shape to investigate genetic architecture at a finer scale. To do this, I took advantage of the Drosophila Genetic Reference Panel (DGRP), a fully sequenced panel of inbred *Drosophila melanogaster* lines sampled from a single natural population in Raleigh, North Carolina (Mackay et al. 2012; Huang et al. 2014). I phenotyped locomotor activity TPCs using two methods to statistically extract the axes of variation; I used the character-state principal component analysis approach similar to the previous two Chapters and the TMV function-valued trait approach (Izem and Kingsolver 2005). Functional annotation of GWAS hits via gene ontology (GO) analysis allowed me to investigate the functional pathways that may contribute to the trade-offs evident in TPC variation.

4.3 Methods

Species Inbreeding and Husbandry

I phenotyped 152 lines from the Drosophila Genetic Reference Panel (DGRP) (Mackay et al. 2012; Huang et al. 2014) obtained from the Bloomington Drosophila Stock Center, Indiana. The DGRP is a set of wild-derived, inbred *Drosophila melanogaster* lines founded from a wild population in Raleigh, NC. Isofemale lines were inbred for 20 generations via full-sib mating. Each line was tested for homozygosity using microsatellite markers and resequencing of several regions on all three chromosomes (Mackay et al. 2012). All flies were maintained on a sugar-yeast-polenta medium at 25^{o}C under a 12:12 hour dark-light cycle.

Genome Re-sequencing

The DGRP lines were sequenced using a combination of Illumina and Roche 454 technology and aligned to the D. mel 5.49 reference genome (www.flybase.org) by Huang et al. (2014) and Mackay et al. (2012). Integrative genotyping strategy was then used to identify single-nucleotide polymorphisms and non-SNP variants (Stone 2012). Furthermore, inversion genotypes were identified by cytogenic analysis of polytene salivary gland chromosomes, and *Wolbachia pipeintis* infection status for each line was determined through PCR assay to identify lateral gene transfer events.

Thermal Performance Assay

Locomotor activity was measured for individual flies briefly exposed to each of the eight test temperatures to estimate TPCs. Activity was measured across the mixed order of temperatures, 38, 25, 29, 35, 32, 20, 40 and 42 ± 0.3^{o}C to maintain the same testing environment among days. The two highest temperatures were measured last to prevent any detrimental effects interfering with any subsequent measurements (Gilchrist 1996). All locomotor activity was measured in a single control temperature cabinet for a total of 20 minutes. To prevent acclimation to the exposed test temperature, flies were held at 25 ± 0.5^{o}C for 40 minutes between each assay recording. Activity was measured between 09:00 and 15:00 to correspond to the light cycle experienced by the flies. Phenotyping occurred across six consecutive days. Each day had one, five to six day-old virgin fly for each sex per line, thereby ensuring all lines were represented across each day. Six males

and six females from three different laying vials were phenotyped per line (two flies per sex from each vial). Each laying vial contained three males and three females that were allowed to mate and lay for 48 hours.

Drosophila Activity Monitors (DAM, TriKinetics, Waltham, Massachusetts, USA) were used to measure locomotor activity using a similar method to Latimer et al. (2011) and in the previous two Chapters. A DAM measures activity as the number of times a fly intersects an infra-red beam that bisects each of the 32 x 5 mm holding tubes. A computer connected to the DAMs records the number of intersections when a fly crosses the beam. To allow flies to adjust to the new environment for phenotyping, they were placed into the 5 mm holding tubes the afternoon prior. Each 5 mm tube contained 1.5 cm of a three-day-old agar-sucrose medium that was caped and stoppered with 0.2 mm of foam on the opposing end.

Statistical Analysis

Quantitative Genetic Analysis

First, I tested for genetic variance in locomotor activity across the eight test temperatures using a multivariate mixed model analysis. To improve normality, locomotor activity was square root transformed (Quinn and Keough 2002). To account for the differences in mean between temperatures, locomotor activity was mean standardised prior to analysis. Males and females were analysed separately. Proc MIXED in SAS (ver 9.3; SAS Institute, Cary, NC, USA) was used to fit the mixed model:

$$\boldsymbol{a} = \boldsymbol{\mu} + \boldsymbol{d} + \boldsymbol{l} + \boldsymbol{v}\,\boldsymbol{(l)} + \boldsymbol{e}, \qquad [4.1]$$

where **a** is a vector of locomotor activity scores at the eight temperatures, **d**, is the day effect, **l** is the effect line, and **v(l)** is the effect of vial nested within line. The terms for line and vial were considered random effects whereas day was fitted as a fixed effect. An unstructured variance-covariance matrix between temperatures (Type=UN) was assumed for the random effects.

Here, I have taken two approaches to characterise TPC shape. The first approach was similar to the previous two Chapters where I used a character-state (classic multivariate) approach to characterise TPC variation. I performed a principal component

(PC) analysis on the among line variance-covariance matrix to reveal the major axes of TPC variance. The matrix was estimated from residuals to account for the effects of day and were estimated from square-root transformed, mean standardised data. The *princomp* function in R was used to perform the PC analysis and calculate the PC scores for each line. The sign of the PC loadings represent changes in TPCs and its shape. The 'faster-slower' axis is represented by PCs with all the temperature loadings having the same sign (i.e. all positive or all negative). The 'hotter-colder' axis is represented by PCs with a sign change between the hot and cold temperatures. The 'generalist-specialist' axis involves PC loadings with two changes in sign; the loadings in the center are a different sign to the loadings at either end of the temperatures. More than two sign changes would represent a more complex change in TPC shape not classified into one of these three major axes of variation.

In contrast to the character-state approach, I also took a function-valued approach to characterise the distribution of genetic variance across the major axes of TPC shape variation. I used the Template Mode of Variation approach (TMV) (Izem and Kingsolver 2005) that describes each inbred line's curve by three parameters, each modeled as deviations from a common shape template curve using a three-parameter shape invariant model Lawton et al. (1972):

$$z_i(t) = \frac{1}{w_i} z\left[\frac{1}{w_i}\left(t - T_{opt_i}\right)\right] + h_i + e, \qquad [4.2]$$

where $z_i(t)$ is the continuous function of locomotor activity of family, *i,* across temperature, *t*. The common shape template is represented by *z*, a quintic polynomial (order 5) in this study, and *h*, T_{opt} and *w* represent the three parameters describing each family's curves, *i*. The vertical shift, *h*, parameterises the 'overall height' of the curves and is independent of temperature. The horizontal shift, T_{opt}, parameterises the location of temperature for maximum performance by detecting a shift in the curve to the left or the right. The width shift, *w*, parametersises the 'generalist-specialist' axes. The estimation of *w* constrains the area under the curve to be constant so that a wider curve has a lower maximum performance.

I analysed the DGRP template curve and individual TPCs using the semi-parametric method as recommended by Izem and Kingsolver (2005) and implemented in the TMV MATLAB package. This produced estimates of the three components of the TPC:

'overall height' (*h*), 'generalist-specialist' (*w*), and optimum temperature (T_{opt}) for each DGRP line. The fourth component of TPC, maximum performance (z_{max}), was estimated using the following formula found within the TMV package,

$$z_{max_j} = w_j \times i + h_j \,, \qquad [4.3]$$

where, i is the intercept for the template curve, w_j is the width axis for the *j*th family and h_j is the height axis for the *j*th family. Additionally, a ratio sum of squares was calculated for each of the T_{opt}, *h* and *w* modes of variation to estimate the amount of among-family variation. A fifth order polynomial was used to fit the template and TPCs as it produced components that had a normal distribution (except *h*) that is critically important for the GWA analysis. A reasonable fit to the TPC curves and estimates for the TMV components was also produced from this polynomial order. Because the current MATLAB code for TMV does not support the fitting of additional fixed effects, TMV was conducted on (untransformed) residual activity scores after correcting for day effects using the MIXED procedure in SAS. Males and females were analysed separately in TMV, allowing each sex to have separate template curves.

Genome Wide Association Analysis (GWA)

GWA was conducted on the DGRP line mean TMV components, *h*, T_{opt}, *w* and z_{max} and the first five PC scores by submitting phenotypic data to the DGRP2 online GWAS pipeline (http://dgrp2.gnets.ncsu.edu) (Huang et al. 2014). In this pipeline, the phenotypic data are first adjusted for the effects of *Wolbachia* infection and the six major inversions [In(2L)t, In(2R)NS, In(3R)P, In(3R)K, and In(3R)Mo]. Then the FastLMM program (v1.09) (Lippert et al. 2011) is used to fit the adjusted phenotypic line values in the linear mixed model

$$y = \boldsymbol{Xb} + \boldsymbol{Zu} + e, \qquad [4.4]$$

where, y is the adjusted TMV component, *h*, T_{opt}, *w* or z_{max}, **X** is the design matrix for the fixed SNP effect $\boldsymbol{b}$, **Z** is the incidence matrix for the random polygenic effect $\boldsymbol{u}$, and e is the residual. The vector of polygenic effects, $\boldsymbol{u}$, has a covariance matrix in the form of $\mathbf{A}\sigma^2$, where σ^2 is the polygenic variance component. For these analyses, 1,895,647 biallelic variants (including snps and indels) with a minor allele frequency (MAF) > 0.05 and a call

rate > 80% were used. A BLOM rank normal transformation (Blom 1958) was performed on the scores for *h* before the GWA analysis (via PROC RANK in SAS, Version 9.3). Owing to a non-normal distribution, this transformation has been shown to improve results in association studies of non-normal phenotypes (Goh and Yap 2009).

Owing to a small sample size in the DGRP compared to human or livestock GWAS, but a relatively large number of markers, I used a significance threshold of $P < 1 \times 10^{-5}$. Whilst lower than a strict Bonferroni threshold, other GWAS studies utilising the DGRP typically use this less stringent threshold (Huang et al. 2012; Jordan et al. 2012; Mackay et al. 2012; Ober et al. 2012; Brown et al. 2013; Chow et al. 2013b; Swarup et al. 2013). When hits in genes are followed up with RNAi knockouts for example, they often demonstrate that the genes identified from GWAS hits using this threshold do indeed detect causal genes underlying quantitative traits (Jordan et al. 2012; Chow et al. 2013a; Grubbs et al. 2013). The effect sizes for significant variants were estimated as one half of the difference between phenotype means for the major and minor allele.

Functional annotation of significant GWA hits

I performed two levels of annotation of the significant GWA hits: DNA sequence-level and gene ontology-level. First, for all variants I determined the genomic position and likely functional consequence of the polymorphism on annotated genes. Variants were classified as intergenic, intronic, exonic, 3'UTR, up or downstream (within 1000bp from a gene), synonymous or non-synonymous, and whether they caused a change in reading frame. This analysis was performed using the DGRP online pipeline (Huang et al. 2014).

Second, for the subset of significant variants that could reliably be determined to reside within or near an annotated gene (i.e. non-intergenic variants), I performed a functional enrichment analysis using gene ontology (GO) terms. This analysis provided an opportunity to explore the likely functional processes that, when perturbed genetically, create variation in TPCs. These analyses essentially evaluate whether specific gene ontology terms for a given set of genes are present more often than expected by chance using hypergeometric tests. The analysis was performed using the gene ontology Enrichment Analysis Software Tool (GOEAST) available online (http://omicslab.genetics.ac.cn/GOEAST/) (Zheng and Wang 2008). The recommended settings, hypergeometric testing, and a $p<0.1$ cut-off were used, and to correct for multiple

testing, the Hochberg FDR correction method was used (Benjamini and Hochberg 1995). I created a network representation of the enriched terms for the four TPC components using Cytoscape (vers. 2.8). This approach has been adopted for use in other functional analysis of GWAS hits (Brown et al. 2013).

Functional enrichment analysis was only performed on the PC scores and for female data TMV components. For male TMV components, after the intergenic variants and genes that do not have functional annotations were removed, *h* only had one gene, and *w* and *z* only had eight genes available for enrichment. This small number of genes is likely to greatly decrease the power of enrichment analysis (Huang et al. 2009).

4.4 Results

Quantitative Genetics

For each DGRP line, locomotor activity TPCs resembled a typical TPC shape. Locomotor activity slowly increased as temperatures approached 35^{o}C, peaked around 38°C-40°C and suddenly dropped (Fig. 4.2). Likelihood ratio tests confirmed significant genetic (co)variance among the DGRP lines for both males (Line effect: LRT χ^2_{36}=508.4, p<0.0001; Fig. 4.2) and females (LRT χ^2_{36}=560.1, p<0.0001; Fig. 4.2). The effect of day was also significant (Males: $F_{5,507}$=7.61, p<0.0001, Females: $F_{5,539}$=12.34, p<0.0001), suggesting some day-to-day variation in the thermal dependence of locomotor activity as expected for behavioural traits. The among-line variance-covariance matrices for both males and females indicated that the genetic covariance for all pairs of temperatures was positive (except for 32°C and 42°C in males), and that genetic correlations between temperatures generally weakened as the difference between the temperatures increased (Table 4.1).

Table 4.1: The among line variance-covariance matrices for locomotor activity across the eight temperatures (°C) in males (top panel), and females (bottom panel). Variances are on the diagonal in bold, the covariances below the diagonal and the correlation between temperatures above the diagonal. Estimates are mean-standardised. The five columns on the far right are eigenvectors 1-5 of the variance-covariance matrix and the percentage they explain.

Males	**20**	**25**	**29**	**32**	**35**	**38**	**40**	**42**	λ_1 (59.65%)	λ_2 (16.60%)	λ_3 (8.25%)	λ_4 (7.46%)	λ_5 (1.81%)
20	**0.210**	0.667	0.489	0.310	0.248	0.198	0.103	0.112	-0.442	-0.462	0.424	-0.612	0.186
25	0.172	**0.319**	0.575	0.440	0.300	0.213	0.169	0.057	-0.627	-0.427	-0.489	0.423	-0.041
29	0.109	0.157	**0.235**	0.707	0.307	0.165	0.209	0.146	-0.509	0.472	0.289	0.014	-0.643
32	0.061	0.106	0.147	**0.183**	0.326	0.100	0.102	-0.063	-0.375	0.616	-0.216	-0.157	0.603
35	0.018	0.027	0.024	0.023	**0.026**	0.659	0.519	0.364	-0.078	0.029	0.176	0.213	0.295
38	0.013	0.017	0.011	0.006	0.015	**0.020**	0.743	0.474	-0.043	-0.026	0.216	0.240	0.223
40	0.008	0.016	0.017	0.007	0.014	0.017	**0.028**	0.714	-0.045	0.007	0.311	0.370	0.199
42	0.011	0.007	0.016	-0.006	0.013	0.015	0.026	**0.049**	-0.031	-0.034	0.526	0.426	0.105
Females									(59.28%)	(13.89%)	(10.81%)	(7.97%)	(3.94%)
20	**0.163**	0.611	0.482	0.406	0.266	0.249	0.224	0.160	-0.407	0.550	0.311	0.647	0.118
25	0.117	**0.227**	0.670	0.504	0.179	0.193	0.180	0.112	-0.574	0.444	-0.284	-0.542	-0.306
29	0.086	0.141	**0.195**	0.773	0.412	0.297	0.270	0.137	-0.548	-0.395	-0.112	-0.076	0.708
32	0.063	0.093	0.132	**0.149**	0.492	0.325	0.260	0.041	-0.423	-0.544	-0.059	0.295	-0.529
35	0.019	0.015	0.032	0.034	**0.031**	0.700	0.638	0.351	-0.105	-0.187	0.290	0.045	-0.219
38	0.014	0.013	0.019	0.018	0.018	**0.020**	0.781	0.503	-0.070	-0.082	0.285	-0.056	-0.170
40	0.015	0.014	0.020	0.017	0.019	0.018	**0.032**	0.787	-0.076	-0.084	0.451	-0.184	-0.121
42	0.014	0.012	0.013	0.003	0.014	0.016	0.031	**0.062**	-0.057	-0.009	0.661	-0.394	0.137

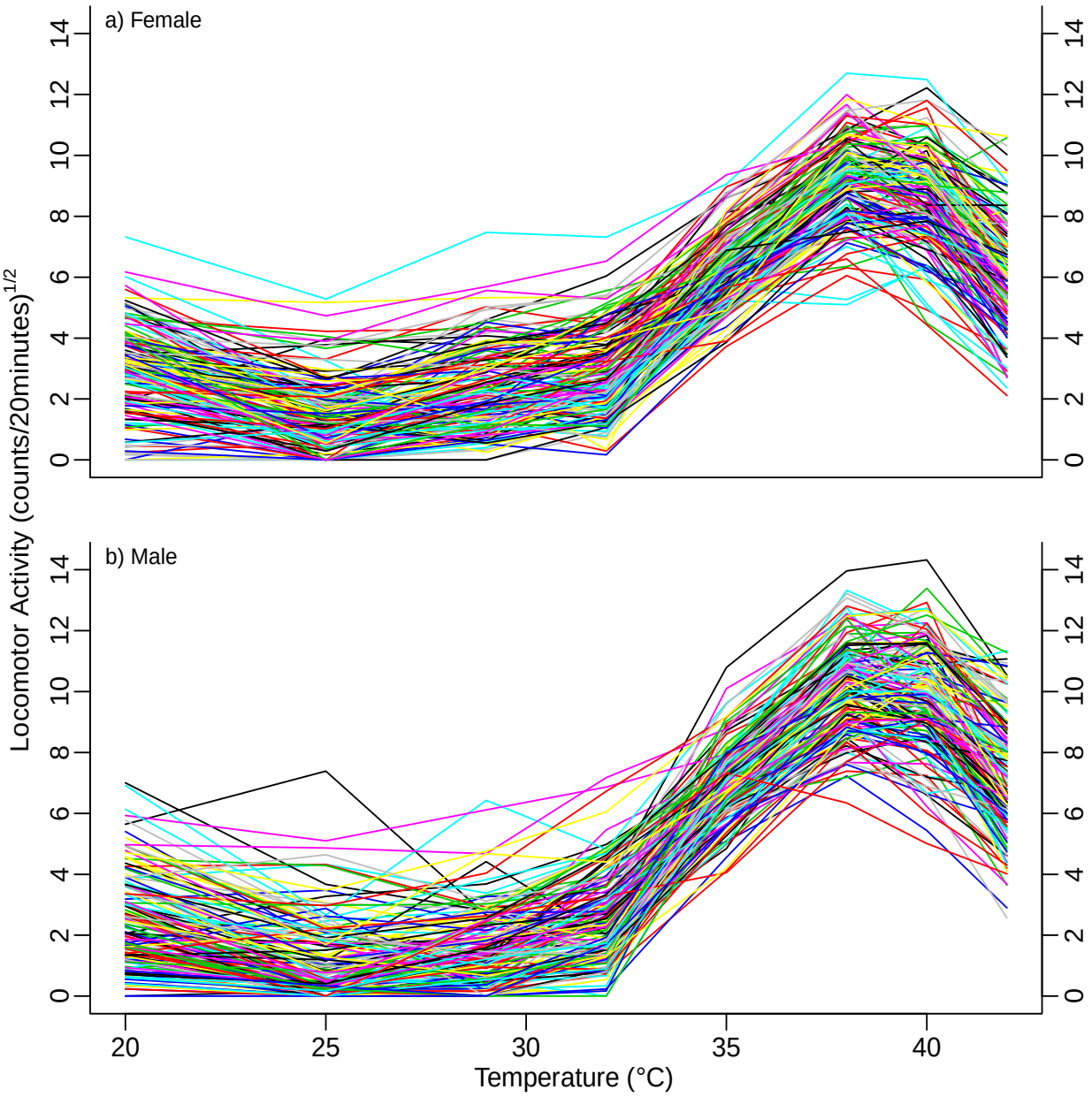


Figure 4.2: DGRP line means for locomotor activity across the eight test temperatures (^{0}C) for a) females and b) males. Each coloured line represents one of the 152 DGRP lines. Locomotor activity was measured as counts per 20 minutes and was squared root transformed.

