Adaptive gene regulatory polymorphisms in natural populations of *Drosophila melanogaster*

Dissertation zur Erlangung des naturwissenschaftlichen Doktorgrades

"Doctor rerum naturalium" (Dr. rer. nat.)

an der Fakultät für Biologie der Ludwig-Maximilians-Universität München

GRADUATE SCHOOL
LIFE SCIENCE MUNICH

vorgelegt von

Timothy James Shankar Ramnarine

aus

San Fernando, Trinidad, West Indies

München, 2021

Erstgutachter: Prof. Dr. John Parsch

Zweitgutachter: Prof. Dr. Richard Merrill

Tag der Abgabe: 04/11/2021

Tag der mündliche Prüfung: 18/02/2022

Erklärung:

Diese Dissertation wurde im Sinne von § 12 der Promotionsordnung von <u>Prof. Dr. John Parsch</u> betreut. Ich erkläre hiermit, dass die Dissertation nicht einer anderen Prüfungskommission vorgelegt worden ist und dass ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

Eidesstattliche Erklärung:

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt wurde.

Timothy James Shankar Ramnarine,

04/11/2021 München

For my family

Table of Contents

List of abbreviations (alphabetical)

Bari-Jheh: *Bari-Juvenile hormone epoxy hydrolase* (transposon)

bp: base pair

CI: confidence interval

CRE: *cis*-regulatory element

DGRP: *Drosophila* Genetic Reference Panel

eQTL: expression quantitative trait loci

fiz: *fezzik* (gene)

GO: gene ontology

GSTs: *glutathione-S-transferase* genes

HWE: Hardy-Weinberg equilibrium

Indel: insertion/deletion polymorphism

miRNA: microRNA

MSB: menadione sodium bisulfite

MtnA: *Metallothionein A* (gene)

ncRNA: non-coding RNA

PCA: principal component analysis

qRT-PCR: quantitative reverse transcription polymerase chain reaction

RNAi: RNA interference

RNA-Seq: RNA sequencing

ROS: reactive oxygen species

SNP: single nucleotide polymorphism

SNP67: single nucleotide polymorphism at position 67 in enhancer region of *fezzik*

UTR: untranslated region

WGCNA: weighted gene co-expression network analysis

List of Publications

Paper 1

Ramnarine, T.J.S., Glaser-Schmitt, A., Catalán, A., and Parsch, J. (2019). "Population genetic and functional analysis of a *cis*-regulatory polymorphism in the *Drosophila melanogaster Metallothionein A* gene." *Genes*, *10*(2), p.147; https://doi.org/10.3390/genes10020147

Paper 2

Ramnarine, T.J.S., Grath, S., and Parsch, J. (2021; in press). "Natural variation in the transcriptional response of *Drosophila melanogaster* to oxidative stress." *G3: Genes, Genomes, Genetics*; https://doi.org/10.1093/g3journal/jkab366

Paper 3

Glaser-Schmitt, A., Wittmann, M.J., Ramnarine, T.J.S., and Parsch, J. (2021). "Sexual antagonism, temporally fluctuating selection, and variable dominance affect a regulatory polymorphism in *Drosophila melanogaster*." *Molecular Biology and Evolution, 38*(11):4891–4907; https://doi.org/10.1093/molbev/msab215

Declaration of Author's Contribution

In this dissertation, I present work that was realized in collaboration with other scientists during my doctoral research conducted from March 2018 to November 2021. Three publications stemmed from the results of my thesis, which comprise Papers 1 – 3.

Paper 1

Ramnarine, T.J.S., Glaser-Schmitt, A., Catalán, A., and Parsch, J. (2019). "Population genetic and functional analysis of a cis-regulatory polymorphism in the *Drosophila melanogaster Metallothionein A* gene." Genes, 10(2), p.147; https://doi.org/10.3390/genes10020147

John Parsch and I were responsible for conceptualization of the study and its design. In this publication, Amanda Glaser-Schmitt, performed the bioinformatic analysis and visualization of *MtnA* expression and oxidative stress tolerance in *Drosophila* Genetic Reference Panel (DGRP) lines and Ana Catalán, performed and visualized the detection of non-African admixture using sequence data. All other experimental work, statistical analysis and, visualization of results was performed by me: fly stock maintenance and culturing, generation of nearly isogenic lines, genotyping and qRT-PCR primer design, fly dissection, nucleic acid extractions (DNA and RNA), cDNA generation, qRT-PCR assays, and population genotyping of the 3'UTR indel polymorphism of *MtnA* for all wild-caught collections. Writing, editing and reviewing the manuscript was shared by all authors.

Paper 2

Ramnarine, T.J.S., Grath, S., and Parsch, J. (2021). "Natural variation in the transcriptional response of *Drosophila melanogaster* to oxidative stress." *G3: Genes, Genomes, Genetics*; https://doi.org/10.1093/g3journal/jkab366

John Parsch and I were responsible for conceptualization of the study and its design. In this publication, Sonja Grath, performed and visualized the weighted gene co-expression network analysis and John Parsch, performed and visualized the sequence and expression divergence analysis between lines. All other experimental work, statistical analysis and visualization of results was performed by me: fly stock maintenance and culturing, oxidative stress assay development and execution, tissue preparation, RNA extraction, identification of differential gene expression from RNA-Seq data and subsequent analyses including: transcriptomic profiling and comparisons between lines and datasets, gene ontology enrichment analysis and evaluation of microRNA target genes. I prepared the initial draft but all authors participated in editing and reviewing the manuscript.

Paper 3

Glaser-Schmitt, A., Wittmann, M.J., **Ramnarine, T.J.S.**, and Parsch, J. (2021). "Sexual antagonism, temporally fluctuating selection, and variable dominance affect a regulatory polymorphism in *Drosophila melanogaster*." *Molecular Biology and Evolution, 38*(11):4891–4907; https://doi.org/10.1093/molbev/msab215

In this publication our aim was to investigate the elements of population dynamics and phenotypic evolution that are responsible for the maintenance of a derived X-linked SNP in *D. melanogaster*. For this publication I assisted in confirmation of species identity, handling, and species-curation of wild caught *D. melanogaster* biannual collections. For the majority of the collections from 2016-2020, I determined allele frequency of the X-linked regulatory SNP (position 67) in the *fezzik* (*fiz*) gene using DNA extraction and PCR followed by a restriction enzyme-based assay. In collaboration with Amanda Glaser-Schmit, I performed starvation tolerance assays and genotyping on post-mortem assay flies for an interaction between sex, *fiz* genotype, and starvation resistance. I and the other authors contributed our input to the initial draft, which was prepared by Amanda Glaser-Schmitt; all authors participated in editing and reviewing the manuscript.

_____   _____

 Timothy James Shankar Ramnarine   Prof. Dr. John Parsch

List of Figures

List of Tables

Abstract

It has long been recognized that changes in gene regulation, specifically mutations in *cis*-regulatory elements that tend to be stable and additive, are important to adaptive processes and phenotypic evolution. Since *cis*-regulatory elements are found in the vicinity of the genes they regulate, the direct effect of changes in these sequences is typically limited to a particular gene that allows for refined, situation-specific control of gene expression but are not exclusive of downstream or *trans*-acting elements. This dissertation focuses on examining mechanisms responsible for maintaining adaptive *cis*-regulatory polymorphisms in two *Drosophila melanogaster* genes: *fezzik* (*fiz*) and *Metallothionein A* (*MtnA*) and their associated effect on gene expression and organismal phenotype.

Previous experiments show that the 3' untranslated region of *MtnA* contains an insertion/deletion (indel) polymorphism, wherein the deletion is rare or absent in the ancestral African range but its deletion frequency appears to follow a latitudinal cline in derived populations worldwide. By genotyping biannual collections of wild caught *D. melanogaster* across a 5-year period, I show that the deletion is maintained at a high frequency (~90%) in a single German population with no evidence for overdominant, seasonally fluctuating or sexually antagonistic selection. Expression analysis on pairs of nearly-isogenic lines and on data from a North American population indicated significant differences in expression associated to the indel. Furthermore, the data from this North American population showed that expression variation was only partially explained by the deletion and the effect on oxidative stress tolerance was significantly associated with menadione sodium bisulfite and not paraquat. Altogether these findings suggested a scenario in which *MtnA* expression and consequently oxidative stress

tolerance is likely a polygenic adaptation that varies with genomic background. Indeed, the effect of the deletion allele on oxidative stress tolerance was dependent on the genomic background with some indication of sign epistasis. Transcriptomic analysis revealed that *MtnA* expression was induced by oxidative stress independent of the indel status, indicating a general role of this gene in stress tolerance as well as suggesting additional levels of context-dependent expression regulation. The transcriptional response to oxidative stress between lines with and without the deletion was mostly similar but interestingly, there were consistently larger numbers of differentially expressed genes associated with the deletion which is possibly related to regulatory cascades resulting from aberrant microRNA epigenetic regulation due to the loss of microRNA binding sites in the deleted region. In general, the response to MSB indicated the significance of functional categories such as general stress response, oxidative stress response, metabolism, apoptosis and autophagy. In particular among the differentially expressed genes with the largest fold-change in response to MSB-induced oxidative stress were several genes related to glutathione metabolism and biosynthesis, suggesting a strong association between this pathway and oxidative stress tolerance.

Another instance of expression divergence between ancestral and cosmopolitan populations being associated with a regulatory polymorphism is represented by a single nucleotide polymorphism (SNP) located 67 base pairs upstream of the start codon, in the enhancer region of the gene *fezzik*, referred to here as "SNP67". SNP67 has two variants segregating in natural populations of *D. melanogaster*: the ancestral "C" variant, and the derived "G" variant that is found outside of the ancestral range at intermediate frequencies and is associated with increased *fiz* expression. Previous studies suggest that this SNP was a recent target of balancing selection; therefore to determine the forces of selection maintaining this SNP

in cosmopolitan populations we genotyped biannually collected wild-caught *D. melanogaster* from a single European (Munich, Germany) population. A model-based approach using allele and genotype frequency data of the SNP67 variants across seasons and sexes was employed. The model indicated that sexually antagonistic and temporally fluctuating selection may help maintain variation at this site, with the derived variant likely being female-beneficial but there was some uncertainty of dominance estimates in the model. Gene expression and body-size phenotypes that were dependent on genomic background and developmental stage indicated that variable dominance may play a role in the maintenance of this polymorphism. Lastly, we identified a novel sex-dependent association between *fiz* expression and starvation resistance that may suggest that this trait is a potential phenotypic target of selection.

Interestingly our findings for the *MtnA* and *fiz* regulatory polymorphisms both indicated that the relationship between gene expression divergence and population-level genetic mechanisms underlying phenotypic evolution is potentially complicated by context-dependent factors such as genomic background or spatial and temporal differences. By integrating extensive experimental work to identify the mechanisms of selection in natural populations along with functional characterizations, a refined understanding of these adaptive regulatory polymorphisms was achieved.

Objectives

In this dissertation the overall aim is to investigate adaptive regulatory polymorphisms in *D. melanogaster* associated with new environmental conditions and their effect on gene expression and phenotype while also exploring the potential mechanisms responsible for their population dynamics. Specifically, this thesis examines two genes, *Metallothionein A* (*MtnA*) and *fezzik* (*fiz*), initially identified in investigations of expression divergence between European (derived) and sub-Saharan African (ancestral) *D. melanogaster* (Hutter et al. 2008; Catalán et al. 2012). Papers 1 and 2 focus the indel polymorphism in the 3' UTR of *MtnA* in which the deletion allele has been shown to increase in frequency with distance from the Equator and significantly contributes to the observed expression divergence between ancestral and derived populations (Catalán et al. 2012; Catalán et al. 2016). Paper 3 builds on previous data and examines the phenotypic association and plausible selection scenario of a SNP in the gene *fiz*, which is at intermediate frequencies in cosmopolitan populations and has been previously linked to expression variation (Hutter et al. 2008; Saminadin-Peter et al. 2012; Glaser-Schmitt et al. 2013; Glaser-Schmitt and Parsch 2018).

In Paper 1, using a qRT-PCR expression assay on generated paired allelic lines with reduced background variation I provide further evidence that the *MtnA* expression divergence between cosmopolitan and ancestral populations is associated with the *MtnA* 3' UTR indel polymorphism. Combining functional and population genetic analyses, including repeated biannual allele frequency measures of a German population, revealed the complexity of this adaptive process and implicated that *MtnA* is just one of many loci involved in a polygenic adaptation. In Paper 2, I continue the investigative work of Paper 1 on the oxidative stress

tolerance adaptation associated with the *MtnA* indel polymorphism. For this purpose, I employ the usage of tolerance assays and transcriptomic profiling in response to oxidative stress of *D. melanogaster* lines from various genomic backgrounds but with known *MtnA* indel status. Using tolerance assays, I demonstrate that the beneficial effect of the deletion polymorphism on oxidative stress tolerance is dependent on genomic background, while the transcriptomic profiles of these lines allowed for the identification of oxidative stress tolerance candidate genes and processes, some of which are correlated with other stress responses and to the indel polymorphism. I also provide evidence for an indirect effect of the deletion polymorphism on gene regulation due to the loss of microRNA binding sites.

Paper 3 continues the investigation of a previously identified case of a *cis*-regulatory SNP (SNP67) in the gene *fiz* and attempts to discover how selection maintains genetic and phenotypic SNP67 variation in natural populations. To assess potential mechanisms of selection that affect this regulatory element, I genotyped wild-caught *D. melanogaster* across multiple years and seasons from a single population. Using a modeling approach, the genotype and allele frequency data allowed for the determination of relevant forms of balancing selection that maintain variation at this site. I also provide evidence for a potential novel phenotypic target of selection using a starvation resistance assay on *fiz* knockdown strains and individuals from two European genetic backgrounds derived from natural populations to demonstrate the sex-dependent correlation between starvation resistance and *fiz* expression.

General introduction

"The essential quality of life is living; the essential quality of living is change;

change is evolution: and we are a part of it."

- John Wyndham, *The Chrysalids*

Nature imposes a constantly shifting gauntlet of challenges that each species must respond to in order to survive and thrive, this is the process of adaptation and evolution. Starting with Charles Darwin and continuing on through the works of various scientists, naturalists, and great thinkers we have achieved our current understanding of these concepts. The combined driving forces of evolution: genetic drift, mutation, selection, and gene flow, shape the genetic composition of all species. In the event of variation in environmental conditions or when a species expands its range, novel biotic or abiotic conditions impose selection pressure through new fitness challenges that evoke changes in the gene pool. Therefore, generally speaking, adaptation is the dynamic biological process by which organisms adjust and pass on genetic information that allows them to fit to their habitat. Accordingly, by studying species that occupy variable environments or with global spread we are able to investigate the components of adaptation events.

Environmental adaptation and evolutionary history

An adaptation is a characteristic that becomes more common in a population through selection because it confers a competitive advantage by affecting survival and/or reproductive success, usually in response to variations in the environment. There are three main modes of selection: (i) balancing, (ii) positive and, (iii) negative or purifying which can have distinctive effects on population variation. In the case of balancing selection, multiple alleles may be maintained in the gene pool by mechanisms such as heterozygote advantage where the heterozygote has a higher relative fitness than homozygotes consequently preventing fixation of a particular allele in the population. Positive selection promotes the prevalence of heritable genetic changes which increase an organism's chances of surviving and reproducing (Ronald and Akey 2005). These beneficial changes usually give rise to a variety of traits which may, for example, involve tolerance or resistance to selective pressures imposed by new biotic (e.g. disease) or abiotic (e.g. temperature) factors encountered during species range expansion. Positive selection leading to fixation of a variant can leave distinctive signatures in the genome such as a selective sweep which is marked by the reduction in genetic variation among linked variants or nucleotide sequences in close proximity to the beneficial allele (Smith and Haigh 1974; McVean 2007). In contrast, negative or purifying selection refers to the system of maintaining biological systems by the purging of alleles that have harmful or deleterious impacts on fitness of the organism. Therefore the distinction between these two categories often involves the variant of interest or the focus of the study: positive selection is used to describe a situation in which a rare variant that improves optimal fitness is observed to increase in prevalence in a population and the term negative selection is used when the focus is on the removal of harmful variants. It is important to note however that when studying a single locus there can be

confounding effects of natural selection and population demographic histories as the latter can impart similar patterns of DNA sequence variation which complicate detection of selection signatures (Ronald and Akey 2005). However, population demographic history is likely to affect patterns of variation at all loci in the genome whereas selection acts on specific loci, therefore identifying genes that appear as outliers in comparison to numerous loci can assist in the identification of genes that are specifically targets of selection (Biswas and Akey 2006).

The evolutionary trajectories of a population are largely determined by two factors: standing genetic variation and evolutionary history (Barrett and Schluter 2008; O'Donnell et al. 2014). Standing genetic variation in a population is the existence of alternative forms of a gene or genetic polymorphisms, also called alleles, that are mostly neutral. However, some alleles may become beneficial or deleterious to survival in the event of changes in the environment (Orr and Betancourt 2001). Environmental changes that affect fitness favor particular alleles by exerting selection pressure on the standing genetic variation. Empirical evidence supporting the idea that selection on standing genetic variation allows for more rapid adaptation of the population to environmental changes compared to adaptation from new mutations, is limited but accumulating (Bitter et al. 2019; Dayan et al. 2019; Lai et al. 2019). Therefore it follows that adaptive response to environmental change is primarily limited by the supply of mutations, which is related to the effective population size and genetic diversity (Barrett and Schluter 2008; Samani and Bell 2010; Rousselle et al. 2020). In this regard, the evolutionary history of a species can provide context for studying potential adaptive processes by correlating population genetics and functional analyses with demographic history.

Genetic basis of adaptation

To observe the genetic components that may contribute to an adaptation we can investigate genomic alignments for variations in genetic sequences from individuals within a derived population and compare these sequences against similar alignments from the ancestral population. Theoretically, the sequences that are the same between populations may indicate shared ancestry with little time for mutations to occur, or there has been no differential selection to drive differences between populations or that these sequences are conserved probably due to functional importance. On the other hand, genomic regions that are variable may contain different types of structural differences that may affect gene function. Structural differences may involve substitutions (single nucleotide polymorphisms), alteration of a sequence length (insertion/deletion polymorphism), sequence rearrangement (translocation or inversion) or multiplicity (duplication or copy number variant) of a gene or part of it, or multiple genes (Figure 1). In particular, single nucleotide polymorphisms (SNPs) and insertion/deletion (indel) polymorphisms are two of the most common forms of genetic variation in most diploid organisms (Dawson et al. 2001; Mills et al. 2006; Huang et al. 2014). SNPs also serve as markers for evolutionary events and are often used to assess the genetic variation of a particular locus in a population (Schmidt et al. 2008; Kapun et al. 2016). However demographic events may also lead to sequence divergence or changes in allele frequencies in a population, thereby underscoring the importance of the demographic history when interrogating genetic sequences for adaptation.

.
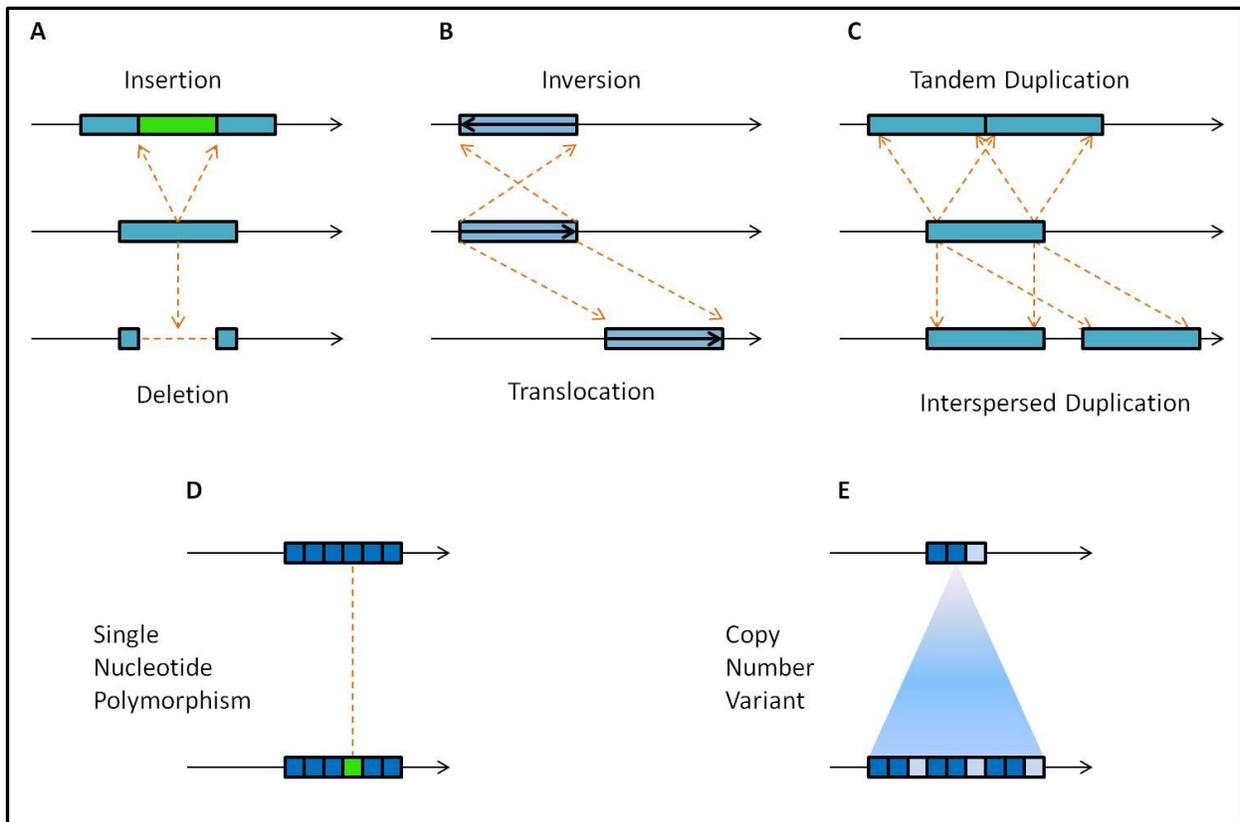
Figure 1. Types of genetic variants: (A) insertion/deletion polymorphism, (B) inversion and translocation, (C) tandem and interspersed duplications (D) Single nucleotide polymorphism and (E) copy number variants, which may be entire repeated genes or partial sequences.