I performed an eigenanalysis on the among-line variance-covariance matrix to investigate the major axes of variance. The eigendecomposition of the variance-covariance matrix showed that the first five principal components captured over 95% of the

variance. The first PC accounted for 59.65% and 59.28% of the total variance for males and females respectively with similar loading patterns in each sex (Table 4.1). All the loadings were in the same direction indicating the ‘faster-slower’ axis of TPC variation (Fig. 4.3a & f). In both sexes, the loadings were stronger between 20^{o}C and 35^{o}C (Fig. 4.3a & f). The remaining PCs explained 46% of the variance with the loadings indicating trade-offs between the temperatures. Female PC2 had a sign change between the hot and cold temperatures, therefore most closely resembling the ‘hotter-colder’ axis of variation (Fig. 4.3g). Female PC3 and male PC5 had a change in sign between the intermediate temperatures with the hottest and coldest temperatures, consistent with a ‘generalist-specialist’ axis of variation (Fig. 4.3e & h). The remaining PCs loadings had more complex patterns, indicating changes in the shape of the TPCs (Fig. 4.3b-d, i, & j).

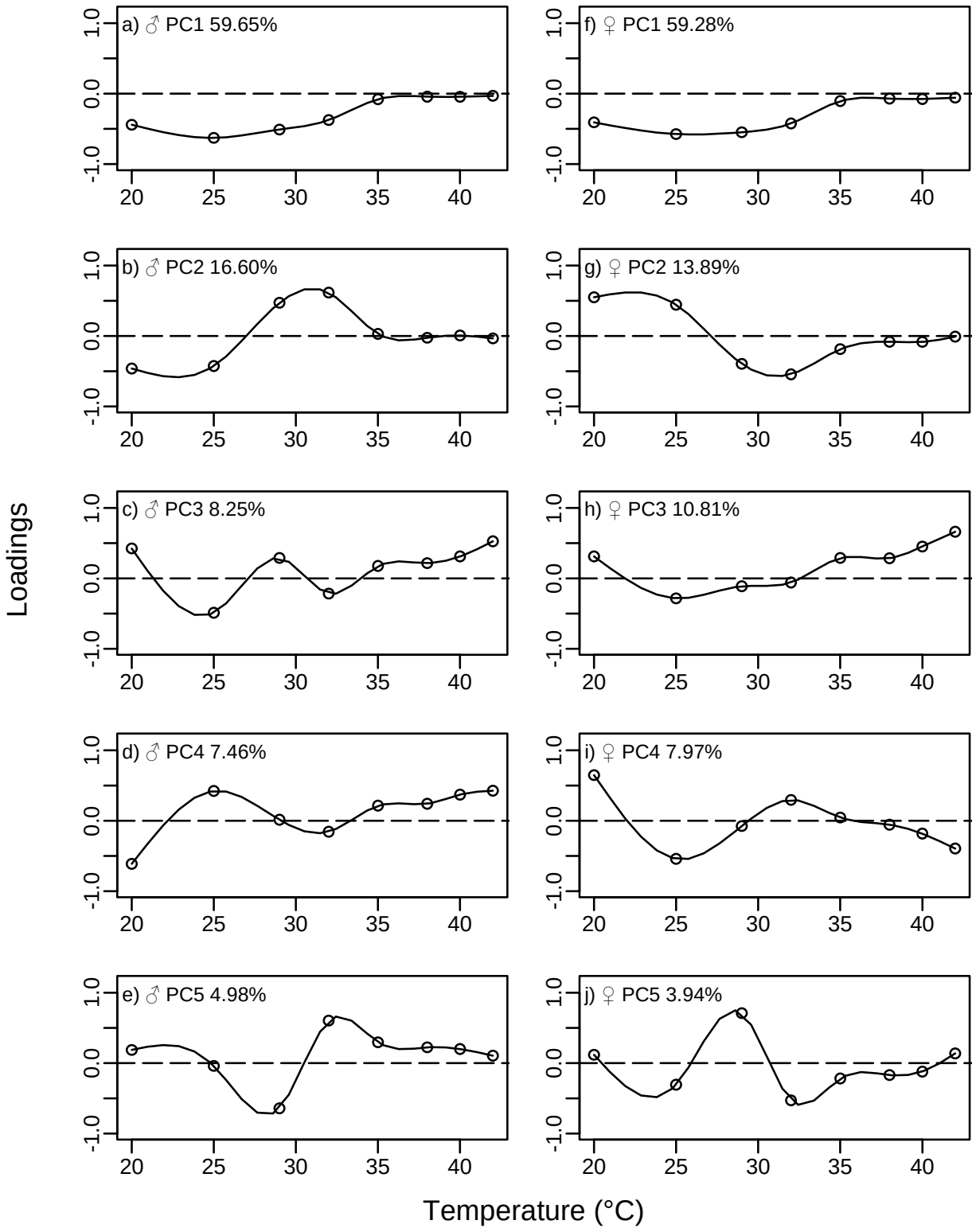


Figure 4.3: PC loadings for the male and females among-line variance-covariance matrices. The first five PCs are shown for each sex (males: a-e, females f-j). Data was mean standardised before PC analysis was performed.

I performed the TMV analysis, on each sex separately to partition the among-line broad-sense genetic variance into the three axes; 'overall height', *h*, 'generalist-specialist', w, and optimum temperature, T_{opt}. The total genetic variance was similar for each sex, at 30.73% and 29.62% for males and females respectively. Most among-line variance was along the 'hotter-colder' and 'generalist-specialist' axes (Table 4.2). Very little among-line variance (less than 1%) was explained by the 'faster-slower' axis, which corresponds to the overall locomotor activity regardless of temperature (Table 4.2).

Table 4.2: Percentage of phenotypic variance due to among-line variance in the three major TMV components for males and females. Values are ratio sum of squares (RSS) expressed as percentage of total variance.

	RSS Values (%)	
	Males	**Females**
Faster-Slower axis (*h*)	0.22	0.28
Hotter-Colder axes (T_{opt})	19.26	17.99
Generalist-Specialist axis (*w*)	11.25	11.35
Total explained by model	30.73	29.62
Error	69.27	70.38

Genome-wide-association analysis

Genome-wide-association analysis was performed to dissect the genetic architecture of the four components of TPC variation, *h,* T_{opt}*, w* and z_{max} and the first five PCs. Before analyses were performed, the components were adjusted for the effects of *Wolbachia* and the six major inversions present in the DGRP, which were tested using analysis of variance. *Wolbachia* only had a significant affect for PC5 in males ($F_{1,150}$=0.031, p=0.016); no TMV components were affected by *Wolbachia* in either sex (Table 4.3). T_{opt} was the only component affected by inversions, where In(3R)Mo inversion was significant for males ($F_{2,149}$=5.874, p=0.004) and In(3R)K inversion was significant for females ($F_{2,149}$=3.974, p=0.021). None of the PCs were affected by any of the inversions in either sex (Table 4.3).

Table 4.3: Effect of inversions and *Wolbachia* on the first five PCs and the four curve components, optimum temperature (T_{opt}), 'generalist-specialist' (*w*), 'overall height' (*h*) and maximum activity at Topt (z_{max}) for females and males. F-values and p-values (in parenthesis) from Type three ANOVA given for *Wolbachia* and inversion genotypes. Significant p-values in bold (p<0.05).

Trait	**Sex**	**In(2L)t (d.f=2,149)**	**In(2R)NS (d.f=2,149)**	**In(3R)P (d.f=2,149)**	**In(3R)K (d.f=2,149)**	**In(3R)Mo (d.f=2,149)**	**Wolbachia (d.f=1,150)**
Optimum Temperature	Male	1.128(0.327)	0.361(0.698)	1.553(0.215)	0.847(0.431)	5.874(**0.004**)	2.261(0.135)
(T_{opt})	Female	0.928(0.398)	0.779(0.461)	0.144(0.866)	3.974(**0.021**)	0.314(0.731)	3.840(0.052)
Generalist-Specialist	Male	0.521(0.595)	0.325(0.723)	2.033(0.135)	0.609(0.545)	2.158(0.119)	0.095(0.759)
(*w*)	Female	0.567(0.569)	0.080(0.923)	0.172(0.842)	2.723(0.069)	0.152(0.859)	0.230(0.632)
Overall Height (*h*)	Male	0.367(0.694)	0.063(0.939)	0.476(0.622)	0.874(0.419)	0.905(0.407)	0.073(0.787)
	Female	0.224(0.799)	1.409(0.248)	0.538(0.585)	1.659(0.194)	0.197(0.821)	0.165(0.685)
Maximum Activity	Male	0.424(0.655)	0.223(0.800)	1.734(0.180)	0.688(0.505)	1.998(0.139)	0.033(0.855)
Level (z_{max})	Female	0.469(0.626)	0.305(0.737)	0.031(0.969)	2.776(0.066)	0.060(0.942)	0.091(0.763)
PC1	Male	0.059(0.943)	0.083(0.919)	2.894(0.059)	1.047(0.354)	0.191(0.826)	0.086(0.770)
	Female	1.209(0.301)	0.069(0.933)	1.107(0.345)	0.966(0.383)	0.201(0.818)	0.481(0.489)
PC2	Male	0.378(0.686)	0.028(0.972)	0.618(0.541)	0.735(0.481)	1.534(0.219)	0.876(0.351)
	Female	1.755(0.177)	0.013(0.988)	0.492(0.612)	0.231(0.794)	0.633(0.532)	0.072(0.788)
PC3	Male	0.400(0.671)	0.369(0.692)	0.523(0.594)	2.358(0.098)	0.770(0.465)	0.328(0.568)
	Female	0.435(0.648)	2.167(0.118)	0.351(0.705)	1.429(0.243)	1.069(0.346)	2.266(0.135)
PC4	Male	0.167(0.847)	1.037(0.357)	0.158(0.854)	0.046(0.955)	0.082(0.921)	1.011(0.316)
	Female	0.400(0.671)	0.369(0.692)	0.523(0.594)	2.356(0.098)	0.770(0.465)	0.328(0.568)
PC5	Male	0.308(0.735)	0.469(0.626)	0.321(0.726)	1.979(0.142)	0.484(0.617)	0.031(0.016)
	Female	1.697(0.187)	0.662(0.517)	0.915(0.403)	0.939(0.393)	0.963(0.384)	0.328(0.861)

GWA analysis was performed on a total of 1,895,647 segregating biallelic SNPs and non-SNP variants. For females, at $p < 1\times10^{-5}$, I identified 20 variants associated with *h*, 222 variants associated with T_{opt}, 66 variants associated with *w,* 56 variants associated with z_{max}, 66 variants associated with PC1, 29 variants associated with PC2, 43 variants associated with PC3*,* 12 variants associated with PC4*,* and 39 variants with PC5. For males, I found 5 variants associated with *h*, 135 variants associated with T_{opt}, 17 variants associated with *w,* 18 variants associated with z_{max}, 87 variants associated with PC1, 53 variants associated with PC2*,* 12 variants associated with PC3, 16 variants associated with PC4, and 23 variants with PC5.

When the p-values were plotted along the chromosomes, the significant variants were distributed broadly throughout the genome, indicating a likely polygenic architecture for all TPC components (Fig. 4.4 & 4.5) and PCs (Fig. 4.6 & Fig. 4.7). For males, the significant hits for PC2, PC3, T_{opt}, *w* and z_{max} predominately occurred on 2R, 3L and the X chromosome, with the addition of 2L for T_{opt} and 3R for PC2 and PC3 (Fig. 4.3 & 4.6). I found that clusters of significant hits spanned all the chromosomes for PC1, PC2, PC3, T_{opt}, *w* and z_{max} in females (Fig. 4.5 & 4.7) and PC1 in males (Fig. 4.6). Noticeably, for both sexes, *w* and z_{max} had a similar spread of p-values (Fig. 4.3 & 4.4). Consistent with the detection of very little among line variance, PC4, PC5, and *h* had few hits (Fig. 4.4-4.7). Varying degrees of deviation from the null hypothesis for each trait for both sexes was illustrated in the quantile-quantile (Q-Q) plots and show little evidence of population stratification (Appendix 4.1 & 4.2).

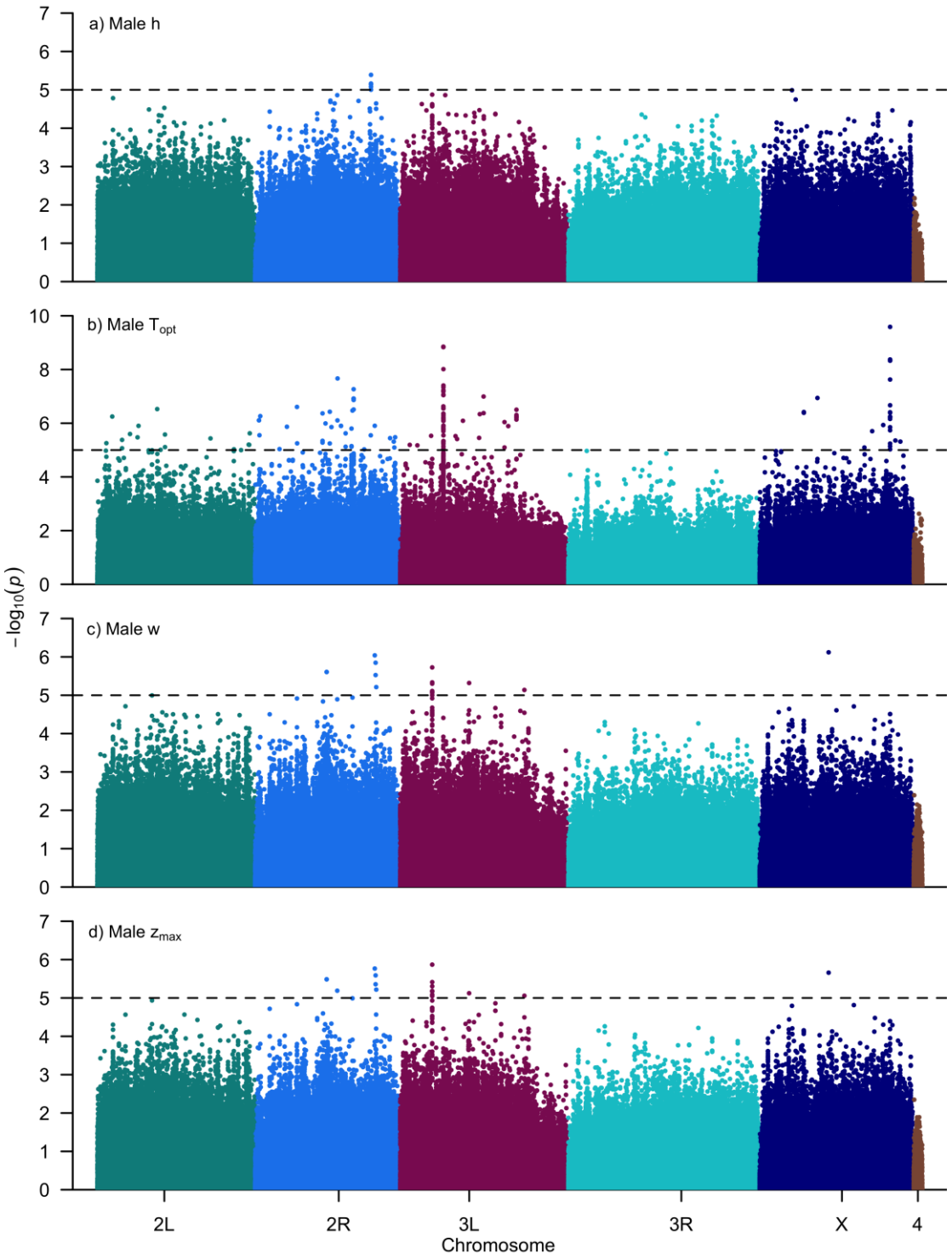


Figure 4.4: Manhattan plots for the association between the variants and each of the four TPC components for males in the DGRP lines. The dashed horizontal line is the nominal threshold of 1 x 10^{-5}. Each colour represents a different chromosomal arm; a) *h* component, b) T_{opt} component, c) *w* component, and d) z_{max} component.

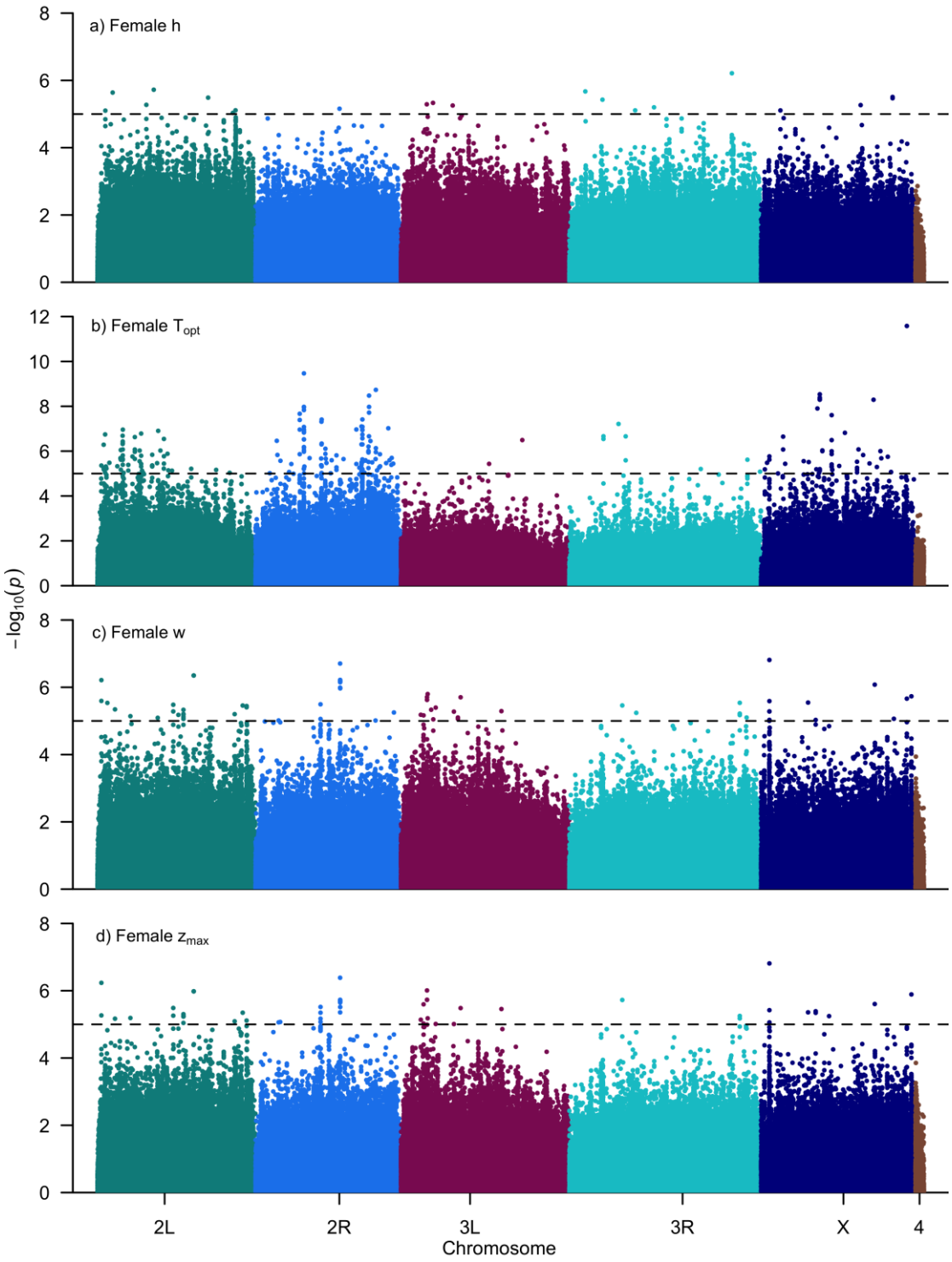


Figure 4.5: Manhattan plots for the association between the variants and each of the four TPC components for females in the DGRP lines. The dashed horizontal line is the nominal threshold of 1 x 10^{-5}. Each colour represents a different chromosomal arm; a) *h* component, b) T_{opt} component, c) *w* component, and d) z_{max} component.

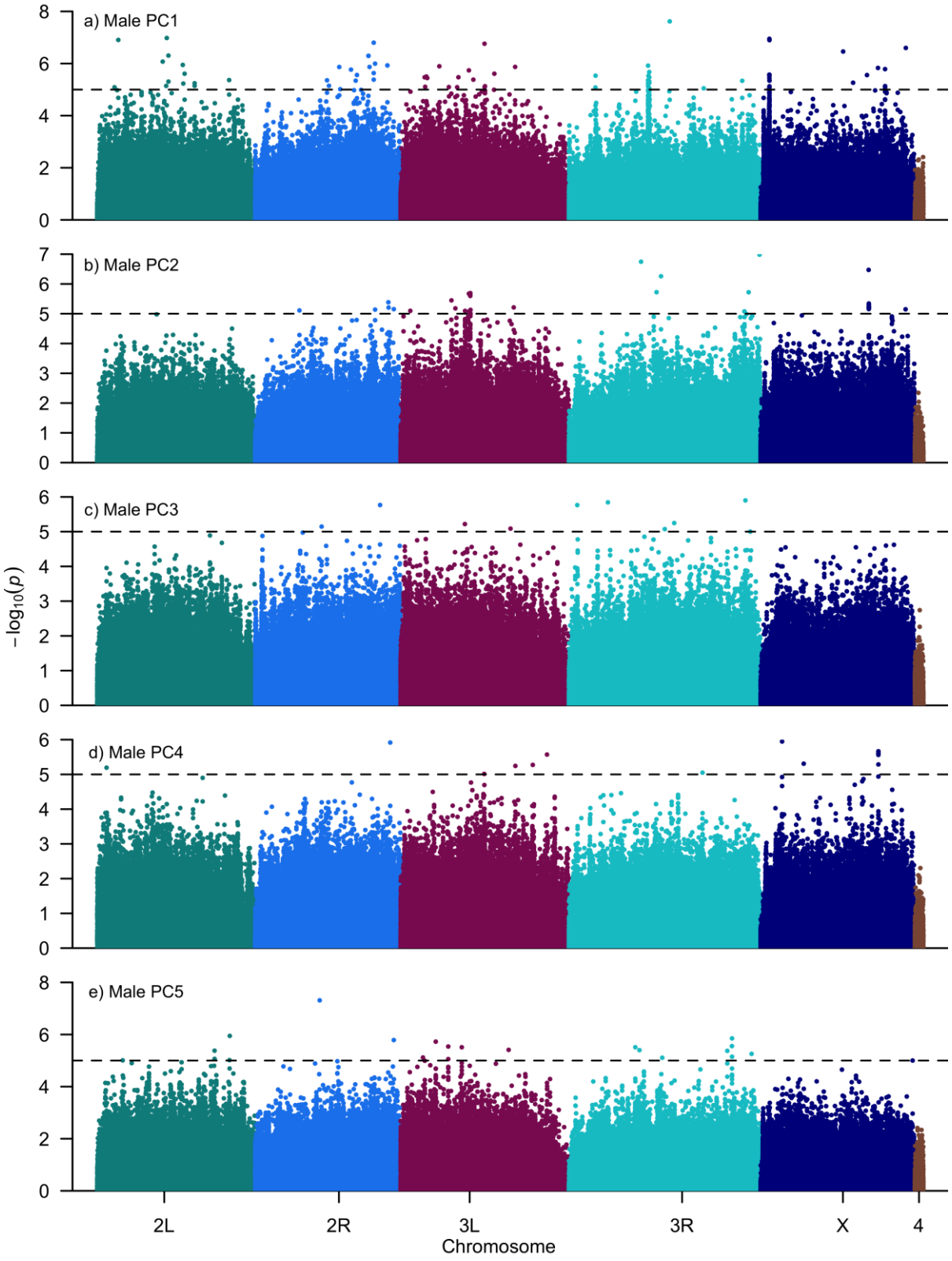


Figure 4.6: Manhattan plots for the association between the variants and each of the first five principal components for males in the DGRP lines. The dashed horizontal line is the nominal threshold of 1 x 10^{-5}. Each colour represents a different chromosomal arm; a) PC1, b) PC2, c) PC3, d) PC4, and e) PC5.

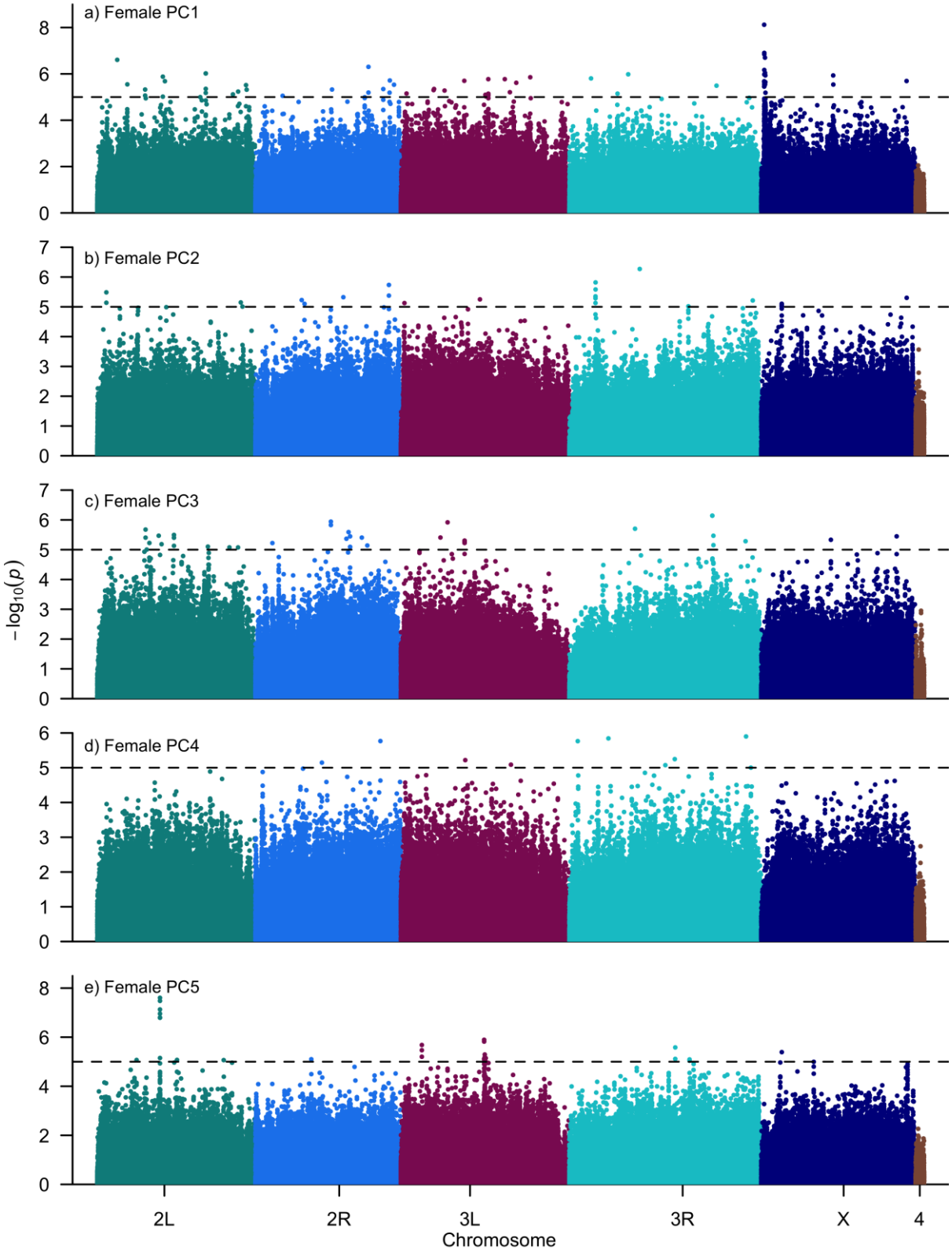


Figure 4.7: Manhattan plots for the association between the variants and each of the first five principal components for females in the DGRP lines. The dashed horizontal line is the nominal threshold of 1 x 10^{-5}. Each colour represents a different chromosomal arm; a) PC1, b) PC2, c) PC3, d) PC4, and e) PC5.

Skew in the direction of minor allele effects

I detected a noticeable skew in the direction in which the minor-frequency alleles affected a trait. Within the PC analysis, the skew was detected in the minor-frequency alleles for PC1 and PC2 in males, and PC1 and PC3 in females (Fig. 4.9). For T_{opt}, a skew towards positive effects was observed suggesting that, at all loci (100% of significant hits for both sexes, Fig. 4.8), the rarer allele increased optimum temperature. A contrasting pattern of a skew towards negative effects was observed for z_{max} (males: z_{max} 94% and *w* 94 %; females z_{max} 96% and *w* 97%, Fig. 4.8), where the rarer allele lowered maximum performance. There was far less skew observed for the *h* component, which had too few hits in males, but for females a similar number of positive an negative values (minor effect>0: 70%, minor effect<0: 30%, Fig. 4.8). For correct interpretation of the effects of *w* values, an explanation of how TMV estimates *w* is required. The measure of *w* from the TMV output incorporates the 'generalist-specialist' axis as it constrains the area under the curve to remain constant, meaning that both changes in the width of the curve and maximum performance occur simultaneously (Izem 2004). Therefore, the interpretation of the *w* value does not directly relate to the range of temperatures; instead, a larger w indicates a specialist and smaller *w* a generalist. Therefore, the observed skew for decreased *w* value (males: *w* 94 %; females *w* 97%, Fig. 4.8) indicates that the rarer alleles resulted in generalists. Across all the components, the rarer alleles increased T_{opt}, decreased z_{max} and resulted in generalists.

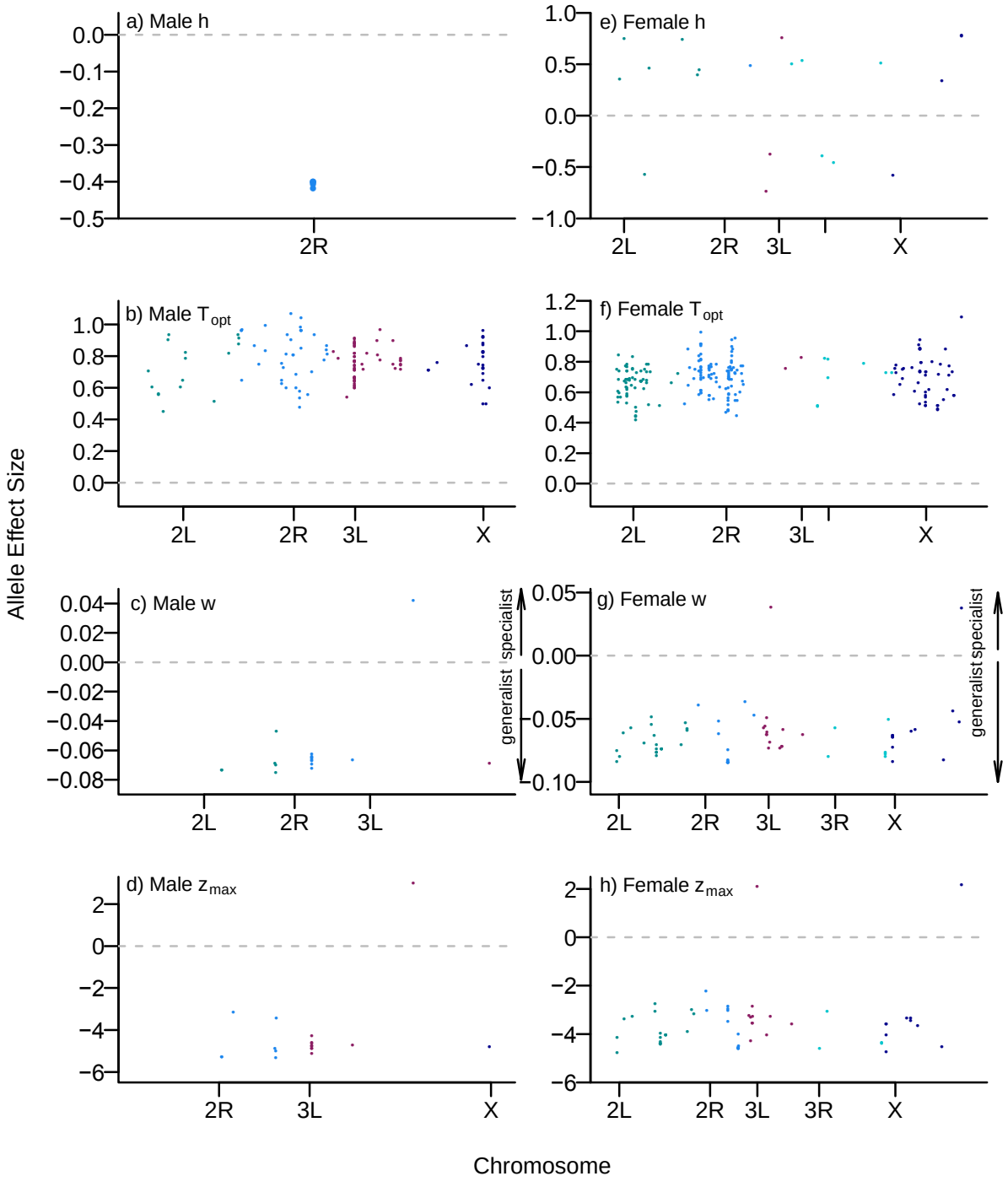


Figure 4.8: Skew in the direction of minor frequency variant effects across the genome for the variants with a p-value larger than 1 x 10^{-5} from the GWA for each sex's four components of TPC. Each point represents a different variant and the colours correspond to the chromosome. *h* is represented in a) for males and d) for females. T_{opt} is represented in b) for males and f) for females. *w* is represented in c) for males and g) for females. z_{max} is presented in d) for males and h) for females.