Importance of gene expression regulation in phenotypic evolution

A persistent challenge in evolutionary genetics is not only identifying genetic variants but also determining the role of these variations in specific adaptive traits. The seminal paper by King and Wilson (1975), which described the similarity in protein sequence between chimpanzees and humans, also recognized that the small degree of molecular divergence could not account for the totality of divergence among closely related species. Rather, evolutionary

changes are more likely to be based in the mechanisms controlling gene expression than protein sequences. The rationale employed here is that protein sequence mutations result in greater pleiotropic effects, which increases the chance of deleterious effects on organismal fitness, thus coding sequence variation will be a less common source of adaptive variation than mutations with less widespread effects such as regulatory variation (Stern 2000; Carroll 2005). Heritable gene expression changes are due to the presence of genetic variants in *cis*-regulatory elements (CREs) such as promoters, enhancers, silencers and insulators or *trans*-regulatory elements (e.g. transcription factors or non-coding RNAs), and these elements are not mutually exclusive. Studies have confirmed, however, that morphological differences among closely related species may be due to changes in gene expression patterns involving specifically CREs (Barrier et al. 2001; Wittkopp et al. 2002; Gompel et al. 2005; Loehlin et al. 2019). CREs affect gene expression allele-specifically resulting in reduced pleiotropic effects and are therefore more likely to be fixed within a population over time (Wray 2007; Wittkopp et al. 2008). Furthermore, CREs have been robustly implicated in rapid evolution and development of adaptive phenotypes in variety of organisms, such as plants (Steige et al. 2017; Groen et al. 2020), yeast (Chen et al. 2010; Renganaath et al. 2020), mice (Johnsen et al. 2009; Mack et al. 2018), fish (Santos et al. 2014; Verta and Jones 2018), and *Drosophila* (Wittkopp et al. 2008; Glaser-Schmitt and Parsch 2018; Hsu et al. 2020). Additionally, *cis*-acting factors are mechanistically important for controlled spatial and temporal expression of a specific gene and downstream elements, allowing for refined context-dependent changes in gene expression limited to a particular tissue, life stage or environmental condition, for example (Prud'homme et al. 2007; Wittkopp and Kalay 2012; Berndt et al. 2015; Weasner et al. 2016; Combs and Fraser 2018). Both *cis*- and *trans*-regulatory changes contribute to gene expression divergence between closely related species but *trans*-

acting factors may alter expression levels of numerous transcripts within a gene network thereby increasing the chance of deleterious pleiotropic effects, this may explain why *cis*-regulatory changes are more common and subsequently account for a majority of interspecific expression difference (Wittkopp et al. 2004).

Gene expression regulation and non-coding RNAs

Approximately 82% of the *D. melanogaster* genome is non-coding (Alexander et al. 2010; Milo et al. 2010), through advances in sequencing technology and subsequent investigations, we now realize the significant regulatory role of non-coding genes that have functions as RNAs (ncRNAs) in eukaryotic transcription and without protein translation in a variety of organisms (Carninci et al. 2005; Hon et al. 2017; Fernandes et al. 2019; Dou et al. 2021). Primarily ncRNAs regulate transcription through various epigenetic mechanisms but may also involve splicing, interaction with RNA polymerase II or the initiation complex, for example. Regulatory ncRNAs are classified into two categories by their size: (i) long non-coding RNAs (lncRNAs) that are greater than 200 nucleotides and can be further divided based on their location with respect to protein coding genes and (ii) small non-coding RNAs that are less than 200 nucleotides.

Small ncRNAs such as microRNAs (miRNAs), small interfering RNAs (siRNAs) and P-element-induced wimpy testis-interacting RNAs (piwi-interacting RNAs or piRNAs) are classified typically based on their interaction with Argonaute (Ago) proteins, which are essential components of the RNA-induced silencing complex (RISC). Small non-coding RNAs modulate gene expression by acting as a template for RISC to recognize the complementary mRNA

transcript, and after successful base-pairing the Ago protein activates, leading to either mRNA transcript cleavage or translation inhibition depending on the degree of sequence complementarity (Murphy et al. 2008; Lucas and Raikhel 2013).

In humans, over 2000 miRNAs have been discovered (Griffiths-Jones et al. 2006) with most protein-coding genes being targeted by one or more miRNAs, highlighting the pervasiveness of this particular form of ncRNA regulatory control (Baek et al. 2008; Selbach et al. 2008). miRNAs are defined as a class of endogenous regulatory RNA molecules, 21-24 nucleotides in length and are typically the most abundant small ncRNAs in transcriptomic profiles (Zhang et al. 2019; Isakova et al. 2020). They are transcribed from DNA sequences as primary miRNAs (pri-miRNAs) that are cleaved into precursor miRNAs (pre-miRNAs) and to form a miRNA duplex that contains the mature miRNA and the miRNA passenger strand, which is usually degraded. miRNAs function as guides for RISC by base-pairing with target mRNA to negatively regulate its expression with the degree of base-pairing determining the type of silencing mechanism (Figure 2). As the degree of base-pairing between miRNA and mRNA is critical to the regulatory process, the binding is affected by polymorphisms in the miRNA target site often resulting in the loss of binding sites or the creation of illegitimate binding sites, consequently yielding aberrant gene expression of target genes (Chen and Rajewsky 2006; Saunders et al. 2007; Gao et al. 2009).
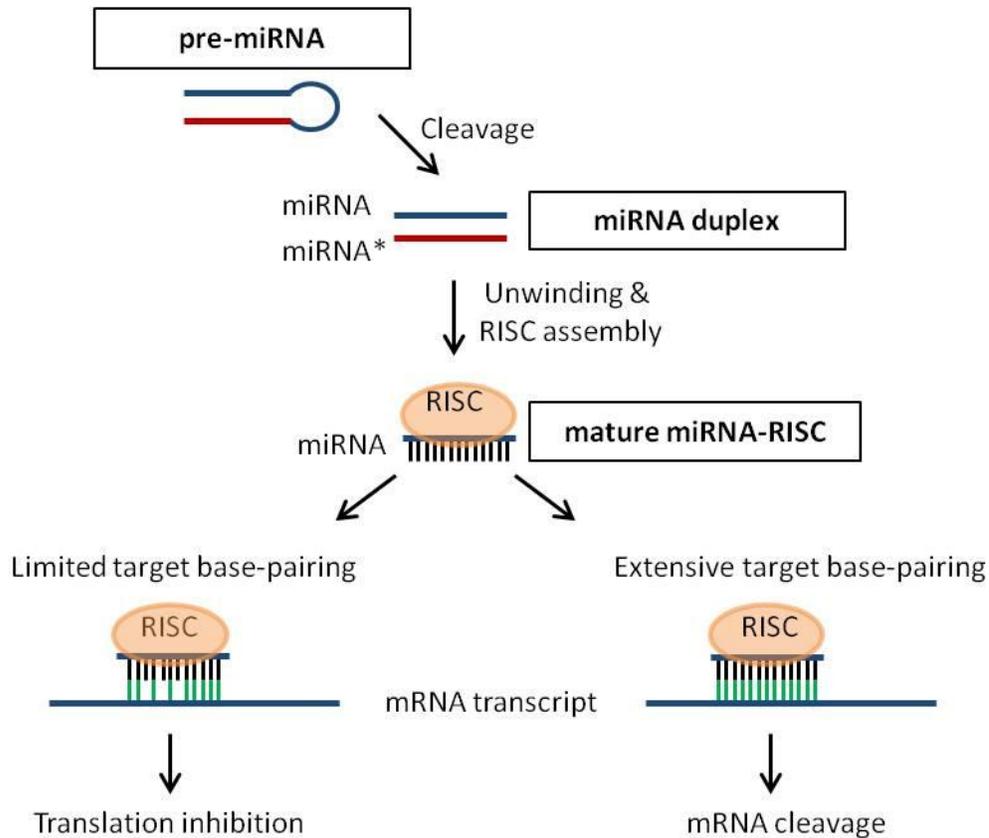
Figure 2. Canonical biogenesis and mechanism of miRNA expression regulation. The mature miRNA duplex is comprised of the guide strand (labelled as "miRNA") and the passenger strand (labelled as "miRNA*"), which is degraded by cellular machinery.

Drosophila melanogaster as a model organism

In terms of experimental feasibility and scalability, *Drosophila melanogaster* is relatively uncomplicated to culture, has a short life cycle and shares extensive genetic similarities to other (more complex) organisms. Moreover, due to its near-global distribution, tractable experimental work across a variety of scientific fields and, supply of genetic information and tools that includes a well-annotated genome, *D. melanogaster* has become a leading model organism for

studying the molecular basis of adaptation (Tamura et al. 2004; Pool et al. 2012; Grenier et al. 2015; Long et al. 2018). Analyses of population genomics indicate that *D. melanogaster* originated in sub-Saharan Africa based on the high level of genetic variation observed in these populations (Ometto et al. 2005; Li and Stephan 2006; Kapopoulou et al. 2018).
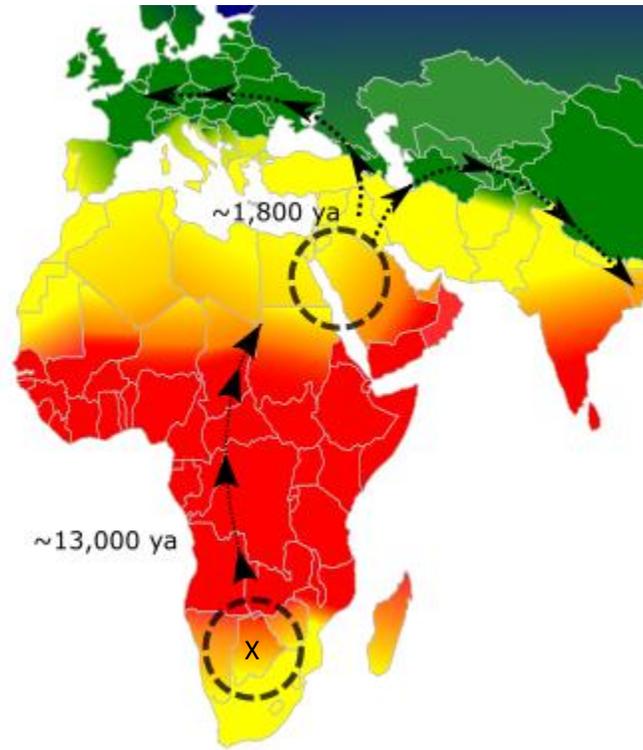
Figure 3. Illustration of demographic history of *D. melanogaster* from Africa to Eurasia. Estimated time in years ago (ya) of species range expansions in *D. melanogaster* from sub-Saharan Africa ("X") to Europe and Asia (Sprengelmeyer et al. 2020). Climatic zones are indicated by color: tropical (red); subtropical (yellow); temperate (green), and polar/subpolar (blue).

A recent study estimates that expansion from the ancestral region began ~13,000 years ago (Figure 3) during a time when the Sahara was becoming less arid (Chevalier and Chase 2015; Sprengelmeyer et al. 2020). Subsequently, persistence of the human-commensal *D. melanogaster* outside of the ancestral range may have been facilitated by the advent of fig cultivation in the Middle East which was estimated to be ~11,000 years ago (Kislev et al. 2006; Sprengelmeyer et al. 2020). More recently, an expansion from the Middle East into Europe and Asia is estimated to have taken place ~1,800 years ago (Figure 3) and colonization of the "new world" occurred within the last ~200 years, (Keller 2007; Duchen et al. 2013; Sprengelmeyer et al. 2020) implying that this species rapidly adapted to new environmental conditions such as seasonality and cold temperatures. The historical biogeography of *D. melanogaster* provides evidence for a strong association between the distribution of this species and human activity from a early time point in this species' history (David and Capy 1988; Lachaise et al. 1988), which may explain instances of admixture in demographic studies of derived populations (Bergland et al. 2016; Mateo et al. 2018; Arguello et al. 2019).

Local adaptation and clinal variation

Different environments select for different genotypes. Given that the demographic history of *D. melanogaster* reveals that this species has colonized a variety of climatic regions, we expect to observe genetic variation in locally adaptive traits. The identification of these traits and the molecular and genetic mechanisms responsible requires in-depth investigational studies that are informed by evolutionary history. Previous studies have compared populations from contrasting environments and/or populations sampled along environmental gradients (clines) to

identify genetic variants and/or gene expression variation associated with spatially or temporally varying selection (Huylmans and Parsch 2014; Reinhardt et al. 2014; Fabian et al. 2015). Subsequently leading to the discovery of polymorphisms linked to a particular environment (Vieira et al. 2000; Lazzaro et al. 2008) and polymorphisms that show allele frequency oscillations indicating an effect of seasonality (Bergland et al. 2014; Machado et al. 2021). These polymorphisms may then be assessed for functional association, such as changes to development, morphology or sensitivity/tolerance to stress (Robinson and Partridge 2001; Pitchers et al. 2013; Rajpurohit et al. 2018). Thermal stress tolerance, for example, has been well-studied and it has been demonstrated to be a trait that is both highly plastic and highly adaptable as gene expression variants may affect thermal tolerance through changes in the dynamic plastic response or anticipatory production of relevant proteins (Sørensen et al. 2001; Sørensen and Loeschcke 2001; Rako et al. 2007). As temperature-based selection increases due to climate change, seasonality or range expansion, the frequencies of heritable differences that exist in the population shift, thereby causing differences in evolutionary trajectories. It follows therefore that the resultant adaptation to thermal stress often follows a latitudinal cline as average temperature ranges decrease with increasing distance from the Equator (Gibert and Huey 2001; Overgaard et al. 2011; Castañeda et al. 2015).