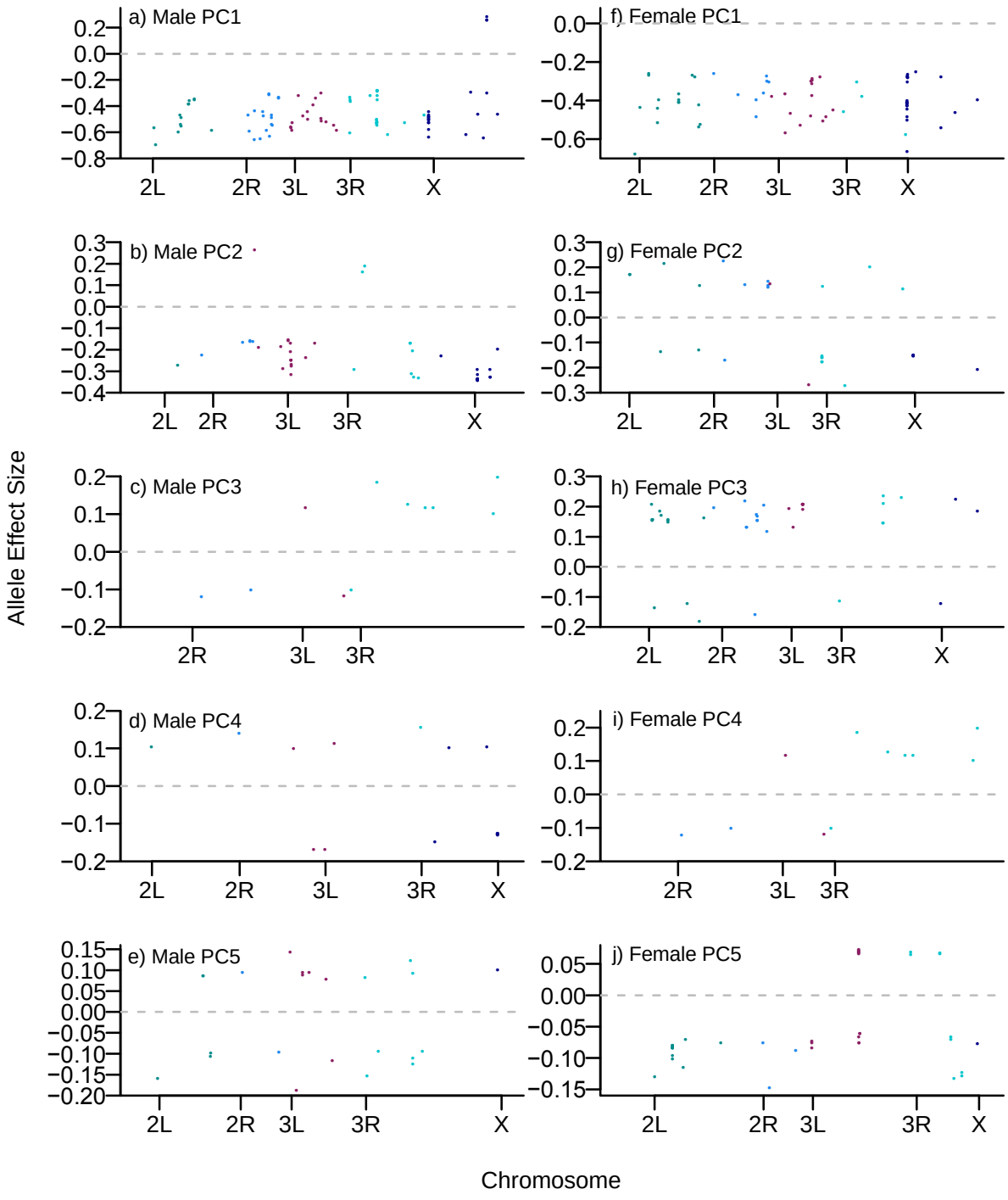


Figure 4.9: Skew in the direction of minor frequency variant effects across the genome for the variants with a p-value larger than 1 x 10^{-5} from the GWA for each sex's first five PCs. Each point represents a different variant and the colours correspond to the chromosome. PC1 is represented in a) for males and d) for females. PC2 is represented in b) for males and f) for females. PC3 is represented in c) for males and g) for females. PC4 is presented in d) for males and h) for females. PC5 is presented in e) for males and j) for females.

Functional Annotation of GWA hits

Upon annotation of the different types of variants, across all components, I found that most variants resided in or near genes for both TMV components and PCs (TMV: males: 76%, females: 83%; PC: males: 81%, females: 79% Fig. 4.6). For the TMV components, I found that 301 out of 364 variants were localised to a total of 223 genes for females and 133 out of 175 significant variants localised to a total of 85 genes for males. For the PCs, I found that 155 out of 191 variants were localised to a total of 138 genes for males and 151 out of 189 significant variants localised to a total of 112 genes for females.

Most variants were located in introns (males: 27-62%, except for male's *h* and PC2; females: 31-79%; Fig. 4.10). A similar number of variants were also found in the intergenic region for PC3 for females (40%) and PC5 for males (27%), in the downstream region for PC2 for males (46%) and the upstream region for PC3 for males (38%) and PC4 for females (38%) (Fig. 4.10). For both sexes, upstream or downstream regions were the next most common for *w* and z_{max} (males: 10-20%, females: 12%-18% Fig. 4.10), third most common for T_{opt} (males: 8-10%, females: 8-9% Fig. 4.10), and most for females *h* (upstream 34% Fig. 4.10). Similar numbers of variants were also found in the intergenic region (males: 7-25%, females: 9-16% Fig. 4.10). The remaining regions were found to generally have fewer variants with 14% or less in the synonymous, non-synonymous, coding change plus codon deletion regions, UTR-3 prime, UTR-5 prime, exon, start gained, and stop gained regions (Fig. 4.10).

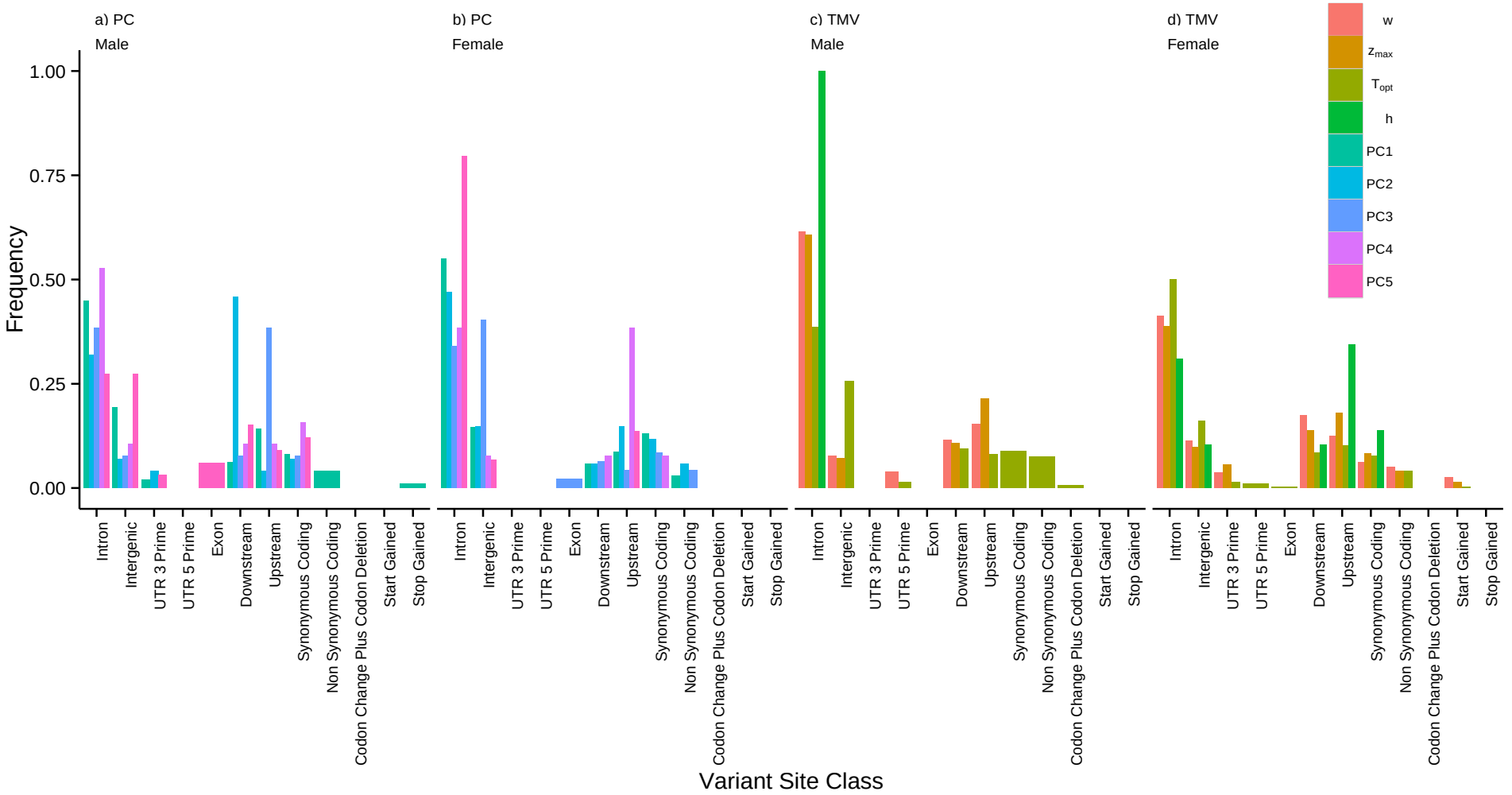
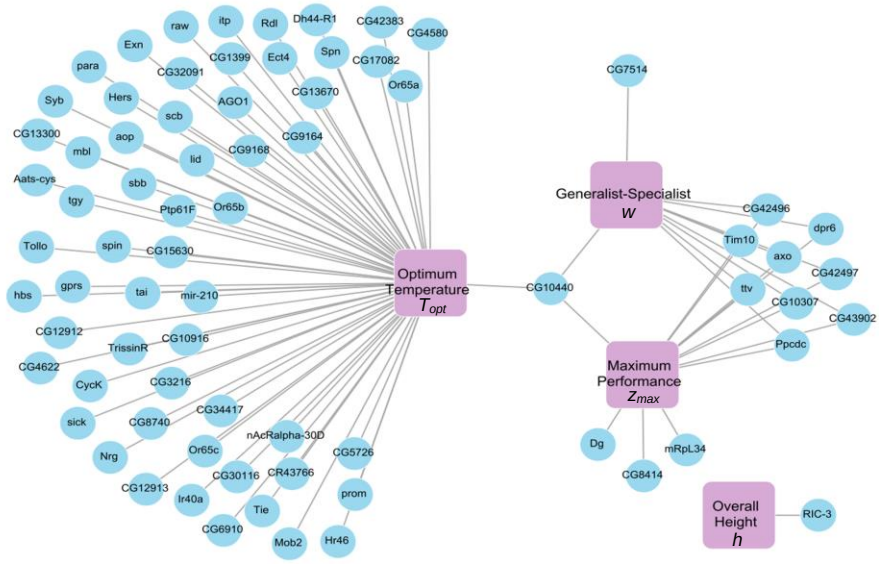


Figure 4.10: The number of significant variants across the different functional site classes for each of the five PCs and four TPC components, a) male PC variants, b) females PC variants, c) male TMV variants, and d) female TMV variants.

For the variants located near or within genes, functional annotations were also performed against the *D. melanogaster* Flybase reference 5.49. Within the TMV analysis, the total number of genes annotated was 85 and 223 for males and females respectively and within the PC analysis, 138 and 112 genes were annotated for males and females respectively. Majority of the genes were unique for each PC axis, whereby each sex only had two genes shared between PCs. For males one gene (*dpr6*) was shared between PC1 and PC2 and one gene (*app*) shared between PC1 and PC4 (Appendix 4.3a). For females, one gene (*kirre*) was shared between PC2 and PC5 and one gene (*Dys*) between PC4 and PC5 (Appendix 4.3b). For the TMV analysis, I found a varying degree of uniqueness for the four TMV components. T_{opt} had the most unique genes (males: 59, females: 118; Fig. 4.11). For females, *h* had the second highest degree of uniqueness, followed by *w* then z_{max} (h:24, z_{max}:3, w:10; Fig 4.11b); for males *h*, *w* and z_{max} had a similar number of genes (h:1, z_{max}:3, w:1; Fig 4.11a). This suggests each TPC component and PC axis has an appreciable degree of (genetic) independence from the other components. The majority of gene sharing occurred between z_{max} and *w* (males: 9, females: 32; Fig. 4.11). Here, most of the genes were shared with 9 out of 14 and 32 out of the 45 genes for males and females (Fig. 4.11). Note that this is to be expected, as *w* is algebraically related to z_{max} through equation 4.3. T_{opt} shared few with genes the other components with one gene for males (*CG10440*) and two genes for females (*CG11409* and *Ets21C*: Fig. 4.11).

a) Males



b) Females

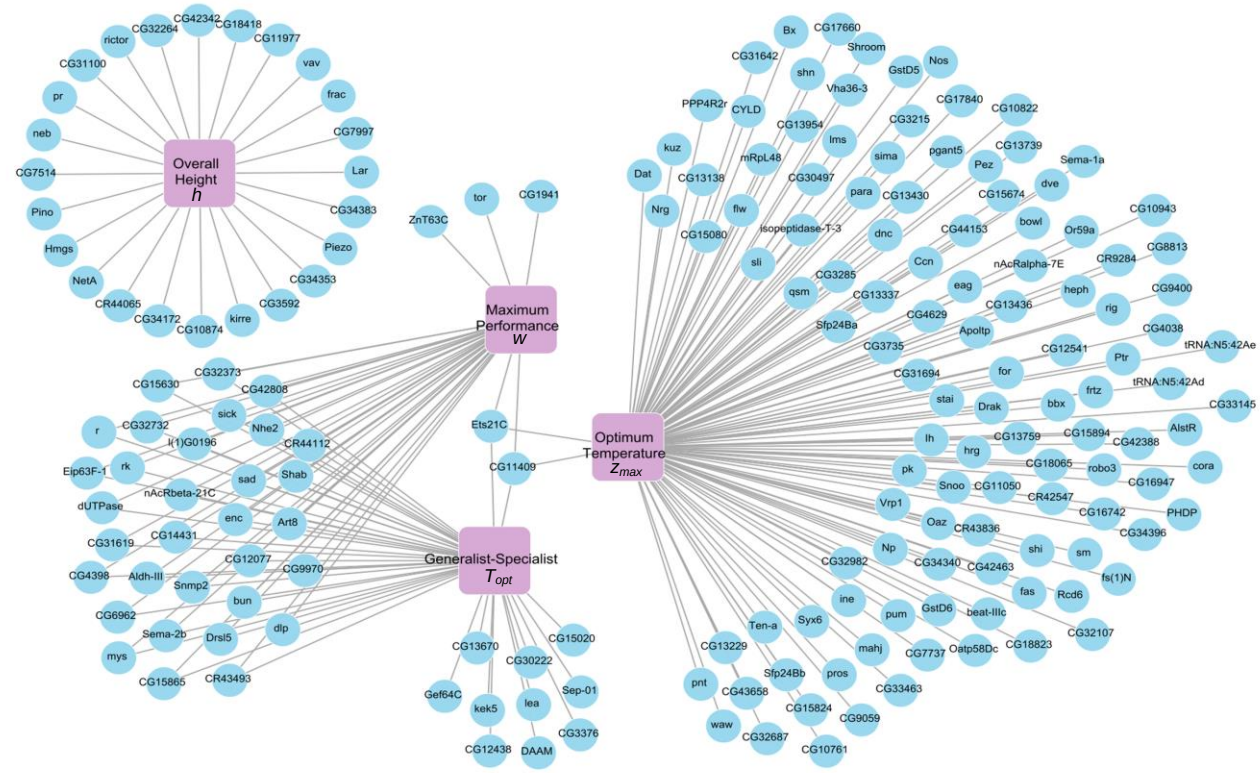


Figure 4.11: Gene network graph for significant variants annotated to a gene for a) males and b) females. Each blue node represents a gene and the purple square nodes represent the four components.

Gene Ontology Exploration

While there was varying overlap in the genes involved in each of the four TMV-defined traits, this alone does not take account of the type of biological processes and functional pathways these genes belong to. There may be an increased degree of overlap at a higher level of organisation. Therefore, I performed a functional enrichment analysis using gene ontology (GO) terms. Not all genes detected via significant associations have been systematically annotated, and therefore were not considered in this functional analysis. The number of genes analysed for females were 20 for *h*, 100 for T_{opt}, 35 for *w,* 29 for z_{max}*, 36 for PC1, 16 for PC2, 18 for PC3, 11 for PC4 and 19 for PC5. For males the number of genes analysed were 51 for PC1, 27 for PC2, 11 for PC3, 12 for PC4 and 17 for PC5.* Male TMV data were not analysed (see methods). The proportion of annotations within and shared among the components varied. At one extreme, majority of the enriched terms for z_{max} and *w* were shared with other components and at the other extreme, the majority of terms for T_{opt} and *h* were not shared (Fig. 4.12). In the PCs for both sexes, the degree of uniqueness for each component had increased; all five PCs had more GO terms that were unique than shared with each other (Fig. 4.13 & 4.14) which is to be expected given the orthongonaility constraint of PC analysis.

Particular types of biological processes, molecular functions and cellular components were enriched. Neurological function and development appeared to be involved in all TMV components. Each TMV component had a GO term that related to neurological function and development. Some specific examples are neurological system processes (T_{opt}), axongenesis (T_{opt}, *h, w* & z_{max}), central nervous system development (*w* & z_{max}), neuron development (T_{opt}, *w, h* & z_{max}), neurongenesis (T_{opt}, *w, h* & z_{max}), neuron perception development (T_{opt}, *h, w* & z_{max}) and regulation of axongenesis (*h* & T_{opt}) (Fig. 4.12). Unlike the TMV components, most but not all PCs had terms relating to neurological function and development. Neurological terms were present for males PC1, PC2 and PC3 (Fig. 4.13) and females PC1, PC3 and PC5 (Fig. 4.14). Response to stimulus was another consistent theme across all TMV components. For example, all four TMV components had response to external stimulus and taxis (directed response to stimulus), *h* had regulation of chemotaxis and T_{opt} had response to chemical stimulus (Fig. 4.12). Response to stimulus terms was only present in two out of the five PCs for both sexes. The term *taxis* was present for PC1 in males and females and PC3 in females; negative regulation of

response to stimulus was present for males PC2 and response to external stimulus term was present for female's PC1 (Fig. 4.13 & 4.14). Another theme across all TMV and PC components (except PC5 in females) was terms relating to the development of organs and anatomical features. Specifically, terms relating to muscle development were found in PC1 and PC2 for males, PC3 for females, *h* and some terms shared between T_{opt} and *h* (Fig. 4.12-4.14).

Some groups of terms I found to only be present in some of the PC and TMV components. These terms related to cognition, behaviours, metabolic processes and signaling processes. Terms relating to cognition were found in PC4 for males, and PC1 and T_{opt} for females and terms relating to behavior were found in PC1, PC3 and T_{opt} for females (Fig. 4.12-4.14). Terms relating to biological processes were enriched in PC3, PC5 for males, and PC3, PC4, *w, h* and T_{opt} for females (Fig. 4.12-4.14). Terms relating to signaling were enriched in all but PC5 for males and in *w,* $T_{opt,}$ z_{max} PC4 and PC5 in females (Fig. 4.12-4.14).

At the molecular function level, all PC and TMV components had GO terms relating to transport and binding of substances; where it was equally spread among the PCs (Fig. 4.13 & 4.14) but not for the TMV components, T_{opt} and *h* had the most amount of unique molecular function terms present (Fig. 4.12). Exploration at the cellular level revealed few enriched terms across all the PC and TMV components (Fig. 4.12-4.14). Some of these terms related to the cell junctions, cell parts, plasma membrane, and channel complexes for PCs and TMV components and also neuron spines for PCs.

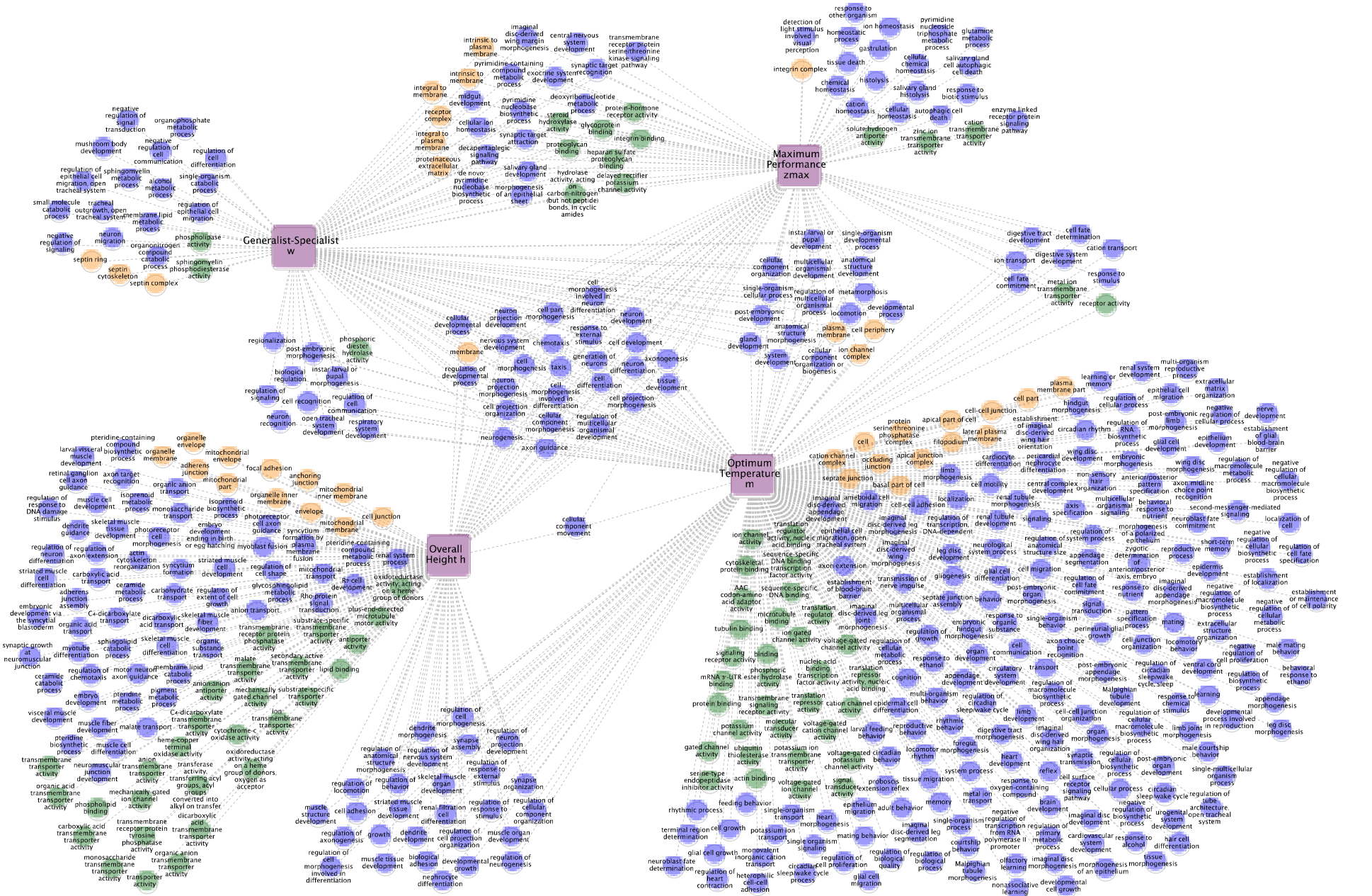

Generalist-Specialist w
Maximum Performance zmax
Optimum Temperature m
Overall Height h

Figure 4.12: Network of significantly enriched terms for genes associated with optimum temperature, T_{opt}, maximum performance, z_{max}, 'generalist-specialist', *w* and 'overall height' *h* for females. The four square purple nodes represent each of the four components, and each circular node represents an enriched GO term with biological processes in blue, molecular functions in green and cellular components in yellow.

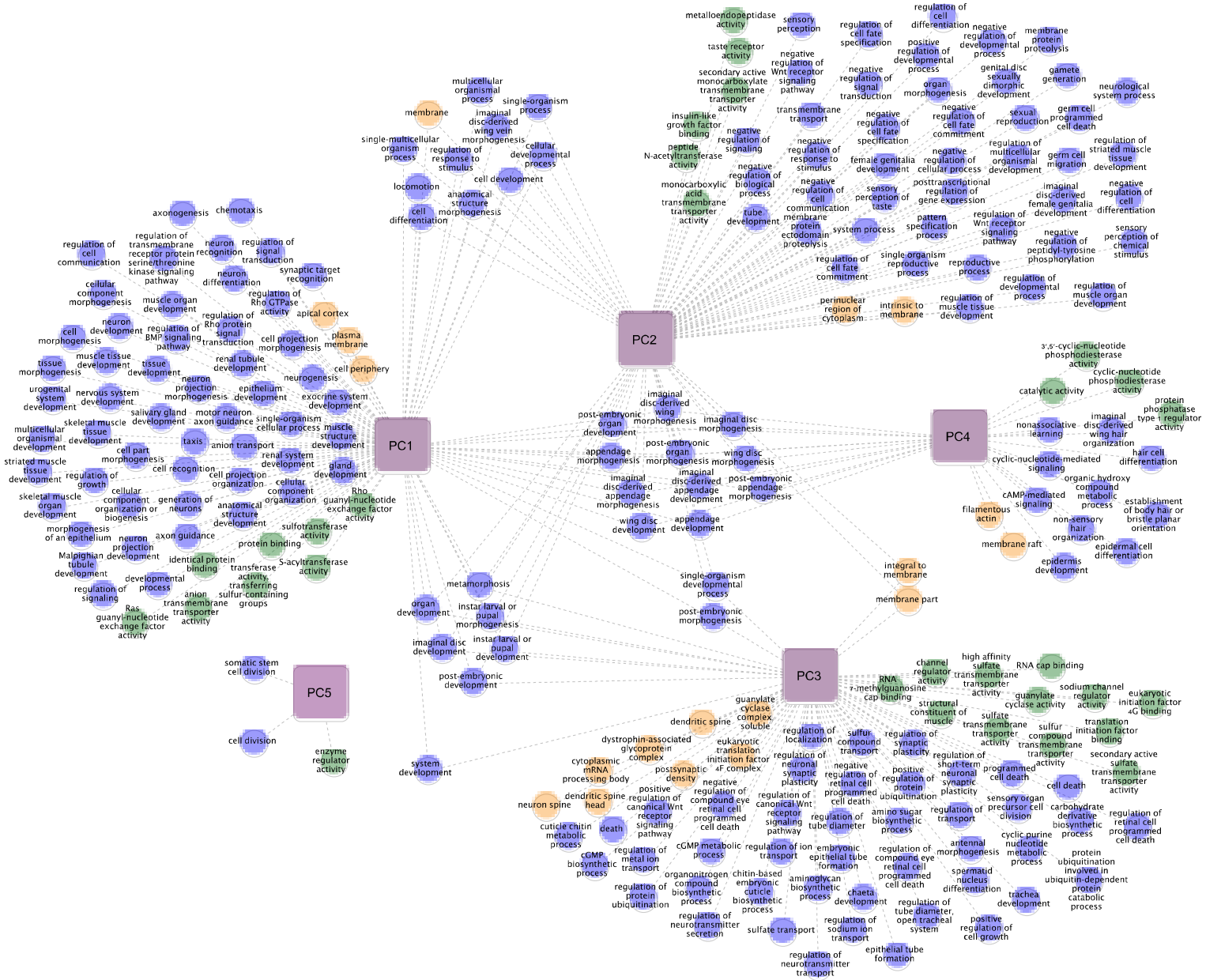


Figure 4.13: Network of significantly enriched terms for genes associated with PC1, PC2, PC3, PC4, and PC5 for males. The five square purple nodes represent each of the five PCs, and each circular node represents an enriched GO term with biological processes in blue, molecular functions in green and cellular components in yellow.

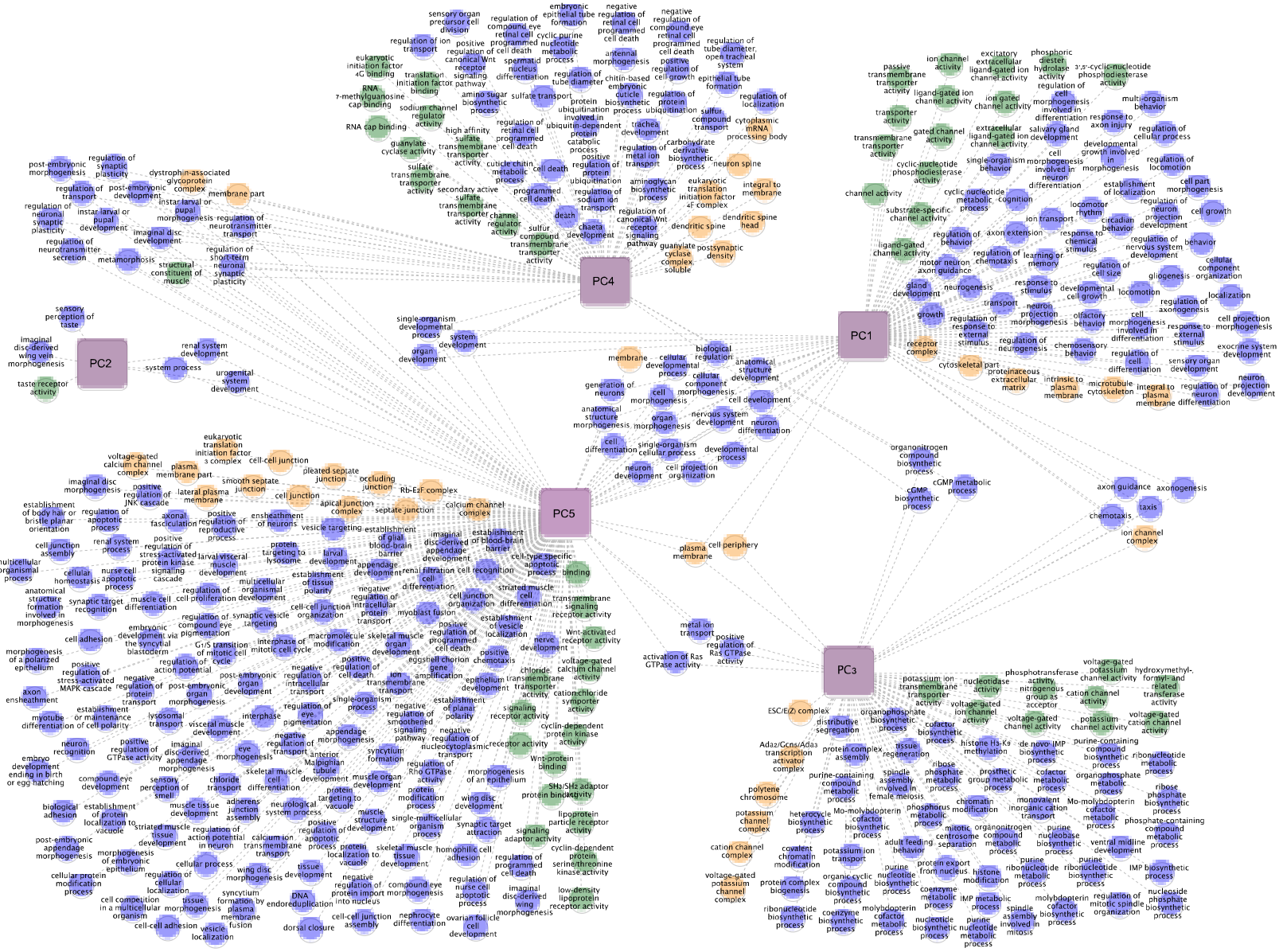


Figure 4.14: Network of significantly enriched terms for genes associated with PC1, PC2, PC3, PC4, and PC5 for females. The five square purple nodes represent each of the five PCs, and each circular node represents an enriched GO term with biological processes in blue, molecular functions in green and cellular components in yellow.

4.5 Discussion

Quantitative Genetics of TPCs

A recurrent theme in quantitative genetic studies of TPC is that genetic variance is distributed unevenly across the three major axes of variation (Kingsolver et al. 2004b; Izem and Kingsolver 2005; Yamahira et al. 2007; Latimer et al. 2011). This was consistent in both the character-state and function-valued analyses in this study. Most variation occurred along a 'faster-slower' axis as defined by the PCs with the remaining PC axes consisting of changes in TPC shape. The 'faster-slower' axis as per the PCs had lower temperature loadings at the hotter temperatures, suggesting this axis defined a combination of both temperature specific effects and increases (or decreases) in overall activity. This pattern of decreased loadings at higher temperatures for PC1 was a consistent finding across the studies in this thesis. As all my analyses were performed on mean standardised trait values it suggests that a class of pleiotropic variant with a larger effects at the cool temperatures to be a true reflection of the connection between the genotype and phenotype for both *D. serrata* and *D. melanogaster*.

Unlike the 'faster-slower axis' defined by the PCs, the function-valued trait analysis showed most of the genetic variance was along the 'hotter-colder' and 'generalist-specialist' axes with little along the 'faster-slower' axis. One possible explanation for this discrepancy would be the differences in the statistical approach. The 'faster-slower' axis as defined by the TMV approach is pure overall height changes without any trade-offs; the equivalent for the PCs would involve equal loadings of shared sign across all temperatures, which was not the case. However, this particular pattern of genetic variance partitioning is consistent with other studies where the template mode of variation statistical approach was used (Izem and Kingsolver 2005; Knies et al. 2006; Latimer et al. 2011). Whilst both statistical approaches have benefits, they may isolate quite different aspects of TPC variation, therefore care needs to taken when comparing the results of studies that have used these different statistical methods. Nonetheless, upon investigation of the GWA results, a degree of consistency was observed.

Genome Wide Association Analysis of TPC variation

This is the first GWAS of natural variation in TPCs and provides an opportunity to understand the types of mutations, genes, and functional pathways that underlie TPC variation. The numbers of variants found for each component tended to correlate with their contributions to genetic variance in the TMV analysis. Thus optimum temperature and PC1, which had the highest genetic variance, also had the largest number of associated variants, followed by 'generalist-specialist', 'overall height' and the remaining PCs. Below I discuss the main evolutionary genetic and functional aspects of this genomic analysis.

Functional Consequences of Associated Variants

At the molecular level, phenotypic evolution can arise through changes in gene expression or changes in coding sequences (Levine and Tjian 2003; King and Wilson 2014). I detected many more variants that could plausibly regulate gene expression than affect protein-coding sequence. Within coding regions, non-synonymous variants are of interest because they alter amino acid sequence and could affect enzyme function. This would be important for TPCs, as temperature has been demonstrated to affect the binding affinity and catalytic activity of enzymes (Fields 2001; Hochachka and Somero 2002). Interestingly, out of all the non-synonymous variants for T_{opt} in males and females, PC1 in males, and PC2, PC3, *w* and z_{max} for females, most had functions related to the binding of a substance and/or catalytic activity. For example the genes, *CG7922*, *ade3*, *Ice1*, *lid, sad, and CG10916* have been implicated in the binding of substances and *CG7922*, *ade3*, *Ice1*, *hrg, CG33145, CG11050*, and *lid* all have catalytic activity functions. In particular, one non-synonymous variant in the gene, *Cyck*, had a function that was related to the regulation of enzyme binding. This supports the concept that the trade-offs observed in enzyme functions contribute to the shape of TPCs (Huey and Kingsolver 1989; Hochachka and Somero 2002 ; Angilletta 2009).

Gene expression regulation has been proposed as an important mechanism allowing individuals to respond to environmental change (Via and Lande 1987; Scheiner 1993; Schlichting and Pigliucci 1995). Therefore I am not surprised to have detected more variants that can be implicated with gene expression than coding changes. The majority of the variants in this study were located in non-coding regions, similar to other studies on DGRP (Jordan et al. 2012; Chow et al. 2013a; Harbison et al. 2013), including three

particular regions: intronic, intergenic and variants upstream or downstream from a gene. Non-coding regions can contain regulatory information, for example, intronic polymorphisms can affect gene expression through the regulation and translation of splicing efficiency. Variants in the upstream regions can also harbour *cis*-regulatory elements. Interestingly, one of the upstream variants for T_{opt} was for the gene, *Drak*, which has been found to have a role in temperature dependent phenotypic plasticity of morphological traits in *D. melanogaster* (Carreira et al. 2013). Intergenic regions can also contain long distance regulators of gene expression, such as enhancers, or non-coding RNA. Non-coding RNA, especially the long non-coding RNAs, have recently been suggested to be important as regulatory factors for molecular processes and cellular functions (Li et al. 2012). They have been demonstrated to function as transcriptional regulators of neighbouring protein coding genes (Feng et al. 2006; Ng et al. 2012) and found to influence traits including locomotor behaviour in *Drosophila* (Li et al. 2012), neural function, and cognition (Mercer et al. 2008; Qureshi and Mehler 2011). Although this study cannot determine how polymorphisms in intergenic regions might be affecting TPC variation, it does suggest these genomic regions should be a focus of follow up studies.