Adaptation to cold and fluctuating temperatures

Insects are, for the most part, considered to be ectotherms, meaning their survival is largely dependent on non-internal physiological sources of heat. Thus, thermal stress is likely to have been and continues to be a major selective determinant of survival and persistence affecting

the evolution and development of most insect species (Chen et al. 2015; Tobler et al. 2015; Lecheta et al. 2020). Temperature can also vary on a daily, seasonal, or spatial scale, thus temperature fluctuations can affect fitness components linked to various physiological and metabolic stress responses (Mitchell and Hoffmann 2010; Williams et al. 2014; Sørensen et al. 2016) such as the generation of reactive oxygen species (ROS), which then leads to oxidative damage (Lalouette et al. 2011; Doelling et al. 2014; Dampc et al. 2020). Given the worldwide spread of *D. melanogaster*, the environmental differences between the tropical ancestral habitat and more recently colonized habitats in the evolutionary history of this species, we may consider the traits related to dealing with temperature and by extension, oxidative stress, to be crucial to range expansion.

The genetic architecture of the regulation of temperature-dependent traits is exceedingly complex. For example, a study showed that in response to cold acclimation nearly one third of the transcriptome and half of the metabolome was differentially regulated (MacMillan et al. 2016). The response also seems to be stage-specific and also variable depending on the methodology of the experiment as some studies demonstrate minimal differential gene overlap between responses to cold shock and chill coma recovery (Teets and Hahn 2018) and no overlap between the genes associated to cold hardiness across the metamorphic boundary (Freda et al. 2017). Furthermore, there have been studies that have identified CREs involved in various phenotypic traits that show clinal variation with temperature in global populations and using the *Drosophila* Genetic Reference Panel (DGRP), a set of fully sequenced inbred lines derived from a natural population from Raleigh, North Carolina, USA (Mackay et al. 2012; Lavington et al. 2014; Juneja et al. 2016; Akhund-Zade et al. 2017). Additionally, in *Drosophila*, genome wide association studies (GWAS) and other quantitative genetic approaches have implicated more

specific but still widely varying biological processes that are highly polygenic and may play a role in temperature-based adaptations such as organ development (Božičević et al. 2016), reproductive behavior (Hsu et al. 2020), and metabolism (Mallard et al. 2018; Barghi et al. 2020). However, due to the complexity of polygenic traits additional studies focused on integrating genomic information with different levels of analysis such as functional characterization, epigenomics, transcriptomics and proteomics are required to provide a greater understanding of polygenic traits.

Paper 1

Population genetic and functional analysis of a *cis*-regulatory polymorphism in the *Drosophila melanogaster Metallothionein A* gene

Timothy J. S. Ramnarine, Amanda Glaser-Schmitt, Ana Catalán, and John Parsch

Paper 2

Natural variation in the transcriptional response of *Drosophila melanogaster* to oxidative stress

Timothy J.S. Ramnarine, Sonja Grath and John Parsch

Paper 3

Sexual antagonism, temporally fluctuating selection, and variable dominance affect a regulatory polymorphism in *Drosophila melanogaster*

Amanda Glaser-Schmitt, Meike J. Wittmann, Timothy J.S. Ramnarine and John Parsch

General Discussion

Advances in the fields of genomics and genetics have revealed that sequence variation that alters the expression of genes strongly contributes to inter- and intra-species differences and adaptive evolution in general. By first identifying instances of regulatory divergence and combining investigations on the effect of this divergence on organismal phenotype with population genetics, discoveries of specific gene function can be made while also providing insight into environmental selection mechanisms. In particular, *cis*-regulatory changes directly affect the expression of a gene but may do so in various ways, such as the disruption or enhancement of transcription factor binding or post-transcriptional modification. Furthermore, *cis*-regulatory mutations may result in dynamic, pleiotropic effects through interactions with downstream elements and *trans*-acting factors, possibly making each instance of *cis*-regulatory divergence unique (Genissel et al. 2008). Therefore studying a single *cis*-polymorphism requires an in-depth evaluation of a plethora of molecular interactions but ultimately leads to a better understanding of the mechanistic pathways and constituent candidate genes involved in adaptive traits. Many fundamental insights into regulatory polymorphisms and their impact on phenotypic evolution are based on whole organisms or focus on adult stages (Buchberger et al. 2019). However a growing body of evidence has demonstrated that the evolution of gene expression and gene regulation is situation-specific wherein the phenotypic impacts of a genetic change are affected by factors such as sex, genomic background, cell/tissue-specificity, developmental stage and, stress imposed by heterogeneous environments (Waskar et al. 2009; Kalay and Wittkopp 2010; Plaisier et al. 2014; Jardine et al. 2021). In addition to regulatory elements at the transcriptional level these context-dependent effects may be facilitated and influenced by the regulatory machinery at the pre-transcriptional genome organisation level and post-

transcriptional level attributable to RNA modification and RNA regulatory molecules (Buchberger et al. 2019).

This thesis focuses on the adaptive regulatory role and phenotypic evolution of an indel polymorphism in the 3' UTR of the gene *MtnA* and a SNP located in the enhancer region of the gene *fiz* in natural populations of *D. melanogaster*. By combining population genetics approaches and functional analyses we further our understanding of the phenotypic traits influenced by these polymorphisms and how selection maintains these polymorphisms in natural populations. In Papers 1 and 2 we demonstrated that the *MtnA* indel is likely part of a polygenic adaptation related to temperature-dependent oxidative stress, which involves compensatory metabolic modifications with some evidence for sign epistasis. Furthermore, we provide preliminary evidence that the *MtnA* indel may indirectly affect the regulation of multiple functional pathways through an epigenetic mechanism. In Paper 3 we identified sexual antagonism, temporal fluctuation and variable dominance as the mechanisms of selection potentially acting on SNP67 and provide empirical evidence for the context-dependent correlation between SNP67 and starvation resistance.

Mechanisms of selection

In Paper 1, an evaluation of genotype and allele frequencies of the *MtnA* 3' UTR indel polymorphism across multiple seasons for the period of 2016-2017 revealed that the deletion frequency (0.91) was not significantly different than the estimated frequency from genotyping individual flies from isofemale lines (0.91) reported by Catalán et al. (2016) and showed no evidence for sexual antagonism, seasonal fluctuation or heterozygote advantage. Repeated measures of this population for the period 2018-2020 indicated a similar frequency (0.90) but still did not show any evidence for the aforementioned mechanisms of selection (Table 1 and 2). The minor shift in deletion frequency over this five year period is mostly due to the June 2020 collection, which had an uncharacteristically low deletion frequency, and consequently showed the only significant instance of a seasonal difference when compared to September 2020 (Fisher's exact test, $P = 0.01$).

Table 1. Frequency of the *MtnA* 3' UTR deletion across seasons and sexes in Munich wild-caught *D. melanogaster* for the years 2018-2020; N = number of chromosomes, Freq = frequency of the *MtnA* 3'UTR deletion.

Collection	N $_{female}$	Freq $_{female}$ (95% CI)	N $_{male}$	Freq $_{male}$ (95% CI)
June 2018	134	0.948 (0.895-0.979)	78	0.910 (0.824-0.963)
Sept 2018	144	0.917 (0.874-0.966)	44	0.955 (0.845-0.994)
June 2019	212	0.915 (0.869-0.949)	138	0.942 (0.889-0.975)
Sept 2019	264	0.890 (0.846-0.925)	60	0.900 (0.795-0.962)
June 2020	206	0.806 (0.745-0.858)	90	0.756 (0.654-0.840)
Sept 2020	172	0.890 (0.833-0.932)	128	0.844 (0.769-0.902)

Table 2. Genotype counts of the *MtnA* 3' UTR indel polymorphism in Munich wild-caught *D. melanogaster* for the years 2018-2020; Del = deletion allele; Non = non-deletion allele; P_{HWE}= *P*-value of a chi-square test (observed vs. expected) of Hardy-Weinberg equilibrium.

Collection	Del/Del	Non/Non	Del/Non	P_{HWE}
June 2018	92	0	14	0.767
Sept 2018	81	1	12	0.774
June 2019	150	1	24	0.999
Sept 2019	127	0	35	0.305
June 2020	97	11	40	0.082
Sept 2020	113	2	35	0.928

Thus the deletion frequency of this population has remained high (~90%) without going to fixation and mostly consistent for ~15 years. The additional measurements included here would suggest that there is likely little effect from genetic drift and that *MtnA* is being subjected to a more complex form of selection. Functional analyses presented in Papers 1 and 2: expression divergence and oxidative stress tolerance, lends support to the idea that these traits are influenced by variation at multiple loci. Meaning, the indel polymorphism was found to only partially account for expression divergence and the magnitude of the effect of the indel polymorphism on both *MtnA* expression and oxidative stress tolerance was found to be background-dependent. Altogether these findings suggest a scenario in which the *MtnA* indel polymorphism is part of a polygenic adaptation in which multiple genetic variants influence a selected trait. Contrastingly, as discussed in Paper 3, the SNP67 polymorphism in *fiz* is unlikely to be maintained by

interactions with other loci because non-parallelism between populations, one of the two criteria of this type of adaptive architecture is absent (Gnad and Parsch 2006; Graze et al. 2014; Barghi et al. 2020). SNP67, which has been maintained in a polymorphic state with the "G" variant at intermediate frequencies in Europe for multiple decades, has a much larger effect on expression divergence between ancestral and cosmopolitan populations in adults and a majority of the expression divergence in larvae in comparison to two other SNPs in the *fiz* enhancer region, at position 1063 and 1147 that showed signatures of a selective sweep (Glaser-Schmitt et al. 2013; Glaser-Schmitt and Parsch 2018). Previous work has proposed that this may be explained by fixation occurring more quickly with advantageous regulatory mutations that have small effects, whereas the fitness optimum may be exceeded by mutations with larger effect leading to maintenance of a polymorphic state by balancing selection (Sellis et al. 2011). In Paper 3, an analysis involving a modelling approach using genotype and allele frequencies of biannually collected wild-caught *D. melanogaster* over a period of five years, revealed that variation in SNP67 in *fezzik* is maintained by a combination of sexual antagonism and temporally varying selection and potentially, spatial variation in dominance that may be dependent on genomic background. More specifically, from the empirical data the "G" variant of SNP67 appears to be more often beneficial in females and mostly recessive, but dominance switching may be an important component shaping allele frequency dynamics at position 67. Sexually antagonistic selection is thought to be of great importance in the maintenance of polymorphisms in sexually dimorphic species and, by decoupling male and female fitness, mutations that can be attributed to sex-specific patterns of selection or sex-by-genotype interactions may be revealed (Connallon and Clark 2014). The classic view of evolutionary theory predicts that the X chromosome should favor female beneficial variants more than male-beneficial variants because the X chromosome

spends twice as much evolutionary time in females as in males (Frank and Crespi 2011; Gardner and Úbeda 2017). More recent mathematical and empirical research suggest that the X chromosome may favor male-beneficial alleles more than alleles at autosomal loci (Patten 2019; Frank and Patten 2020). These seemingly contrasting ideas may be resolved when we consider the inclusive-fitness interests of a single gene rather than a whole genotype (Hitchcock and Gardner 2020). In other words, the fitness components of a gene are modulated by the biological context.

Based on the maintenance of genetic variation in cosmopolitan populations and rarity in the ancestral range, it is possible to infer that both of these polymorphisms represent separate examples of environmental adaptations that are conditionally favored in cosmopolitan populations by different mechanisms of selection (Table 3). Alternatively, though difficult to distinguish, it may be that repeated migration events contribute to the allele frequency. However, to correlate genetic variation with variable selection patterns involves the identification of the affected organismal trait(s), which can also prove particularly difficult in polygenic adaptations.

Table 3. Summary of the current population genetics view of the two regulatory variants investigated in this thesis. [a]expression change of derived allele relative to ancestral allele; [b]average derived allele frequency from biannual (June and September) collections of wild-caught *D. melanogaster* from a population in Munich, Germany during the years 2016-2020.

Gene name	*Metallothionein A*	*fezzik*
Chromosome	3R (autosomal)	X (sex)
Regulatory polymorphism	3' UTR indel	Enhancer region SNP
Derived allele	Deletion	G
Ancestral allele	Non-deletion	C
Expression (derived/ancestral)[a]	Increase	Increase
Derived allele prevalence[b]	High (~90%)	Intermediate (~43%)
Type of selection/context-dependence detected	Complex; likely polygenic Genomic background Epigenetic interactions	Sexual antagonism Temporal fluctuation Variable dominance Developmental stage Genomic background

Relating sequence variation with organismal phenotype

The difficulty in mapping genotype to phenotype can be attributed to a number of factors, including a lack of phenotypic descriptions, a scarcity of genotype data, the underlying intricacy of the networks that control cellular processes, and pleiotropic effects. Furthermore, phenotypic alterations are more often due to the degree, rather than presence or absence of a trait (Wittkopp et al. 2008; Gibert et al. 2016; Ramirez-Corona et al. 2021) implying that single-gene knockdown experiments, for example, can provide useful but limited evidence of gene function and network interactivity. Recent technological advancements in the acquisition of genome-wide data and the statistical power to correlate quantitative trait loci with the variation of the trait, promise to improve predictions of genotype to phenotype associations. However, robust functional analyses in controlled settings still need to be performed in tandem with sequence analysis to confirm and refine the association of adaptive regulatory changes with expression divergence and subsequently the affected trait(s).

Regarding the indel polymorphism in *MtnA*, the deletion's position in the 3' UTR suggests that its effect on expression is most likely post-transcriptional, possibly through the deletion of microRNA binding sites (Catalán et al. 2016). Metallothioneins show expression in a variety of tissues and are known to be involved in heavy metal homeostasis and tolerance and protection against oxidative stress (Egli et al. 2006; Chintapalli et al. 2007; Atanesyan et al. 2011; Gaudet et al. 2011; Catalán et al. 2016; Luo et al. 2020). In Paper 1, expression was primarily quantified through qRT-PCR, while functional associations were investigated in Paper 2 through transcriptomic profiling by RNA-Seq and an improved tolerance assay based on the findings using DGRP data in Paper 1. In both Paper 1 and 2, experimental work was performed on multiple natural populations with and without reduced background variation to further define

the functional role of this adaptive regulatory polymorphism. In these efforts, the technique used to generate nearly-isogenic lines was effective in minimizing the contribution of other background variants to strengthen the case of expression divergence due to the indel polymorphism. However, the variable effect of the deletion on oxidative stress tolerance wherein the deletion was beneficial in German, lines but not significant (or even deleterious), in others, provides evidence for fitness-related sign epistasis. In other words, interactions between the indel polymorphism and other loci may have negative or positive effects on phenotype that are dependent on the genomic background and the environment in which certain combinations occur and, consequently, constrain fixation in certain genetic backgrounds (Weinreich et al. 2005; Hoekstra et al. 2013; Nghe et al. 2018). This may partially explain the clinal trend of the deletion allele in global populations reported by Catalán et al. (2016) and the stable deletion frequency measured in our five year collection from Munich, Germany. Interestingly, increased oxidative stress tolerance showed substrate-specificity in the analysis of DGRP data that may suggest that the molecular pathways affected by *MtnA* expression and the indel are affected by the mode of chemical action of MSB. Indeed previous studies in mice (Bauman et al. 1991; Sato and Bremner 1993) and yeast (Liu and Thiele 1996; Kim et al. 2011) have demonstrated that metallothioneins are capable of free-radical scavenging activity, and induction of metallothionein synthesis along with other antioxidant enzymes are essential to protecting against cellular damage through menadione-mediated oxidative damage.

The causal SNPs in the *fiz* enhancer are in close proximity to the transcription start site, thus they are likely to affect expression by influencing transcription factor binding and consequently transcription initiation or rate (Glaser-Schmitt and Parsch 2018). In general *fiz* expression is high in both sexes but shows male-biased expression in adult somatic tissues,

specifically the Malpighian tubule and head (Gnad and Parsch 2006; Huylmans and Parsch 2014; Newell et al. 2016; Leader et al. 2018). It is likely that this sex-biased expression is due to gene-specific regulation rather than dosage compensation, as *fiz* is not located within close proximity to any dosage compensation component binding site (Bachtrog et al. 2010; Straub et al. 2013; Huylmans and Parsch 2015). Furthermore, in previous publications, *fiz* has been shown to have diverse functional roles in oxidoreductase activity, ecdysone metabolism, larval growth, and body size (Iida et al. 2007; Gaudet et al. 2011; Glaser-Schmitt and Parsch 2018) with expression divergence being associated with insecticide resistance and cold tolerance (Glaser-Schmitt and Parsch 2018) and, specifically in Paper 3, a novel sex-dependent, female beneficial association to starvation resistance. In these assays we were only able to detect a significant effect of *fiz* expression on starvation resistance when expression was knocked down in males and increased from an already high level in females. We proposed that, native *fiz* expression and the effect on starvation resistance is dependent on relative fitness optima for each sex. It should be noted that starvation resistance is not technically classified as a life-history characteristic, but it is an essential component of fitness since it aids survival (Flatt 2020). Taking into consideration the diverse mechanisms that are affected by *fiz* expression, in order to draw definitive conclusions about the adaptive role of SNP67 in natural environments would require further investigations of the correlation between starvation resistance and other affected fitness components.

Conserved transcriptional response to oxidative stress

The resulting gene ontology terms associated to MSB-induced oxidative stress implicated several metabolic processes as being relevant to oxidative stress tolerance but specifically there was up-regulation of genes related to glutathione. This may be partially due to chemical action of

MSB, which has been shown to be affect the expression of glutathione metabolic genes directly (Chang et al. 1992; Kavitha and Chandra 2014; Thomas et al. 2016), but it is also likely that this is part of an important mechanistic consequence in protecting the organism against oxidative challenge. For example, oxidative stress may cause a release of zinc ions that regulates metallothionein and glutathione expression that cooperatively work to scavenge reactive oxygen species (Maret 1994; Jiang et al. 1998; Ruttkay-Nedecky et al. 2013). Further support for the involvement and relevance of metallothioneins may be extrapolated from our dataset, which shows the strong induction of three metallothionein genes (*MtnA*, *MtnD* and, *MtnE*) in response to oxidative stress.

Additionally, in response to oxidative stress we observed the categories of general stress response, proteolysis, apoptosis, and autophagy that consist of significantly differentially expressed genes and/or represented by strongly connected nodes in co-expression pathways. The majority of these genes are not known for their direct association to oxidative stress but may be a consequence of oxidative challenge and cytotoxicity resulting in DNA/protein damage and subsequent removal of damaged cellular components (Simonsen et al. 2008; McClung et al. 2010; Pickering et al. 2013; Reynolds-Peterson et al. 2020). Interestingly, we also observe a small group of genes related to animal organ development that show a divergent expression pattern compared to similar genes under cold stress (MacMillan et al. 2016). This is likely due to the metabolic signalling properties of reactive oxygen species (Owusu-Ansah and Banerjee 2009; Landis et al. 2012; Engelhart et al. 2020) and therefore represents a potential stress-specific response on developmental phenotype.

Environmental adaptation in *Drosophila melanogaster*

Expansion of *D. melanogaster* out of Africa into global climates exposed this species to a variety of new biotic and abiotic stresses. During this expansion, adaptations to colder temperatures and temperature ranges likely played a significant role, as *D. melanogaster* is a chill-susceptible species. Sustained low temperatures cause water and ion loss in adult flies, leading to cell membrane disruption, cell death, tissue damage, deficient physical performance and mortality (Yi et al. 2007; MacMillan et al. 2015b). Thus, at the biochemical level, evolved differences in cold tolerance often relate to modifications to energy and metabolite consumption and processing (MacMillan et al. 2015a). Furthermore, adaptations to cold stress have been linked to a variety of organismal phenotypes such as alterations to metabolic processes and development pathways and in some instances ROS detoxification and starvation resistance (Lalouette et al. 2011; MacMillan et al. 2016; Glaser-Schmitt and Parsch 2018; Pathak et al. 2018).

The results of the transcriptomic analysis in Paper 2 displayed a strong similarity to a transcriptomic dataset involving cold stress reported by von Heckel et al. (2016) and emphasize the importance of metabolic processes, particularly genes related to glutathione metabolism, to both stress responses. In a study by MacMillan et al. (2016), cold acclimated *D. melanogaster* were shown to be under increased oxidative challenge compared to control conditions, with the strongest functional associations between cold tolerance and metabolism of glutathione and proline. Glutathione metabolism and in particular, *glutathione-S-transferase* (*GST*) genes were suggested to be important in avoiding or repairing oxidative damage at low temperatures (MacMillan et al. 2016). On the other hand, proline metabolism seems to be specific to the experimental measure of cold tolerance employed as there was divergent alterations for cold

acclimation and chill coma recovery time (CCRT), which is the time taken for adults to become active again after being knocked down by exposure to near-freezing temperatures (MacMillan et al. 2016). Mutations in metabolite allocation or in metabolism-related genes in general are likely to incorporate trade-offs in neighboring pathways but may be important in responses that maintain fitness components across heterogeneous environments (Marden et al. 2003; Hoffmann et al. 2005; Mockett and Sohal 2006; MacMillan et al. 2016; Buchanan et al. 2018). Therefore genetic variants that impact metabolism may be mechanistically affected by selection acting upon the affected pleiotropic traits. In turn, the benefit or hindrance to survival that these variants provide varies in relation to other loci in genomic backgrounds and according to the conditional effects of selection that change in a spatial or temporal context.

An additional example of an environmental adaptation involving oxidative stress is represented by the insertion of a transposable element into the intergenic region of *Juvenile hormone epoxide hydrolase* (*Jheh*) genes, referred to as the *Bari-Jheh* transposon, which shows evidence for a partial selective sweep in non-African *D. melanogaster* (González et al. 2009), as it is found more frequently in populations outside of the ancestral range. The *Bari-Jheh* transposon is attributed with adding extra antioxidant response elements upstream of *Jheh1* and *Jheh2* genes leading to up-regulation of these genes and increased oxidative stress tolerance (Guio et al. 2014). Thus it is likely that oxidative stress is a determinant of environmental adaptive processes, however it is important to note that the specific relationship between oxidative stress and the causal environmental factor may not be obvious, as ROS may be introduced by a number of biotic and abiotic factors such as UV light, radiation, infection or exposure to chemicals or toxins (Landis et al. 2012; Wu et al. 2012; Ng et al. 2017). Therefore, an understanding of the relationship between oxidative stress tolerance and all latitudinally and

locally changing environmental sources of selection, as well as important morphological features and molecular pathways, is critical for understanding and drawing accurate conclusions about the adaptation process.