Biased Directions of Minor Allele Effects

Natural selection may play an important part in shaping TPCs variation, for example, selection has been proposed as an explanation for lower standing genetic variance at hotter temperatures than cooler ones (Berger et al. 2013). This study provides an opportunity to explore the evolutionary forces that may shape standing variation by examining the frequencies and direction of phenotypic effects of trait-associated alleles. For instance, any major skew in the directional effect for minor frequency alleles associated with a trait may tell how selection acts on that trait. I observed quite a strong minor frequency allele skew for PC1 T_{opt}, w and z_{max} for both sexes and PC2 for males. As PC1 resembles the 'faster-slower' axis suggests that selection maybe occurring to try and alter the level of activity regardless of the temperature. Unfortunately, due to the lack of directionality in the PCs, I can not discern which direction selection may have occurred. Generalists with a lower z_{max} and higher T_{opt} appeared to have been selected against in this population in favour of specialists with higher z_{max} but lower T_{opt} TPCs. The skew in PC2 supports this whereby it resembles a variation in TPC shape that is likely to encompass changes in w and T_{opt}. In optimisation models, selection on T_{opt} is predicted to

match the local environment and width of curves to match the degree of temperature fluctuations between and within generations (Lynch and Gabriel 1987; Gilchrist 1995). If this were the case, I would expect to see an opposite pattern of skew for species that can perform in hotter environments, which emphasises a need for similar studies to be conducted in other species such as thermal specialists.

Pleiotropy

Pleiotropy is predicted to be a major component of the genetic architecture of TPCs (Angilletta 2009; Knies et al. 2009; Latimer et al. 2011). Two levels of pleiotropy were found in this study; pleiotropy occurred at the variant-level and also the gene-level. At both levels, a large number of pleiotropic effects were identified between *w* and z_{max}; however, as these two components are algebraically connected, this was expected. Therefore, more interesting would be the existence of pleiotropic effects between the PCs and the two trade-off axes, 'hotter-colder' (T_{opt}) and 'generalist-specialist' (w). At the variant-level, no variants were shared among the PCs for either sex and only one single variant in females had a minor allele that increased T_{opt} and also resulted in a broader curve (lower w). This 'hotter-is-broader' aspect was seen for growth rate TPCs in bacteriophages (Knies et al. 2009) where the authors suggest it may have been a result of adaptation to high temperatures or high temperature variation. My study suggests these types of effects are extremely rare for TPCs in *D. melanogaster*. Gene-level pleiotropy was also very low between the PCs and between T_{opt} and *w*. Only one gene for males and two genes for females showed such a pattern between T_{opt} and *w*, and only two genes where shared between two PCs for each of the sexes. The low apparent pleiotropy was not surprising for the PC analysis and suggests that each PC axis is a separate unity with trade-offs present only among the set of genes that occur within that particular axis. In contrast, the low pleiotropy was surprising for the TMV components and could be due to the way the TPCs were analysed. The TMV analysis explicitly incorporates specific trade-offs within components. For example, *w* includes both a change in the breadth of temperatures for performance as well as the level of performance. Therefore, pleiotropic effects may exist within each of the components, but this study could not detect them because they were effectively combined into one trait. A GWA analysis using the multivariate mapping method I used in Chapter 3 may have revealed more pleiotropic effects; however, it was not

computationally feasible to implement here given the very large numbers of variants to be tested.

Gene Ontology Functional Analysis

In thermal adaptation, trade-offs are believed to be due to differences in biochemical and cell structure performance across temperatures (Hochachka and Somero 2002 ; Angilletta 2009). My study suggests that the underlying biochemistry and physiology for TPC is complex, especially for the three physiological areas suggested to shape TPCs: oxidation-limitation, enzyme function and membrane structure (Angilletta 2009). The gene ontology analysis revealed patterns consistent with the involvement of oxidation-limitation for 'faster-slower' variation. In particular, the 'faster-slower' axis had gene ontology terms relating to the transport of molecules involved in the citric-acid cycle (malate). All the female TMV axes and majority of the PC axes had enriched terms related to enzymes, where terms were present in all but PC5 for males and all but PC2 and PC3 for females. The terms included the activity of steroid hydroxylase, cytochrome-c oxidase, oxidoreductase and phosphoric ester hydrolase for TMV and cyclic-nucleotide phosphodiesterase, metalloendopeptidase, quanylate cyclae, sulfotransferase, and nucleotidase for the PC axes. Membrane, plasma membrane or membrane part structures, which could lead to trade-offs through differences in stability between temperatures, were terms enriched for all TMV components and most PCs (except PC5 for males and PC2 for females) axes.

For behavioural traits such as locomotor activity, it was expected that individuals must be capable of detecting and then responding to an environmental temperature stimulus. I found ontology terms linked to responses to stimuli for PC1 and PC2 for males and PC1, PC3, and all the TMV components for females. While only one gene, *dnc,* associated with PC1 for females, PC4 for males and T_{opt}, is currently annotated in responding specifically to a temperature-based stimulus (GO term: thermosensory behaviour), independent studies have recently linked temperature-based stimulus responses to other detected genes that linked to the 'response to stimulus'. These included a suite of olfactory receptor genes *or65a, or65b, or65c* and *or59a,* and two glutathione-S-transferases genes gstD6 and gstD5 (Riveron et al. 2013).

The ability to respond to a stimulus also requires sensing the stimulus from the peripheral system and relaying the information through the central nervous system. For instance, Brown et al. (2013) found that locomotory behavioral responses to olfactory cues were associated with neural functions. I also found that neurological function had a key role. Genes were also identified with functions relating to the peripheral system that included *Nrx-IV*, *Appl*, and *bun for PCs, and shn mbl, and Tollo* for TMV components; and for the development of nervous system and neurological function *Ets21c, Lar, and frac* for TMV components and *NetB, Lar and* sdk for the PCs plus many others. Therefore, the development and efficiency of an organism's central nervous system may be processes important to TPC variation. In addition to the nervous system, signaling would also aid the relay of information. As expected I found terms related to signaling enriched in some of the axes, specifically the 'hotter-colder', 'generalist-specialist', 'maximum performance', PC4 and PC5 axes in females and PC5 in males. An interesting result for the 'faster-slower' axis (PC1 in males and *h)* was the presence of many terms related to muscle development and function. This makes sense, as the 'faster-slower' axis encompasses overall performance regardless of temperature, and as a locomotor performance trait was used, muscles would be required for movement. Unlike the TMV components, terms relating to the muscle development and function were also present in other axes that affected TPC shape. This demonstrates that perhaps muscle development maybe also important for other variation in TPC shape. As a general note of caution, the functional annotation of genes in *D. melanogaster* is a constantly developing area and annotations to genes are constantly being added and revised over time. It is therefore difficult to gauge the level of uncertainty in these analyses. For now they serve as a useful guide into the likely functions that can be tested with targeted follow up studies.

I found varied levels of overlap in annotated gene functions between the different TPC components and to a lesser extent for the PCs. There was a high degree of sharing between z_{max} and the 'generalist-specialist' axis, *w*. In contrast, term-sharing was more limited for the 'hotter-colder' and 'faster-slower' axes and the PCs. It may be the case that the 'hotter-colder', 'faster-slower' and PC axes have a higher degree of evolutionary autonomy than other axes. I found that the 'faster-slower' axis defined by the PCs in this chapter (PC1) and in previous chapters to also contain a level of 'hotter-colder' type variation. If this were the case for the TMV defined 'faster-slower' axis, *h*, I would have expected to see a greater number of shared functions between the TMV 'hotter-colder' and

‘faster-slower’ axes. However, shared functions between ‘hotter-colder’ and ‘faster-slower’ TMV axes were not found. As mentioned earlier, this may be due to the different statistical approaches whereby the TMV method has more likely efficiently isolated pure ‘faster-slower’ variation, disregarding any trade-offs. Recently developed statistical approaches, such as simplicity measures to explore the nearly null-space (Gaydos et al. 2013), may provide a useful complement to eigenanalysis in extracting this kind of variation.

Conclusion

In this study, I detected genetic variance for the thermal dependence of locomotor activity for *Drosophila melanogaster*. I found genes associated with five principal components that explained more than 95% of variance and with the three main axes of TPC variation defined by a function-valued trait analysis. Annotation of associated variants suggests that gene expression is likely an important process shaping TPC variation. At the functional level, different levels of independence were found between components, suggesting that some modes of variation, especially ‘hotter-colder’ and the axes defined by the PCs, might have greater evolutionary independence than others. Exploration of the genetic architecture and functions of TPCs remains in its infancy and further research is still needed to further understand the genomic processes involved in thermal adaptation.

Chapter 5: General Discussion

The empirical studies in this thesis have contributed to the field of thermal adaptation through evaluation of the evolutionary potential and genetic architecture of an organism's ability to perform across different environmental temperatures. Specifically, I have conducted an integrative genetic dissection of thermal performance curve (TPC) variation for locomotor activity in two *Drosophilids*. Below, I briefly acknowledge some limitations of the work and suggest directions for future research. I conclude discussing some broad observations from the work.

5.1 Limitations and future directions

One aspect that should be considered when interpreting the findings of this thesis is that the studies focused on a single trait—locomotor activity—and on a single type of continuous reaction norm (CRN). As the shape of CRNs, including TPCs, have been suggested to differ according to trait type and environmental factor (Angilletta 2009; Rocha and Klaczko 2012; Murren et al. 2014), some of my genetic results may be trait and CRN specific. Although the number of studies that have examined the genetic architecture of CRNs at the same fine scale examined in this thesis is low, some similarities may exist. For example, a QTL analysis on photosynthetic light curves indicated elevation QTLs; they found QTLs that increased photosynthetic rate across all CO_2 and irradiance levels (Gu et al. 2012). Another QTL study on growth curves for Japanese medaka fish fin suggest a contribution by QTLs that vary in their pleiotropic effects across the ontogeny, which could lead to changes in curve shape as opposed to elevation (Kawajiri et al. 2014). Such a finding is similar to my results for Chapters 2 and 3, where I detected pleiotropic effects on TPC shape and elevation, but the relative contribution to each differed between studies where more QTLs contributed to the shape of growth curves in medaka. Further, Kawajiri et al. (2014) annotated the genes found within the associated QTL for medaka and found functions related to the central nervous system, ion transport, substrate binding and membrane; a very similar finding to my results for Chapter 4. However, it was not possible to determine if there was a greater contribution to gene expression as opposed to coding sequence based on these analyses, therefore, the relative contributions of coding verse regulatory variation to CRNs remains an open question. Until more studies are conducted on the genetic architecture of CRNs, especially at the finer scale permitted in a GWAS, I

can only speculate on the generality of my results The existence of living genomic resources for animals and plants, such as the DGRP, may facilitate controlled comparative analyses of genetic architectures across a broad range of CRNs within the same organism.

A broader taxonomic breadth to genomic studies may also be required. While this thesis focused on two species of *Drosophila,* other species including ectothermic vertebrates, like the zebrafish, offer exciting opportunities for the genomic dissection of TPCs. As different species have different distributions and inhabit different thermal environments, differences in genetic architecture may become apparent. For instance, the two species used in this study inhabit different ranges of climates. *Drosophila serrata* is endemic to the Australasian region, occurring from Papua New Guinea and down along the east coast of Australia (Lemeunier et al. 1986; Kellett et al. 2005). *Drosophila melanogaster,* however, is a cosmopolitan species that is found across the world (Lemeunier et al. 1986; David et al. 2007). The recent development of a panel of 105 sequenced lines of *D. serrata* (A. Reddiex and S. Chenoweth, manuscript in preparation) may offer an opportunity to conduct a comparative GWAS between these different species.

Populations of the same species that occur in different climates, such as tropical and temperate environments, may also have different TPC genetic architectures. Studies have identified population divergence in TPCs (Wilson 2001; Yamahira et al. 2007; Latimer et al. 2011; Berger et al. 2013; Gaitan-Espitia et al. 2013) but have not investigated the genetic basis of divergence. Exploration of the genetic architecture among different populations was attempted in Chapter 3 by looking at recombinant inbred lines derived from two populations. However, weak divergence was detected, suggesting they may have not been the best candidate populations to compare. Comparisons of the genetic architecture for a different group of lines that more closely represents the natural population (e.g. isofemales collected from natural populations and inbred similar to the DGRP lines) may be more informative of how selection has shaped TPCs. For instance, would different patterns of skew in the directions of allelic effects occur between populations with different thermal minima and maxima or different levels of variability in temperature?

In thermal adaptation, acclimation during development or for a short period of time (e.g. 5-10 days) is often found to influence an organism's ability to respond to temperature (Wilson and Franklin 1999; Glanville and Seebacher 2006; Cooper et al. 2010; Lachenicht et al. 2010). In particular, recent studies have started to use acclimation as a way to better understand how fluctuating environments during development affect an organism's ability to respond to thermal environments and has been suggested to contain different genetic constraints (Cooper et al. 2010). Whilst my aim was not to explore the effects of acclimation, I acknowledge that if the flies were exposed to a different thermal regime during their development, such as fluctuating temperatures, a difference in TPC shape may have been observed. For example, the low genetic variance found for the 'faster-slower' axis in Chapter 4 may be characteristic of non-developmental rather than developmental thermal reaction norms, as found in Kingsolver et al. (2004a) study. If the partitioning of the genetic variance between the axes differs between developmental and non-developmental thermal reaction norms, the underlying genetic architecture may vary with the activation of different genes.

One particular challenging aspect to the study of function-valued traits, such as TPCs, is the statistical approach used for their analysis. Many different methods have been used to investigate TPCs including regression analysis, ANOVAs (Butler et al. 2013), eigenanalysis (Kingsolver et al. 2004b), and the template mode of variation (Izem and Kingsolver 2005). Over the years, debates have occurred over which methods are best, Bulté and Blouin-Demers (2006) arguing regression is the most optimal, whilst others argued that method choice is data and question specific (Angilletta 2006; Stinchcombe and Kirkpatrick 2012). If a curve is fit, then arguments also arise over the best function to fit; Angilletta (2006) argues that the Gaussian is the best while others have used different models such as the four parameter *spain* model (Krenek et al. 2011) and the *Briere1* and *Briere2* models (Shi and Ge 2010).

In this thesis, I have used two different approaches, the eigendecomposition (character-state) and the template mode of variation (function-valued trait) approaches. Whilst both have been suggested as adequate tools for function-valued trait analysis (Stinchcombe and Kirkpatrick 2012), in Chapter 4 I highlight that these two approaches may distill TPC variation in different ways. This suggests that studies on TPC may benefit from a combination of statistical approach to capture the full picture of TPC variation.

However, both approaches may not always be applied together for some data, whereby each approach is more suited to different circumstances depending on whether a common shape exists for all individuals in a data set (Stinchcombe and Kirkpatrick 2012). For example, the eigenanalysis is more flexible when very different shapes occur between individuals, as was the case for Chapters 2 and 3. The eigendecomposition approach was able to detect genetic variance that was less constrained to specific directions but could not explore axes with very low genetic variance. The template mode of variation methods was powerful to explore the shape of the TPCs and at detecting predefined axes that had very low genetic variance. However, it was restrictive when looking for pleiotropic effects across the temperatures. I can see two complementary approaches that could be applied in future research to avoid these issues. First, would be to conduct an eigenanalysis, similar to Chapters 2 and 3, but in addition to this, also look at the nearly null spaces of **M** or **G** to understand regions of absolute genetic constraint. Gaydos et al.'s (2013) simplex method may be useful for such analyses. Second, would be to fit curves to each genotype, and then width, maximum performance, and optimum temperature could be measured without imposing area (under the curve) constraints. Such a non-parametric approach may allow pleiotropic effects to be more easily mapped. Such methods have been used by Knies et al. (2009) and Krenek et al. (2011). Whilst it would be interesting to then explore pleiotropic effects at a whole genome-scale using either of these suggested methods, both have limitations. Fitting individual curves would require an adequate sample size within each genotype to ensure the correct curve is fitted, which can itself pose logistical constraints (Stinchcombe and Kirkpatrick 2012) especially when a large number of genotypes are needed for sufficient power in a GWAS. The multivariate QTL mapping approach used in Chapter 3 could be applied to GWAS data to gain a more complete picture of the distributions of allelic affects across temperatures, but the approach is not without significant computational overhead.

5.2 Conclusions

Standing variation is likely shaped by natural selection

A common observation in quantitative genetic studies of TPC variation has been that the genetic variance is not distributed equally, either across individual temperatures along the curves (Gilchrist 1996; Kingsolver et al. 2004b; Berger et al. 2013), or across specific axes of variation (Izem and Kingsolver 2005; Knies et al. 2006; Knies et al. 2009;

Latimer et al. 2011). There are two non-mutually exclusive explanations for these patterns. First, differences in genetic variance might reflect different mutational inputs to each temperature or axis of variation. For example, some axes could have greater mutational target sizes or accrue generally larger mutational effects than others. My mutation accumulation study (Chapter 2) showed that while mutational contributions were detectable for all three common axes of TPC variation, mutational variances differed greatly. However, the distribution of genetic variance across the major axes of variation was not the same as the distribution seen for standing variation in *D. serrata* (Latimer et al. 2011), the TMV analysis in my study of *D. melanogaster* (Chapter 4) or, more generally, prior studies of TPCs (Izem and Kingsolver 2005; Knies et al. 2006). Specifically, there was limited genetic variance for the 'faster-slower' axis, whereas the mutational variance was dominated by this axis.