Previous work has suggested that the increase in *fiz* expression that results in reduced larval growth rate, subsequently smaller adult body size, reduced wing loading and improved flight dynamics in colder temperatures, is potentially related to energy or metabolic conservation in response to temperate climate colonization, where temperature range and seasonality represent strong selective forces (Frazier et al. 2008; Glaser-Schmitt and Parsch 2018). Furthermore, *fiz* expression was also found to be positively associated with cold tolerance, but in females only (Glaser-Schmitt and Parsch 2018). In Paper 3, the elucidation of a novel functional correlation between *fiz* expression and genotype to starvation resistance, which also occurs in a sex-dependent manner, seems to match the proposed pleiotropic profile of an ecologically varying, temperature-based adaptation that affects development and metabolism (Mensch et al. 2008; Glaser-Schmitt et al. 2013; Hoekstra et al. 2013; Glaser-Schmitt and Parsch 2018). Previous work in experimental evolution has also shown that developmental timing and body size are correlated with starvation resistance, specifically implicating hormone signalling and metabolic processing as important pathways (Hardy et al. 2018; Kawecki et al. 2021). Thus, a possible way of relating *fiz* expression to organismal phenotype may involve the action of the steroid hormone ecdysone, a central regulator of insect developmental transitions (Takeuchi et al. 2005; Iida et al. 2007; Gaudet et al. 2011). Normally, ecdysone antagonizes insulin signaling and suppresses larval growth rate but does not alter developmental time (Caldwell et al. 2005; Colombani et al. 2005). As part of a previous study with *fiz* knockdown, developmental timing was not affected but larval growth rate increased (Glaser-Schmitt and Parsch 2018). Furthermore, temperature,

along with other environmental cues, has been associated to other polymorphisms that may affect insulin signaling or the interaction between insulin and the steroid hormone ecdysone (Robinson and Partridge 2001; Fabian et al. 2012; Paaby et al. 2014; Durmaz et al. 2019) thereby emphasizing the importance of these hormones as major mediators of life-history adaptations.

Epigenetic regulation by indel-associated miRNAs in response to oxidative stress

In Paper 2, we hypothesized that the mechanism by which the *MtnA* 3' UTR deletion affects expression: post-transcriptional modification through the action of miRNAs, may also produce a consistent transcriptomic effect across genomic backgrounds (Chen and Rajewsky 2006; Catalán et al. 2016). Therefore, the transcriptomic profile was interrogated for the paired allelic lines in each background, excluding the Zambian lines, which were all homozygous for the non-deletion allele. These pairwise comparisons showed that the response to oxidative stress in deletion and non-deletion lines was mainly similar but a consistently larger number of genes was differentially expressed in deletion lines. Furthermore, the target genes of the miRNAs predicted to bind within *MtnA* 3' UTR deleted region show greater down-regulation under stress in comparison to non-deletion lines, which support possible aberrant effects on expression from miRNAs (Figure 4).
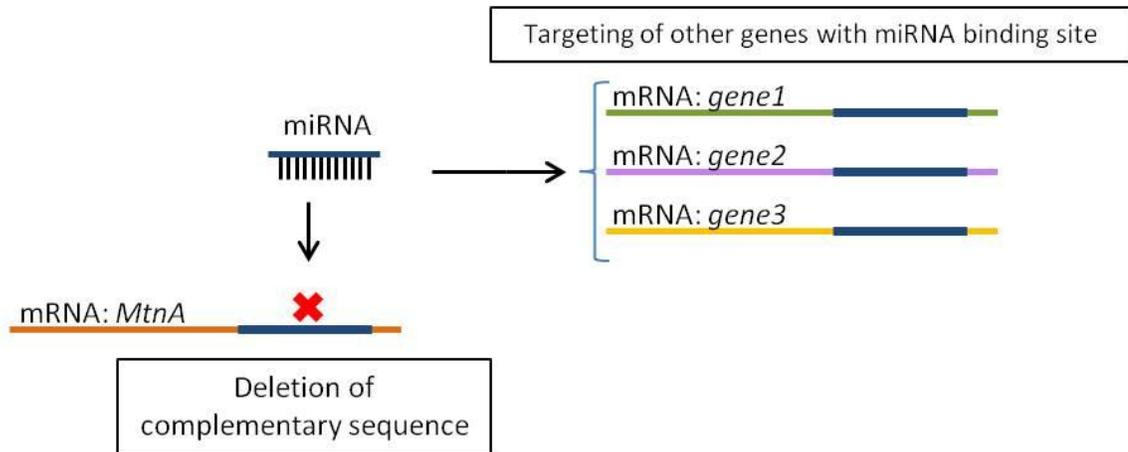
Figure 4. Schematic representation of the effect of the indel polymorphism in the *MtnA* 3' UTR on post-transcriptional modification of miRNA target genes.

Several of these miRNA target genes are implicated in regulatory processes but specifically three of these genes were linked to oxidative stress response. Among these genes is *alphabet* (*alph*) that functions as a negative regulator of various components of stress-activated protein kinase (SAPK) pathways (Baril et al. 2009; Gaudet et al. 2011; Ashton-Beaucage et al. 2014). In particular, *alph* inactivation has been directly implicated in increases to oxidative stress tolerance and life span. Furthermore *alph* is involved in the negative regulation of the c-Jun N-terminal kinase (JNK) cascade that functions in the control of various cellular processes, including proliferation, development, metabolism, immune responses, and apoptosis (Agnes et al. 1999; Jasper et al. 2001; Wang et al. 2003; Delaney et al. 2006; Pinal et al. 2018). The other two genes are *period* (*per*) and *Adar* (*Adenosine deaminase acting on RNA*). In addition to affecting oxidative stress tolerance, divergent expression of these genes has been linked to changes to metabolic processes, behavior and lifespan (Gaudet et al. 2011). Therefore, the disparity in the number of differentially expressed genes between deletion and non-deletion lines

may be due to regulatory cascades affected by the miRNA-associated down-regulation of particular genes. It should be noted, however, that miRNA target predictions are still subject to a high false positive rate but are being improved by functional studies in *Drosophila* and other organisms. These studies are aimed at defining the biological roles of miRNAs as well as the effect of target gene polymorphisms on miRNA regulatory pathways (Saunders et al. 2007; Liu et al. 2014; Agarwal et al. 2018) thereby reducing the reliance on computational methods for miRNA target prediction.

In humans, polymorphisms such as SNPs and indels that alter complementarity between the mRNA target and miRNA have been linked to disease pathologies and variation in physiological and behavioral phenotypes (Chang and Mendell 2007; Moszyńska et al. 2017; Rivera-Barahona et al. 2017). However the interaction between miRNAs and their targets is affected by more than sequence complementarity, as it has been shown that the repressive effects of miRNAs are modulated by stress (Ashraf et al. 2006; Bhattacharyya et al. 2006; Schratt et al. 2006) and miRNAs may regulate other non-coding RNAs including their own transcripts (Zhao et al. 2008), which may imply that miRNAs interact with their targets in a reversible manner. Overall, the depth and application of knowledge within the field non-coding RNAs and the mechanisms of expression regulation on gene networks is currently limited, thus necessitating future experimental work that demonstrates these interactions and the proposed effects mentioned above.

Final Remarks

This thesis continues the investigation of the roles of *cis*-regulatory polymorphisms in two genes, *MtnA* and *fiz*, as examples of environmental adaptations that potentially affect organismal phenotype and survival through metabolic processes.

In the case of *MtnA*, it appears that the indel polymorphism in derived populations is a result of selection on an ecological factor related to oxidative stress tolerance, or more specifically, metal ion homeostasis and glutathione metabolism in response to oxidative challenge. However the exact mechanism of selection and the oxidative stress-causative ecological factor maintaining this polymorphism in a German population over a period of five years are yet to be determined. The previously demonstrated clinal patterns of allele frequencies in global populations (Catalán et al. 2016) and the similarities between oxidative stress and cold stress transcriptomic profiles strongly suggest that temperature plays a key role. Furthermore, the *MtnA* indel may represent a complex case of epigenetic regulatory control through the loss of miRNA binding sites and the consequent reversal of negative regulation processes such as those enacted by the *alph* gene in the JNK cascade, a major signal transduction network that coordinates the induction of protective genes in response to oxidative stress. Using nearly-isogenic lines created in the laboratory we were able to reduce the effect of genetic background and confirm the effect of the indel on expression divergence. However, conclusions about the effect of expression divergence and organismal phenotype were complicated by indications of sign epistasis. Therefore, it is likely that future studies focusing on the identification of additional variants across genomic backgrounds that may interact with the *MtnA* polymorphism and

tolerance experiments using combinations of these variants in a common background are necessary to further define this mechanism.

In the case of *fiz*, expression divergence was primarily linked to SNP67 in which the "G" allele is associated to an increase in *fiz* expression that reduces growth, body size and wing loading and has a sex-dependent effect on cold tolerance (Glaser-Schmitt and Parsch 2018). It is likely that the effect of *fiz* expression on regulation of growth rate and body size determination is facilitated by the modulation of levels of ecdysone, a steroid hormone and an insulin antagonist that is involved in the regulation of insect developmental stages levels and expression of growth factors (Glaser-Schmitt and Parsch 2018). The sex-dependent effect of *fiz* expression on cold tolerance led to the initial hypothesis that climatic variation involving temperature in the derived species range led to selection for reduced wing loading (Glaser-Schmitt and Parsch 2018). A further sex-dependent association between *fiz* expression and starvation resistance was demonstrated in this thesis. Starvation resistance may be a by-product of changes in overall body size and mating-related sex differences, with females needing more resources for egg production, thereby necessitating enhanced starvation resistance (Wayne et al. 2006; Ballard et al. 2008). However, the exact mechanism by which *fiz* expression affects starvation resistance remains unknown. Using a modelling approach it was determined that variation at SNP67 is likely maintained through a combination of temporally fluctuating and sexually antagonistic selection. Furthermore, dominance of the "G" allele was variable, as it was dependent on developmental stage and genomic background. Additionally, the temporal fluctuation was not purely seasonal. A possible explanation may be that variation in dominance and interaction with other loci may result in modulation of selection coefficients at SNP67. Alternatively, the effect of seasonality may be influenced by additional environmental factors that are related to food availability or

nutritional stress at developmental stages that vary with climate or anthropological factors such as agriculture or land development.

Overall, population genetics approaches combined with genomic and transcriptomic data can elucidate the intricate interactions between the forces driving sequence evolution and expression divergence and how this relates to phenotypic targets of selection. By functionally investigating two specific instances of regulatory variants in natural populations of *D. melanogaster*, this combinatorial approach provided new and exciting mechanistic insights into the evolutionary process of environmentally-based adaptation. These insights were further informed by taking aspects of demographic and life history into consideration, leading to a refinement of our understanding of the complexity and context dependency of gene regulation as it pertains to this species' expansion from the ancestral range and eventual global colonization. Although the mechanisms responsible for the maintenance of these polymorphisms and the functional pathways affected by expression divergence in these genes are different, there are some unifying attributes. For example, they both appear to be components of adaptive processes linked to climatic variation that involve metabolic adjustments, thereby implicating that environmentally-adaptive responses are strongly influenced and driven by metabolic regulation.

Works Cited

Agarwal V, Subtelny AO, Thiru P, Ulitsky I, Bartel DP. 2018. Predicting microRNA targeting efficacy in *Drosophila*. Genome Biol. 19(1):152. doi:10.1186/s13059-018-1504-3.

Agnes F, Suzanne M, Noselli S. 1999. The *Drosophila* JNK pathway controls the morphogenesis of imaginal discs during metamorphosis. Development. 126(23):5453–5462. doi:10.1242/dev.126.23.5453.

Akhund-Zade J, Bergland AO, Crowe SO, Unckless RL. 2017. The Genetic Basis of Natural Variation in *Drosophila* (Diptera: Drosophilidae) Virgin Egg Retention. J Insect Sci. 17(1). doi:10.1093/jisesa/iew094. [accessed 2021 Aug 22]. https://doi.org/10.1093/jisesa/iew094.