A second explanation for differences in genetic variance between temperatures or TPC components is that a balance between natural selection and mutation may shape standing variance (Berger et al. 2013). It is quite possible that the mismatches between standing and mutational variation seen for the 'faster-slower' axis are the consequence of natural selection against deleterious 'faster-slower' type alleles. Because 'faster-slower' variation is likely to involve alleles with effects independent of temperature, they may be more efficiently purged by natural selection than alleles with temperature-dependent effects. I speculate that low standing variation for the 'faster-slower' axis may be a general phenomenon induced by natural selection. Higher standing variance for the 'hotter-colder' and 'generalist-specialist' axes could therefore be a by-product of natural selection purging 'faster-slower' variance faster (or more efficiently) than particularly large mutational contributions to the 'hotter-colder' and 'generalist-specialist' axes. For example, the QTL analysis in Chapter 3 was somewhat consistent with the mutational study; generalist-specialist QTL were relatively uncommon and tended to have smaller effect sizes than 'faster-slower' QTLs. The eigendecomposition of *D. melanogaster* TPC variation also revealed PC axis loadings that decreased towards warmer temperatures for the 'faster-slower' axis.

It should be noted that the mismatch between standing and mutational variation was not always strong. For example, when considering specific temperatures rather than TPC components, Berger et al. (2013) argued that low standing variance at hot

temperatures in *Sepsid* dung flies may have been the result of strong selection as opposed to low mutational variation. In Chapter 2, I found that the mutational variance was very low at hot temperatures, which was also present in Chapter 3—QTL effects tended to be quite small at hotter temperatures—and in Chapter 4—the loading effect sizes were lower at the hotter temperatures. These observations suggest that this specific pattern could be a genuine feature of the genotype-phenotype map rather than a mutation-selection balance.

Another line of evidence supporting a role for natural selection in shaping standing variation came from the GWAS on *D. melanogaster*. I observed some quite consistent patterns of skew in the direction in which the minor-frequency allele affected a trait. So long as the sample of associated variants is sufficiently large and linkage disequilibria between them are weak, as is the case for most DGRP variants (Huang et al. 2014), a bias in directionality would likely be the result of natural selection against the minor allele. In the GWAS I found that there was a strong skew for all shape components and for some of the principal component axes. For curve height, minor frequency alleles tended to increase activity, which was also the direction of effect for most, presumably deleterious, mutations in Chapter 2. For the 'generalist-specialist' axis, rare alleles tended to produce broader TPCs with lower maximal performance levels. There was also apparent selection along the 'faster–slower' axis, with a bias in favor of lower optimum temperatures.

Different classes of pleiotropic alleles likely contribute to thermal trade-offs

Multivariate genetic constraints slow and/or bias the direction of phenotypic evolution during adaptation by reducing the availability of genetic variance in the direction favored by natural selection. Such constraints are thought to be a consequence of pleiotropy. Two different classes of pleiotropic alleles may underlie the trade-offs involved in thermal adaptation: antagonistic alleles that affect performance in different directions at different temperatures, and also alleles that affect performance in the same direction across temperatures, but with varying strength. I observed both of these in Chapters 2 and 3. In particular, the 'hotter-colder' axis appears due to both antagonistic pleiotropy and, perhaps to a larger extent, pleiotropic effects that vary in size but not direction. In contrast, the 'generalist-specialist' axis appeared to involve mainly antagonistic pleiotropic effects. The existence of many pleiotropic alleles that might increase locomotor activity across all temperatures but to varying degrees is interesting because it suggests that although the

‘faster-slower’ and ‘hotter-colder’ axes can be distinguished statistically, they may not necessarily be genetically independent.

Towards an understanding of the molecular basis of TPC variation

By conducting a genome-wide association study on a well-understood model system, I was able to begin to provide a molecular understanding of natural variation in TPCs. It has been suggested that changes in gene expression may aid organisms in responding to different environmental conditions (Scheiner 1993; Schlichting and Pigliucci 1995). The GWAS results from Chapter 4 implicate changes in gene expression as a major contributor to TPC variation as opposed to changes in protein coding sequences. This was illustrated by the majority of significant GWAS hits residing near, but in the non-coding regions of genes, and therefore likely to have regulatory functions. Although I observed changes in the amino acid sequence (non-synonymous variants), these were modest in number. Interestingly, those I did detect appear to have implications for enzyme function, which has been predicted to affect the ability to perform at different temperatures (Somero 1995; Fields 2001; Hochachka and Somero 2002 ; Angilletta 2009).

The functional annotation of TPC-associated genes revealed that the underlying physiological and biochemical processes affecting TPC variation were quite complex. There were three key physiological functions previously hypothesised to affect TPCs, including enzyme function, oxidation-limitation, and membrane structure (Hochachka and Somero 2002 ; Angilletta 2009). I provided a link to these functions, through the exploration of gene annotations. Specifically, membrane structure was associated with all the axes of TPCs, and oxidation-limitation was present in gene functions for the ‘faster-slower’ axes, such as enriched terms for molecules relating to the citric-acid cycle. Enzyme functions were enriched for the genes in the ‘faster-slower’, ‘hotter-colder’, and ‘generalist-specialist’ axes. They were also found to be largely present for functional genes that were annotated to non-synonymous variants, suggesting the enzymes functions may be impacted by amino acid changes. Additionally, the neurological system was evident to have a key role in the shape of TPCs because it was present across all TPC axes. As genome annotations improve and genomic resources become available for other species where the study of TPCs is tractable, further studies should be conducted to permit a better understanding of the functional aspects of TPCs.

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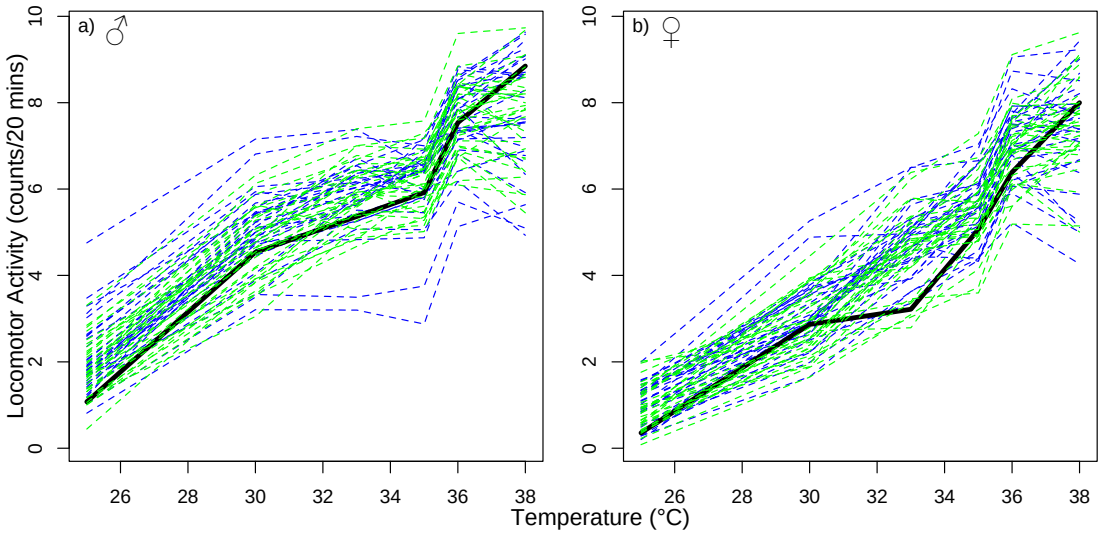
Appendix 2



Appendix 2.1: The mean locomotor activity TPCs for a) females and b) males of each mutation accumulation line. Locomotor activity has been square root transformed and was measured as counts per twenty-minute testing period. The founder line is in thick solid black, the control mutation accumulation lines are in blue and the sexual selection mutation accumulation treatment lines are in green.

Appendix 2.2: Univariate mixed effects linear models testing for the effects of mating treatment, block, line and vial on locomotor activity at each of the six test temperatures in male and female *D. serrata*. The test for differences in mutational variance between treatments involves estimating a model with independent variance components for the Line(Treatment) effect in each treatment and testing for a significant improvement of fit using likelihood ratio tests. Significance of all random effects was evaluated using likelihood ratio tests. P-values are shown in italics (significant tests (P < 0.05) are highlighted in bold) and variance components for random effects are in parentheses.

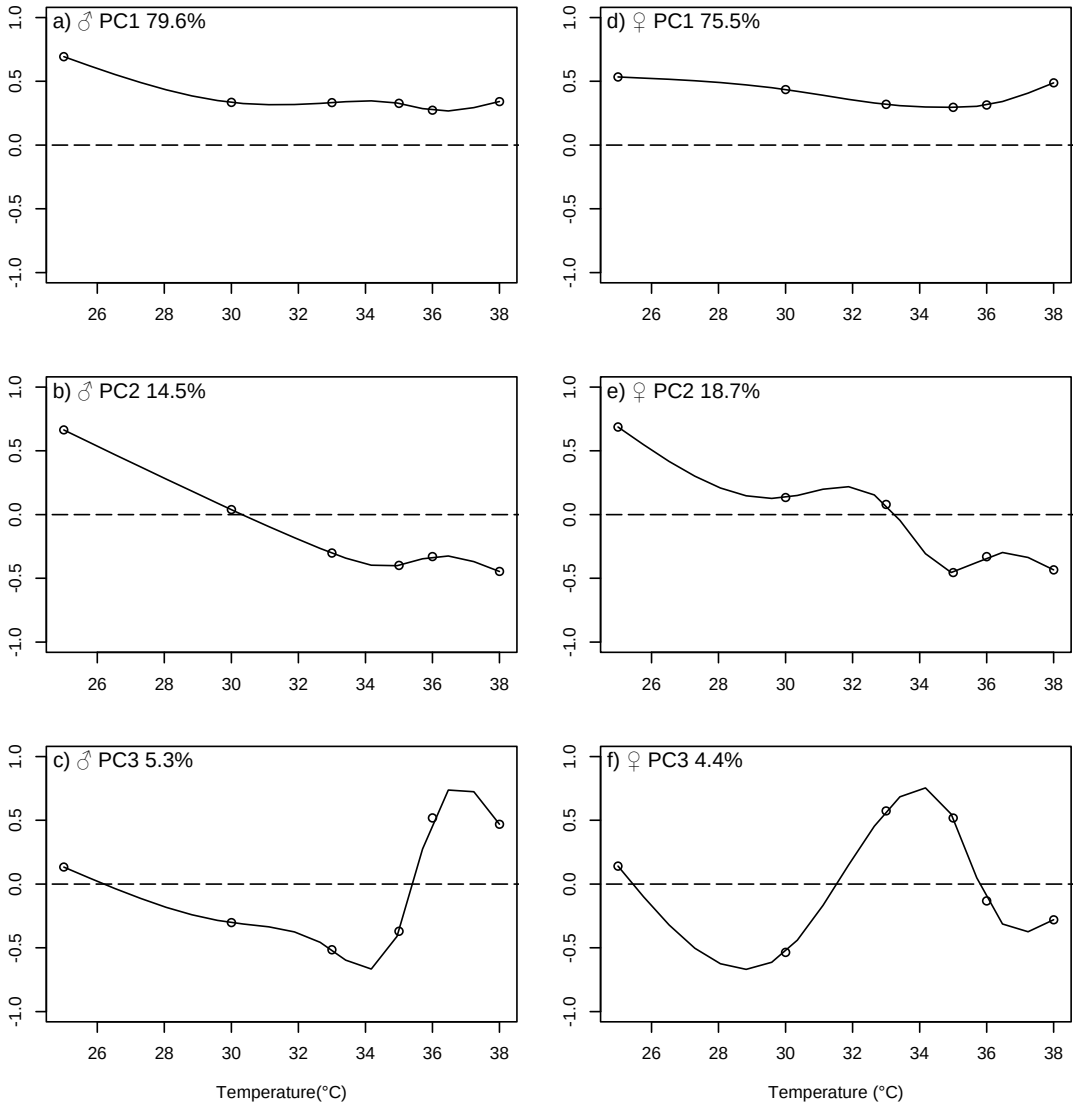
Effects	**Test Statistic**	**Temperature**					
		25°C	**30°C**	**33°C**	**35°C**	**36°C**	**38°C**
Males							
Block	$F_{1,630}$	7.48, <0.0001	5.28, <0.0001	3.81, <*0.0001*	11.57, <*0.0001*	4.71, <0.0001	3.95, <0.0001
Treatment	$F_{1,64}$	1.62, *0.208*	2.61, *0.111*	0.28, *0.599*	0.36, *0.553*	0.16, *0.689*	0.05, *0.826*
Block × Treatment	$F_{1,630}$	0.97, *0.474*	01.37, *0.181*	1.03, 0.422	1.16, 0.316	1.55, 0.1088	1.38, 0.179
Line(Treatment)	χ^2_1	28.0, <0.0001	22.6, <0.0001	34.7, <0.0001	38.48, <0.0001	45.4, <0.0001	42.7, <0.0001
		(0.304)	(0.445)	(0.394)	(0.440)	(0.521)	(1.039)
Vial(Line(Treatment))	χ^2_1	0.0, 1.000	0.226, *0.635*	0.0, 1.000	0.040, *0.841*	0.0, 1.000	0.977, *0.323*
		(0.000)	(0.043)	(0.000)	(0.010)	(0.000)	(0.109)
Residual		(1.826)	(2.216)	(1.693)	(1.309)	(1.609)	(2.875)
Independent line variances between treatments	χ^2_1	2.561, *0.109*	0.123, *0.725*	1.4, *0.238*	3.5, *0.060*	0.98, *0.755*	0.74, *0.390*
Females							
Block	χ^2_1	4.65, <0.0001	7.00 <0.0001	5.76, <0.0001	7.21, <0.0001	6.75, <0.0001	3.50, <0.0001
Treatment	$F_{1,64}$	1.07, *0.305*	1.55, *0.217*	0.20, *0.657*	1.06, *0.308*	1.03, *0.313*	0.17, *0.678*
Block × Treatment	χ^2_1	1.14, 0.329	0.59, 0.834	0.91, 0.529	1.40, 0.166	1.49, 0.131	0.09, 0.358
Line(Treatment)	χ^2_1	1.29, 0.254	9.46, *0.002*	19.17, <*0.0001*	33.8, <0.0001	32.1, <0.0001	45.3, <0.0001

		(0.047)	(0.267)	(0.410)	(0.386)	(0.416)	(1.015)
Vial(Line(Treatment))	χ^2_1	1.73, *0.188*	2.83, 0.092	0.0, *1.000*	0.0, *1.000*	0.0, *1.000*	0.0, *1.000*
		(0.073)	(0.163)	(0.000)	(0.000)	(0.000)	(0.000)
Residual		(1.389)	(2.32)	(2.696)	(2.205)	(2.540)	(3.329)
Independent line variances between treatments	χ^2_1	0.009, *0.924*	0.018, *0.892*	0.857, *0.354*	0.02, 0.892	0.005, *0.945*	2.23, 0.136

Appendix 2.3: Eigenvalues and vectors of the mutational variance-covariance matrices, **M**, for locomotor activity across six temperatures for *Drosophila serrata*. Estimation was based on an unstructured variance-covariance matrix using PROC MIXED in SAS 9.3

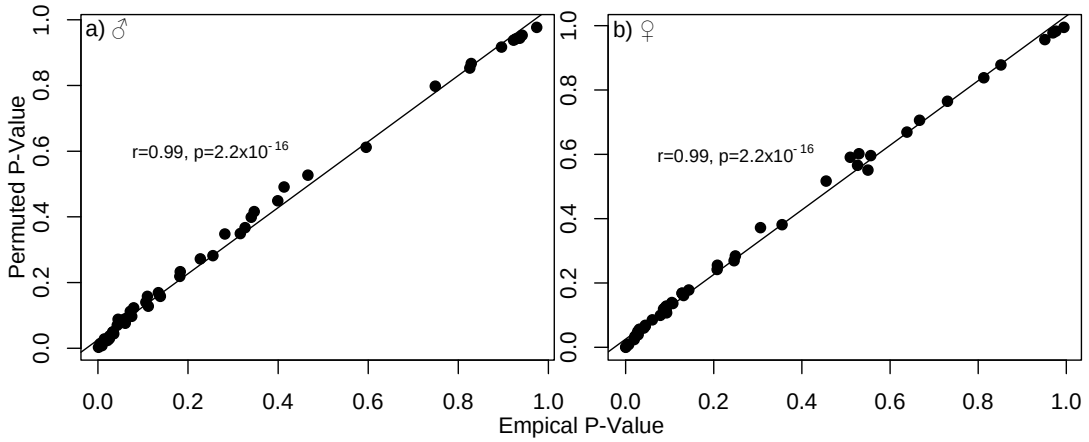
Males	$\mathbf{m_1}$	$\mathbf{m_2}$	$\mathbf{m_3}$	$\mathbf{m_4}$	$\mathbf{m_5}$	$\mathbf{m_6}$
Eigenvalue	1.740×10^{-3}	4.677×10^{-4}	6.342×10^{-5}	2.522×10^{-5}	1.012×10^{-5}	-4.650×10^{-6}
25°C	0.819	-0.382	0.323	-0.218	-0.126	0.129
30°C	0.398	-0.006	-0.356	0.818	0.074	-0.200
33°C	0.276	0.212	-0.632	-0.380	0.497	0.298
35°C	0.214	0.425	-0.302	-0.284	-0.635	-0.445
36°C	0.168	0.390	0.422	-0.086	0.555	-0.571
38°C	0.145	0.691	0.317	0.226	-0.143	0.575
Females						
Eigenvalue	1.506×10^{-3}	4.914×10^{-4}	1.306×10^{-3}	2.380×10^{-5}	2.045×10^{-5}	-1.181×10^{-5}
25°C	0.688	-0.453	0.169	-0.156	-0.426	0.295
30°C	0.496	0.097	-0.807	0.154	0.207	-0.165
33°C	0.382	0.002	0.510	0.556	0.314	-0.432
35°C	0.288	0.334	0.199	-0.774	0.247	-0.325
36°C	0.191	0.405	0.138	0.095	0.429	0.767
38°C	0.125	0.714	0.045	0.186	-0.658	-0.072

(Sas Institute, Cary NC). Eigenanalyses were conducted on mean standardised values.