Alexander RP, Fang G, Rozowsky J, Snyder M, Gerstein MB. 2010. Annotating non-coding regions of the genome. Nat Rev Genet. 11(8):559–571. doi:10.1038/nrg2814.

Arguello JR, Laurent S, Clark AG. 2019. Demographic History of the Human Commensal *Drosophila melanogaster*. Genome Biol Evol. 11(3):844–854. doi:10.1093/gbe/evz022.

Ashraf SI, McLoon AL, Sclarsic SM, Kunes S. 2006. Synaptic Protein Synthesis Associated with Memory Is Regulated by the RISC Pathway in *Drosophila*. Cell. 124(1):191–205. doi:10.1016/j.cell.2005.12.017.

Ashton-Beaucage D, Udell CM, Gendron P, Sahmi M, Lefrançois M, Baril C, Guenier A-S, Duchaine J, Lamarre D, Lemieux S, et al. 2014. A Functional Screen Reveals an Extensive Layer of Transcriptional and Splicing Control Underlying RAS/MAPK Signaling in *Drosophila*. PLoS Biol. 12(3):e1001809. doi:10.1371/journal.pbio.1001809.

Atanesyan L, Günther V, Celniker SE, Georgiev O, Schaffner W. 2011. Characterization of *MtnE*, the fifth metallothionein member in *Drosophila*. J Biol Inorg Chem JBIC Publ Soc Biol Inorg Chem. 16(7):1047–1056. doi:10.1007/s00775-011-0825-4.

Bachtrog D, Toda NR, Lockton S. 2010. Dosage compensation and demasculinization of X chromosomes in *Drosophila*. Curr Biol CB. 20(16):1476–1481. doi:10.1016/j.cub.2010.06.076.

Baek D, Villén J, Shin C, Camargo FD, Gygi SP, Bartel DP. 2008. The impact of microRNAs on protein output. Nature. 455(7209):64–71. doi:10.1038/nature07242.

Ballard JWO, Melvin RG, Simpson SJ. 2008. Starvation resistance is positively correlated with body lipid proportion in five wild caught *Drosophila simulans* populations. J Insect Physiol. 54(9):1371–1376. doi:10.1016/j.jinsphys.2008.07.009.

Barghi N, Hermisson J, Schlötterer C. 2020. Polygenic adaptation: a unifying framework to understand positive selection. Nat Rev Genet. 21(12):769–781. doi:10.1038/s41576-020-0250-z.

Baril C, Sahmi M, Ashton-Beaucage D, Stronach B, Therrien M. 2009. The PP2C Alphabet is a negative regulator of stress-activated protein kinase signaling in *Drosophila*. Genetics. 181(2):567–579. doi:10.1534/genetics.108.096461.

Barrett RDH, Schluter D. 2008. Adaptation from standing genetic variation. Trends Ecol Evol. 23(1):38–44. doi:10.1016/j.tree.2007.09.008.

Barrier M, Robichaux RH, Purugganan MD. 2001. Accelerated regulatory gene evolution in an adaptive radiation. Proc Natl Acad Sci. 98(18):10208–10213. doi:10.1073/pnas.181257698.

Bauman JW, Liu J, Liu YP, Klaassen CD. 1991. Increase in metallothionein produced by chemicals that induce oxidative stress. Toxicol Appl Pharmacol. 110(2):347–354. doi:10.1016/S0041-008X(05)80017-1.

Bergland AO, Behrman EL, O'Brien KR, Schmidt PS, Petrov DA. 2014. Genomic Evidence of Rapid and Stable Adaptive Oscillations over Seasonal Time Scales in *Drosophila*. PLOS Genet. 10(11):e1004775. doi:10.1371/journal.pgen.1004775.

Bergland AO, Tobler R, González J, Schmidt P, Petrov D. 2016. Secondary contact and local adaptation contribute to genome-wide patterns of clinal variation in *Drosophila melanogaster*. Mol Ecol. 25(5):1157–1174. doi:10.1111/mec.13455.

Berndt AJE, Tang JCY, Ridyard MS, Lian T, Keatings K, Allan DW. 2015. Gene Regulatory Mechanisms Underlying the Spatial and Temporal Regulation of Target-Dependent Gene Expression in *Drosophila* Neurons. PLOS Genet. 11(12):e1005754. doi:10.1371/journal.pgen.1005754.

Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W. 2006. Relief of microRNA-Mediated Translational Repression in Human Cells Subjected to Stress. Cell. 125(6):1111–1124. doi:10.1016/j.cell.2006.04.031.

Biswas S, Akey JM. 2006. Genomic insights into positive selection. Trends Genet. 22(8):437–446. doi:10.1016/j.tig.2006.06.005.

Bitter MC, Kapsenberg L, Gattuso J-P, Pfister CA. 2019. Standing genetic variation fuels rapid adaptation to ocean acidification. Nat Commun. 10(1):5821. doi:10.1038/s41467-019-13767-1.

Božičević V, Hutter S, Stephan W, Wollstein A. 2016. Population genetic evidence for cold adaptation in European *Drosophila melanogaster* populations. Mol Ecol. 25(5):1175–1191. doi:10.1111/mec.13464.

Buchanan JL, Meiklejohn CD, Montooth KL. 2018. Energetic stress and infection generate immunity-fecundity tradeoffs in *Drosophila*. [accessed 2021 Sep 19]. https://www.biorxiv.org/content/10.1101/318568v1.

Buchberger E, Reis M, Lu T-H, Posnien N. 2019. Cloudy with a Chance of Insights: Context Dependent Gene Regulation and Implications for Evolutionary Studies. Genes. 10(7):492. doi:10.3390/genes10070492.

Caldwell PE, Walkiewicz M, Stern M. 2005. Ras activity in the *Drosophila* prothoracic gland regulates body size and developmental rate via ecdysone release. Curr Biol CB. 15(20):1785–1795. doi:10.1016/j.cub.2005.09.011.

Carninci P, Kasukawa T, Katayama S, Gough J, Frith MC, Maeda N, Oyama R, Ravasi T, Lenhard B, Wells C, et al. 2005. The Transcriptional Landscape of the Mammalian Genome. Science. 309(5740):1559–1563. doi:10.1126/science.1112014.

Carroll SB. 2005. Evolution at Two Levels: On Genes and Form. PLOS Biol. 3(7):e245. doi:10.1371/journal.pbio.0030245.

Castañeda LE, Rezende EL, Santos M. 2015. Heat tolerance in *Drosophila subobscura* along a latitudinal gradient: Contrasting patterns between plastic and genetic responses. Evolution. 69(10):2721–2734. doi:10.1111/evo.12757.

Catalán A, Glaser-Schmitt A, Argyridou E, Duchen P, Parsch J. 2016. An Indel Polymorphism in the *MtnA* 3' Untranslated Region Is Associated with Gene Expression Variation and Local Adaptation in *Drosophila melanogaster*. PLoS Genet. 12(4). doi:10.1371/journal.pgen.1005987. [accessed 2020 Jun 30]. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4847869/.

Catalán A, Hutter S, Parsch J. 2012. Population and sex differences in *Drosophila melanogaster* brain gene expression. BMC Genomics. 13(1):654. doi:10.1186/1471-2164-13-654.

Chang M, Shi M, Forman HJ. 1992. Exogenous glutathione protects endothelial cells from menadione toxicity. Am J Physiol-Lung Cell Mol Physiol. 262(5):L637–L643. doi:10.1152/ajplung.1992.262.5.L637.

Chang T-C, Mendell JT. 2007. microRNAs in Vertebrate Physiology and Human Disease. Annu Rev Genomics Hum Genet. 8(1):215–239. doi:10.1146/annurev.genom.8.080706.092351.

Chen J, Nolte V, Schlötterer C. 2015. Temperature Stress Mediates Decanalization and Dominance of Gene Expression in *Drosophila melanogaster*. PLOS Genet. 11(2):e1004883. doi:10.1371/journal.pgen.1004883.

Chen K, van Nimwegen E, Rajewsky N, Siegal ML. 2010. Correlating Gene Expression Variation with *cis*-Regulatory Polymorphism in Saccharomyces cerevisiae. Genome Biol Evol. 2:697–707. doi:10.1093/gbe/evq054.

Chen K, Rajewsky N. 2006. Natural selection on human microRNA binding sites inferred from SNP data. Nat Genet. 38(12):1452–1456. doi:10.1038/ng1910.

Chevalier M, Chase BM. 2015. Southeast African records reveal a coherent shift from high- to low-latitude forcing mechanisms along the east African margin across last glacial–interglacial transition. Quat Sci Rev. 125:117–130. doi:10.1016/j.quascirev.2015.07.009.

Chintapalli VR, Wang J, Dow JAT. 2007. Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease. Nat Genet. 39(6):715–720. doi:10.1038/ng2049.

Colombani J, Bianchini L, Layalle S, Pondeville E, Dauphin-Villemant C, Antoniewski C, Carré C, Noselli S, Léopold P. 2005. Antagonistic Actions of Ecdysone and Insulins Determine Final Size in *Drosophila*. Science. 310(5748):667–670. doi:10.1126/science.1119432.

Combs PA, Fraser HB. 2018. Spatially varying *cis*-regulatory divergence in *Drosophila* embryos elucidates *cis*-regulatory logic. PLOS Genet. 14(11):e1007631. doi:10.1371/journal.pgen.1007631.

Connallon T, Clark AG. 2014. Evolutionary inevitability of sexual antagonism. Proc R Soc B Biol Sci. 281(1776):20132123. doi:10.1098/rspb.2013.2123.

Dampc J, Kula-Maximenko M, Molon M, Durak R. 2020. Enzymatic Defense Response of Apple Aphid *Aphis pomi* to Increased Temperature. Insects. 11(7):436. doi:10.3390/insects11070436.

David JR, Capy P. 1988. Genetic variation of *Drosophila melanogaster* natural populations. Trends Genet. 4(4):106–111. doi:10.1016/0168-9525(88)90098-4.

Dawson E, Chen Y, Hunt S, Smink LJ, Hunt A, Rice K, Livingston S, Bumpstead S, Bruskiewich R, Sham P, et al. 2001. A SNP Resource for Human Chromosome 22: Extracting Dense Clusters of SNPs From the Genomic Sequence. Genome Res. 11(1):170–178. doi:10.1101/gr.156901.

Dayan DI, Du X, Baris TZ, Wagner DN, Crawford DL, Oleksiak MF. 2019. Population genomics of rapid evolution in natural populations: polygenic selection in response to power station thermal effluents. BMC Evol Biol. 19(1):61. doi:10.1186/s12862-019-1392-5.

Delaney JR, Stöven S, Uvell H, Anderson KV, Engström Y, Mlodzik M. 2006. Cooperative control of *Drosophila* immune responses by the JNK and NF-κB signaling pathways. EMBO J. 25(13):3068–3077. doi:10.1038/sj.emboj.7601182.

Doelling ARW, Griffis N, Williams JB. 2014. Repeated freezing induces oxidative stress and reduces survival in the freeze-tolerant goldenrod gall fly, *Eurosta solidaginis*. J Insect Physiol. 67:20–27. doi:10.1016/j.jinsphys.2014.05.024.

Dou J, Cánovas A, Brito LF, Yu Y, Schenkel FS, Wang Y. 2021. Comprehensive RNA-Seq Profiling Reveals Temporal and Tissue-Specific Changes in Gene Expression in Sprague–Dawley Rats as Response to Heat Stress Challenges. Front Genet. 12:651979. doi:10.3389/fgene.2021.651979.

Duchen P, Živković D, Hutter S, Stephan W, Laurent S. 2013. Demographic Inference Reveals African and European Admixture in the North American *Drosophila melanogaster* Population. Genetics. 193(1):291–301. doi:10.1534/genetics.112.145912.