Appendix 2.4: Reanalysis of data in figure 3.3 of the main text including only those MA lines that showed a unimodal activity curve (males 24 lines, females 16 lines). Eigenvector loadings are shown for the male and female mutational variance-covariance matrices, $\mathbf{M_m}$ and $\mathbf{M_f}$. **M** was mean standardised (sex-specific means) before eigenanalyses were conducted. The first three eigenvectors are shown for each sex (males: a-c, females e-f) and all are normalised to unit length. The linear model used to re-estimate the among line variances and covariances is the same as eqn. 3, but with the vial term removed owing to the smaller numbers of lines available for analysis (i.e $\boldsymbol{a = \mu + b + l + \varepsilon}$). The TYPE = FA0(6) option was specified for the random effect of line in SAS Proc MIXED upon re-estimation to improve model convergence.

Appendix 3



Appendix 3.1: The correlation between the F-approximated and permutation based p-values for the multivariate QTL analysis in a) males and b) females. Each point represents a different marker.

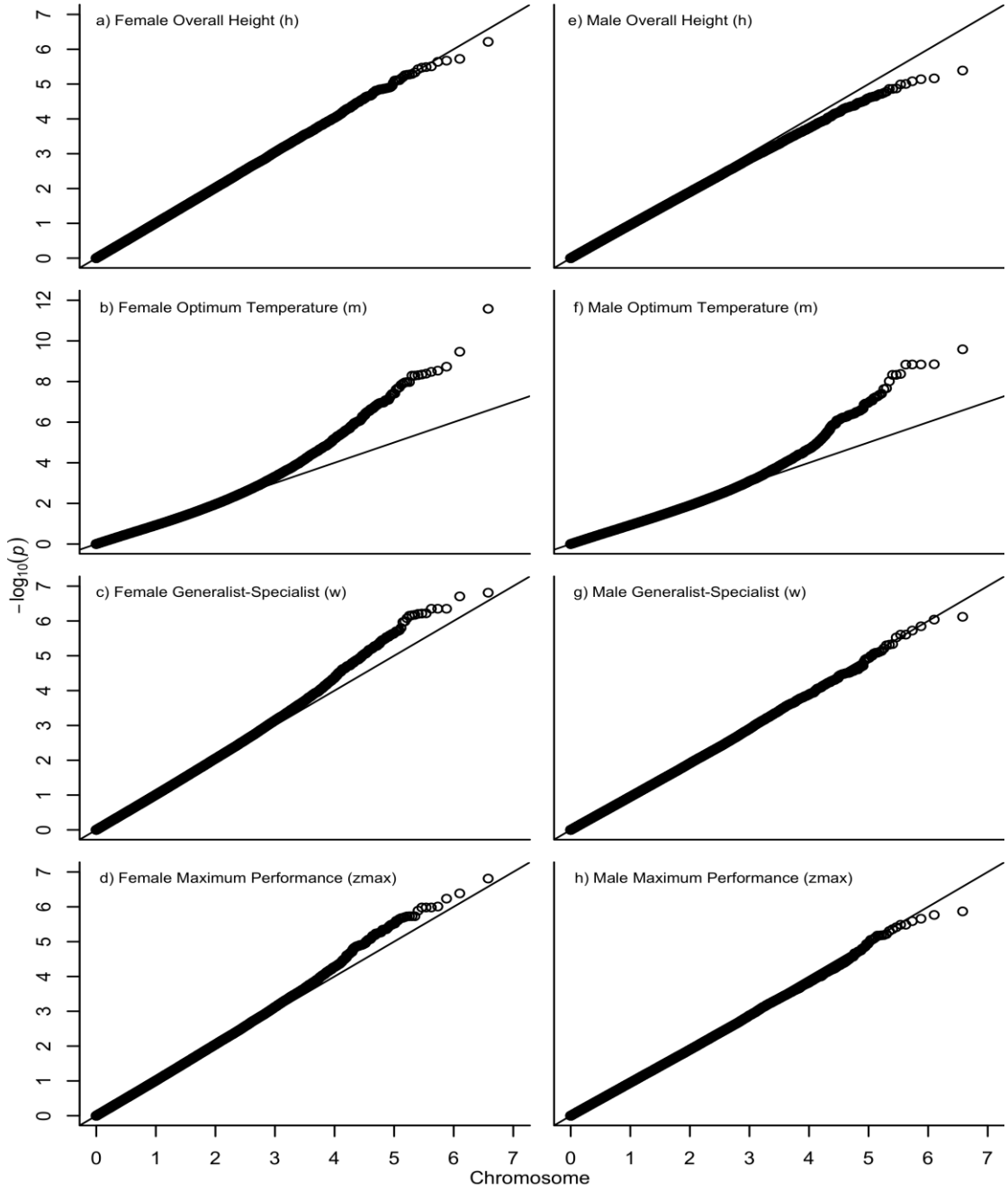
Appendix 3.2: Molecular markers used in the QTL analysis of locomotor activity thermal performance for *D. serrata*. Chromosomal arm locations correspond to the top BLAST hit to *D. melanogaster* using FlyBase. Allele frequency is the count of Eungella vs. Forster alleles for each marker. Segregation distortion is the chi-squared test on the allele frequency to investigate if frequency was an even 50:50 split for each marker.

Marker	Marker Type	D. melanogaster Chr. Arm	Allele Frequency Eung.|Fors	Segregation Distortion χ^2 (p-value)
1	Microsatellite	2R	32|47	2.8481(0.0915)
2	Microsatellite	2L	34|46	1.8000(0.1797)
3	Microsatellite	2R	31|50	4.4568(0.0348)
4*	Microsatellite	2R	78|5	64.2048(1.12×10^{-15})
5	Microsatellite	3R	37|40	0.1169(0.7324)
6	Microsatellite	Not Mapped	32|45	2.1948(0.1385)
7	Microsatellite	3R	53|28	7.7160(0.0055)
8	Microsatellite	X	33|30	0.1429(0.7055)
9	Microsatellite	2L	39|42	0.1111(0.7389)
10	Microsatellite	2L	38|42	0.2000(0.6547)
11	Microsatellite	2R	21|41	6.4516(0.01108)

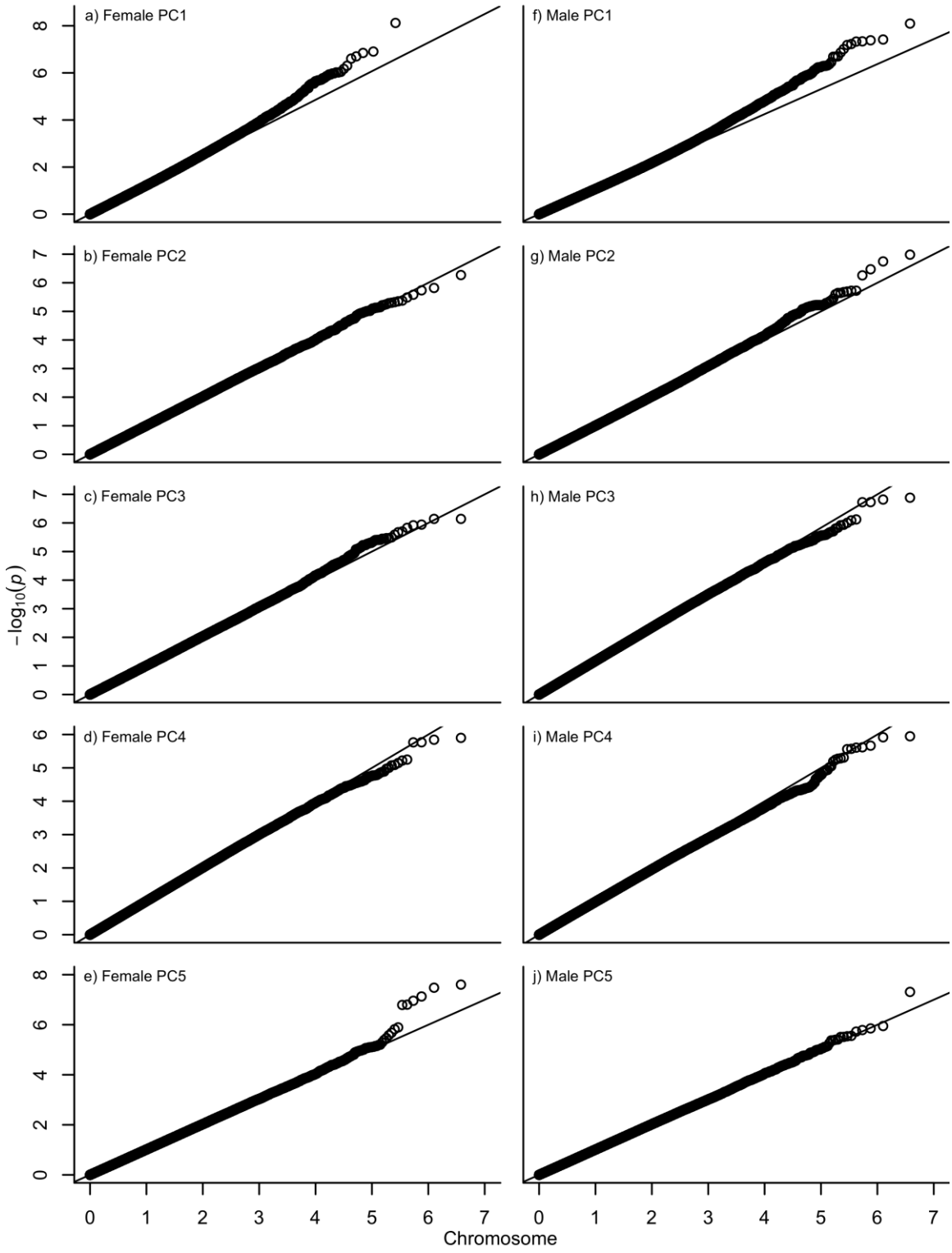
12	Microsatellite	2R	22|61	18.3253(1.86 x10^{-5})
13	Microsatellite	3L	16|61	26.2987(2.92x10^{-7})
14	Microsatellite	2L	31|50	4.4568(0.0347)
15	Microsatellite	X	39|38	0.0130(0.9092)
16	Microsatellite	2L	16|54	20.6286(5.558x10^{-6})
17	Microsatellite	2L	45|36	1.0000(0.3173)
18	Microsatellite	3L	34|37	0.1268(0.7218)
19	SNP	3R	35|44	1.0253(0.3112)
20	SNP	3R	37|44	0.6049(0.4367)
21	Microsatellite	2L	43|35	0.8205(0.365)
22	Microsatellite	2L	32|46	2.5128(0.1129)
23*	Microsatellite	2R	7|56	38.1111(6.68x10^{-10})
24	Microsatellite	3R	26|40	2.9697(0.08483)
25**	Microsatellite	?	23|52	11.2133(8.121x10^{-4})
26	Microsatellite	3L	40|43	0.1084(0.7419)
27	Microsatellite	2R	20|59	19.2532(1.14x10^{-5})
28	DCAPS	2R	40|37	0.1169(0.7324)
29	DCAPS	3L	34|45	1.5316(0.2159)
30	DCAPS	3L	50|31	4.4568(0.03476)
31	DCAPS	2L	36|41	0.3247(0.5688)
32	DCAPS	3L	24|55	12.1646(4.87x10^{-4})
33	DCAPS	2L	25|57	12.4878(4.096x10^{-4})
34	DCAPS	3R	37|43	0.4500(0.50233)
35	EST derived SNP	3R	36|45	1.0000(0.3173)
36	EST derived SNP	3L	40|39	0.0127(0.9104)
37	EST derived SNP	X	40|44	0.1905(0.6625)
38	EST derived SNP	3L	37|47	1.1905(0.2752)
39	EST derived SNP	2L	23|57	14.4500(1.439x10^{-4})
40	EST derived SNP	X	40|42	0.0488(0.8252)
41	EST derived SNP	3L	42|37	0.3165(0.5737)
42	EST derived SNP	3R	28|54	8.2439(0.0041)
43	EST derived SNP	2L	37|41	0.2051(0.6506)
44	EST derived SNP	3R	24|58	14.0976(1.735x10^{-4})
45	EST derived SNP	3R	16|58	23.8378(1.05x10-6)
46	EST derived SNP	2L	25|58	13.1205(2.92x10-4)
47	EST derived SNP	2L	38|41	0.1139(0.73576)
48	EST derived SNP	3L	23|57	14.4500(1.439x10^{-4})
49	EST derived SNP	2R	30|50	5.0000(0.0253)

* Markers were removed from QTL analysis due to extreme segregation distortion. **Dser6 marker from (Magiafoglou et al. 2002)

Appendix 4

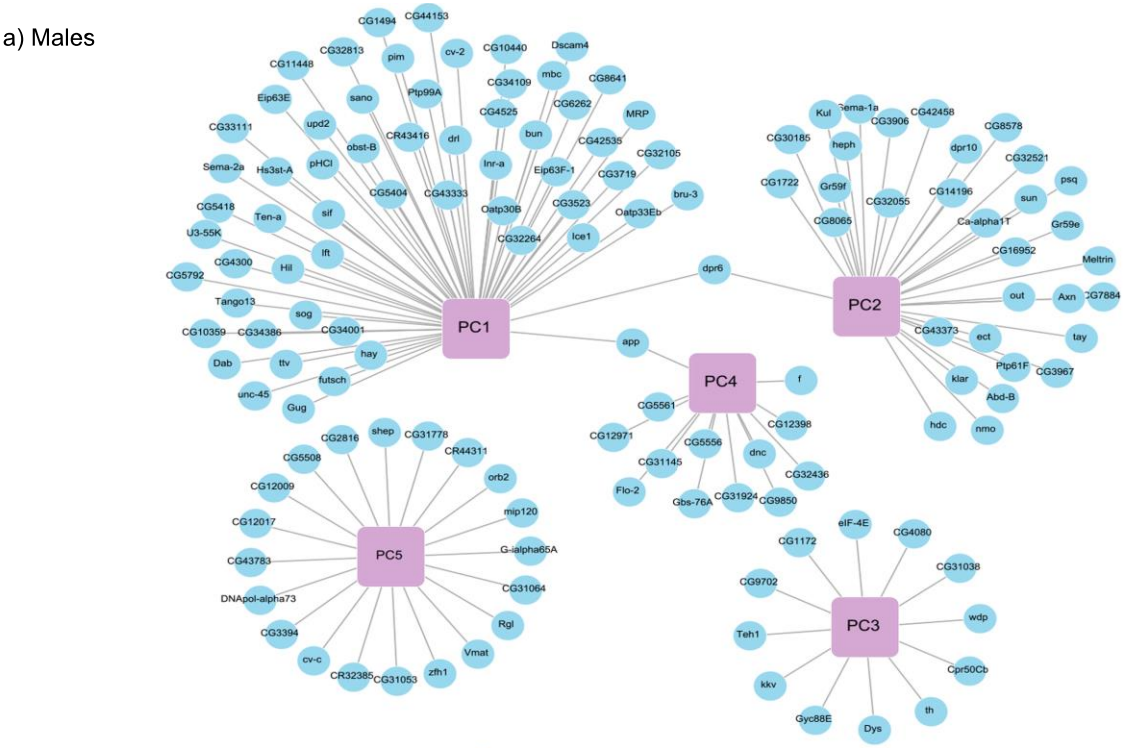


Appendix 4.1: Quantile–quantile plots of the observed P-values against the expected P-values under the null hypothesis that no true associations exist with each of the TPC component for each sex. The straight line is the distribution expected if the observed values equal the expected values. Graph was plotted using the coding from R package qqman.

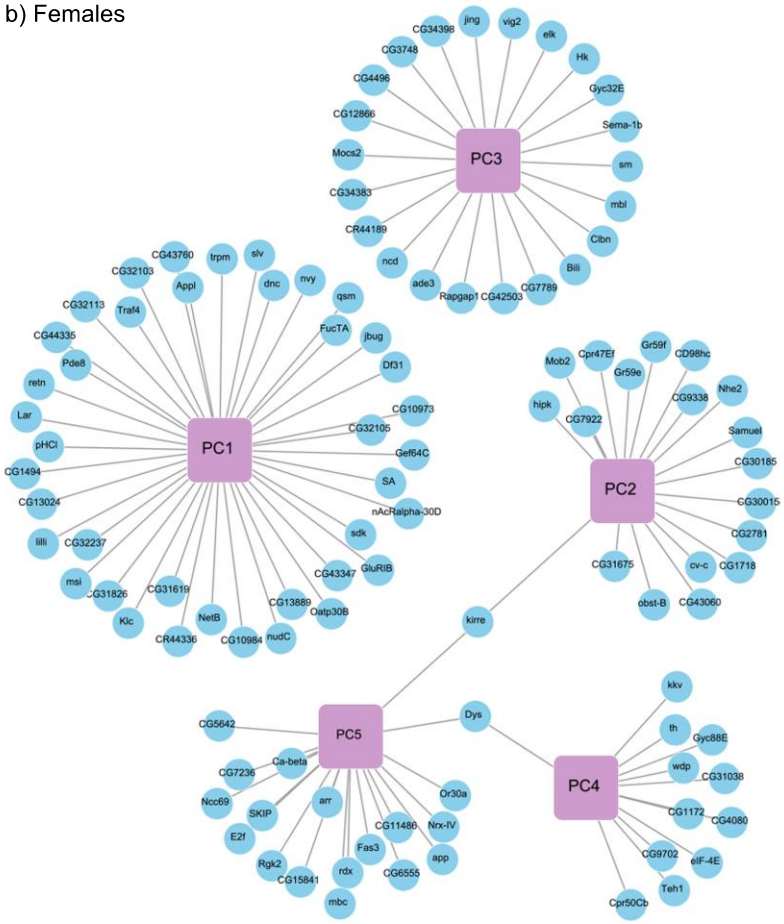


Appendix 4.2: Quantile–quantile plots of the observed P-values against the expected P-values under the null hypothesis that no true associations exist with the first five principal component scores for each sex. The straight line is the distribution expected if the observed values equal the expected values. Graph was plotted using the coding from R package qqman.

a) Males



b) Females



Appendix 4.3: Gene network graph for significant variants from the principal component analysis annotated to a gene for a) males and b) females. Each blue node represents a gene and the purple square nodes represent the first five principal components.