Durmaz E, Rajpurohit S, Betancourt N, Fabian DK, Kapun M, Schmidt P, Flatt T. 2019. A clinal polymorphism in the insulin signaling transcription factor foxo contributes to life-history adaptation in *Drosophila**. Evolution. 73(9):1774–1792. doi:10.1111/evo.13759.

Egli D, Domènech J, Selvaraj A, Balamurugan K, Hua H, Capdevila M, Georgiev O, Schaffner W, Atrian S. 2006. The four members of the *Drosophila* metallothionein family exhibit distinct yet overlapping roles in heavy metal homeostasis and detoxification. Genes Cells. 11(6):647–658. doi:10.1111/j.1365-2443.2006.00971.x.

Engelhart DC, Azad P, Ali S, Granados JC, Haddad GG, Nigam SK. 2020. *Drosophila* SLC22 Orthologs Related to OATs, OCTs, and OCTNs Regulate Development and Responsiveness to Oxidative Stress. Int J Mol Sci. 21(6):2002. doi:10.3390/ijms21062002.

Fabian DK, Kapun M, Nolte V, Kofler R, Schmidt PS, Schlötterer C, Flatt T. 2012. Genome-wide patterns of latitudinal differentiation among populations of *Drosophila melanogaster* from North America. Mol Ecol. 21(19):4748–4769. doi:10.1111/j.1365-294X.2012.05731.x.

Fabian DK, Lack JB, Mathur V, Schlötterer C, Schmidt PS, Pool JE, Flatt T. 2015. Spatially varying selection shapes life history clines among populations of *Drosophila melanogaster* from sub-Saharan Africa. J Evol Biol. 28(4):826–840. doi:10.1111/jeb.12607.

Fernandes JCR, Acuña SM, Aoki JI, Floeter-Winter LM, Muxel SM. 2019. Long Non-Coding RNAs in the Regulation of Gene Expression: Physiology and Disease. Non-Coding RNA. 5(1):17. doi:10.3390/ncrna5010017.

Flatt T. 2020. Life-History Evolution and the Genetics of Fitness Components in *Drosophila melanogaster*. Genetics. 214(1):3–48. doi:10.1534/genetics.119.300160.

Frank SA, Crespi BJ. 2011. Pathology from evolutionary conflict, with a theory of X chromosome versus autosome conflict over sexually antagonistic traits. Proc Natl Acad Sci. 108(Supplement 2):10886–10893. doi:10.1073/pnas.1100921108.

Frank SA, Patten MM. 2020. Sexual antagonism leads to a mosaic of X-autosome conflict. Evolution. 74(2):495–498. doi:10.1111/evo.13918.

Frazier MR, Harrison JF, Kirkton SD, Roberts SP. 2008. Cold rearing improves cold-flight performance in *Drosophila* via changes in wing morphology. J Exp Biol. 211(13):2116–2122. doi:10.1242/jeb.019422.

Freda PJ, Alex JT, Morgan TJ, Ragland GJ. 2017. Genetic Decoupling of Thermal Hardiness across Metamorphosis in *Drosophila melanogaster*. Integr Comp Biol. 57(5):999–1009. doi:10.1093/icb/icx102.

Gao Y, He Y, Ding J, Wu K, Hu B, Liu Y, Wu Y, Guo B, Shen Y, Landi D, et al. 2009. An insertion/deletion polymorphism at *miRNA-1α*-binding site in the *interleukin-1α* 3′ untranslated region confers risk for hepatocellular carcinoma. Carcinogenesis. 30(12):2064–2069. doi:10.1093/carcin/bgp283.

Gardner A, Úbeda F. 2017. The meaning of intragenomic conflict. Nat Ecol Evol. 1(12):1807–1815. doi:10.1038/s41559-017-0354-9.

Gaudet P, Livstone MS, Lewis SE, Thomas PD. 2011. Phylogenetic-based propagation of functional annotations within the Gene Ontology consortium. Brief Bioinform. 12(5):449–462. doi:10.1093/bib/bbr042.

Genissel A, McIntyre LM, Wayne ML, Nuzhdin SV. 2008. *Cis* and *trans* regulatory effects contribute to natural variation in transcriptome of *Drosophila melanogaster*. Mol Biol Evol. 25(1):101–110. doi:10.1093/molbev/msm247.

Gibert J-M, Mouchel-Vielh E, De Castro S, Peronnet F. 2016. Phenotypic Plasticity through Transcriptional Regulation of the Evolutionary Hotspot Gene tan in *Drosophila melanogaster*. PLoS Genet. 12(8):e1006218. doi:10.1371/journal.pgen.1006218.

Gibert P, Huey RB. 2001. Chill-coma temperature in *Drosophila*: effects of developmental temperature, latitude, and phylogeny. Physiol Biochem Zool PBZ. 74(3):429–434. doi:10.1086/320429.

Glaser-Schmitt A, Catalán A, Parsch J. 2013. Adaptive divergence of a transcriptional enhancer between populations of *Drosophila melanogaster*. Philos Trans R Soc B Biol Sci. 368(1632):20130024. doi:10.1098/rstb.2013.0024.

Glaser-Schmitt A, Parsch J. 2018. Functional characterization of adaptive variation within a *cis*-regulatory element influencing *Drosophila melanogaster* growth. PLOS Biol. 16(1):e2004538. doi:10.1371/journal.pbio.2004538.

Gnad F, Parsch J. 2006. Sebida: a database for the functional and evolutionary analysis of genes with sex-biased expression. Bioinformatics. 22(20):2577–2579. doi:10.1093/bioinformatics/btl422.

Gompel N, Prud'homme B, Wittkopp PJ, Kassner VA, Carroll SB. 2005. Chance caught on the wing: *cis*-regulatory evolution and the origin of pigment patterns in *Drosophila*. Nature. 433(7025):481–487. doi:10.1038/nature03235.

González J, Macpherson JM, Petrov DA. 2009. A Recent Adaptive Transposable Element Insertion Near Highly Conserved Developmental Loci in *Drosophila melanogaster*. Mol Biol Evol. 26(9):1949–1961. doi:10.1093/molbev/msp107.

Graze RM, McIntyre LM, Morse AM, Boyd BM, Nuzhdin SV, Wayne ML. 2014. What the X Has to Do with It: Differences in Regulatory Variability between the Sexes in *Drosophila* simulans. Genome Biol Evol. 6(4):818–829. doi:10.1093/gbe/evu060.

Grenier JK, Arguello JR, Moreira MC, Gottipati S, Mohammed J, Hackett SR, Boughton R, Greenberg AJ, Clark AG. 2015. Global Diversity Lines–A Five-Continent Reference Panel of Sequenced *Drosophila melanogaster* Strains. G3 GenesGenomesGenetics. 5(4):593–603. doi:10.1534/g3.114.015883.

Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. 2006. miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res. 34(Database issue):D140–D144. doi:10.1093/nar/gkj112.

Groen SC, Ćalić I, Joly-Lopez Z, Platts AE, Choi JY, Natividad M, Dorph K, Mauck WM, Bracken B, Cabral CLU, et al. 2020. The strength and pattern of natural selection on gene expression in rice. Nature. 578(7796):572–576. doi:10.1038/s41586-020-1997-2.

Guio L, Barrón MG, González J. 2014. The transposable element *Bari-Jheh* mediates oxidative stress response in *Drosophila*. Mol Ecol. 23(8):2020–2030. doi:10.1111/mec.12711.

Hardy CM, Burke MK, Everett LJ, Han MV, Lantz KM, Gibbs AG. 2018. Genome-Wide Analysis of Starvation-Selected *Drosophila melanogaster*-A Genetic Model of Obesity. Mol Biol Evol. 35(1):50–65. doi:10.1093/molbev/msx254.

von Heckel K, Stephan W, Hutter S. 2016. Canalization of gene expression is a major signature of regulatory cold adaptation in temperate *Drosophila melanogaster*. BMC Genomics. 17(1):574. doi:10.1186/s12864-016-2866-0.

Hitchcock TJ, Gardner A. 2020. A gene's-eye view of sexual antagonism. Proc R Soc B Biol Sci. 287(1932):20201633. doi:10.1098/rspb.2020.1633.

Hoekstra LA, Siddiq MA, Montooth KL. 2013. Pleiotropic Effects of a Mitochondrial–Nuclear Incompatibility Depend upon the Accelerating Effect of Temperature in *Drosophila*. Genetics. 195(3):1129–1139. doi:10.1534/genetics.113.154914.

Hoffmann AA, Hallas R, Anderson AR, Telonis-Scott M. 2005. Evidence for a robust sex-specific trade-off between cold resistance and starvation resistance in *Drosophila melanogaster*. J Evol Biol. 18(4):804–810. doi:10.1111/j.1420-9101.2004.00871.x.

Hon C-C, Ramilowski JA, Harshbarger J, Bertin N, Rackham OJL, Gough J, Denisenko E, Schmeier S, Poulsen TM, Severin J, et al. 2017. An atlas of human long non-coding RNAs with accurate 5′ ends. Nature. 543(7644):199–204. doi:10.1038/nature21374.

Hsu S-K, Jakšić AM, Nolte V, Lirakis M, Kofler R, Barghi N, Versace E, Schlötterer C. 2020. Rapid sex-specific adaptation to high temperature in *Drosophila*. Tautz D, Ebert D, Reisser C, editors. eLife. 9:e53237. doi:10.7554/eLife.53237.

Huang W, Massouras A, Inoue Y, Peiffer J, Ràmia M, Tarone AM, Turlapati L, Zichner T, Zhu D, Lyman RF, et al. 2014. Natural variation in genome architecture among 205 *Drosophila melanogaster* Genetic Reference Panel lines. Genome Res. 24(7):1193–1208. doi:10.1101/gr.171546.113.

Hutter S, Saminadin-Peter SS, Stephan W, Parsch J. 2008. Gene expression variation in African and European populations of *Drosophila melanogaster*. Genome Biol. 9(1):R12. doi:10.1186/gb-2008-9-1-r12.

Huylmans AK, Parsch J. 2014. Population- and Sex-Biased Gene Expression in the Excretion Organs of *Drosophila melanogaster*. G3 GenesGenomesGenetics. 4(12):2307–2315. doi:10.1534/g3.114.013417.

Huylmans AK, Parsch J. 2015. Variation in the X:Autosome Distribution of Male-Biased Genes among *Drosophila melanogaster* Tissues and Its Relationship with Dosage Compensation. Genome Biol Evol. 7(7):1960–1971. doi:10.1093/gbe/evv117.

Iida K, Cox-Foster DL, Yang X, Ko W-Y, Cavener DR. 2007. Expansion and evolution of insect GMC oxidoreductases. BMC Evol Biol. 7:75. doi:10.1186/1471-2148-7-75.

Isakova A, Fehlmann T, Keller A, Quake SR. 2020. A mouse tissue atlas of small noncoding RNA. Proc Natl Acad Sci. 117(41):25634–25645. doi:10.1073/pnas.2002277117.

Jardine MD, Ruzicka F, Diffley C, Fowler K, Reuter M. 2021. A non-coding indel polymorphism in the fruitless gene of *Drosophila melanogaster* exhibits antagonistically pleiotropic fitness effects. Proc R Soc B Biol Sci. 288(1950):20202958. doi:10.1098/rspb.2020.2958.

Jasper H, Benes V, Schwager C, Sauer S, Clauder-Münster S, Ansorge W, Bohmann D. 2001. The Genomic Response of the *Drosophila* Embryo to JNK Signaling. Dev Cell. 1(4):579–586. doi:10.1016/S1534-5807(01)00045-4.

Jiang L-J, Maret W, Vallee BL. 1998. The glutathione redox couple modulates zinc transfer from metallothionein to zinc-depleted sorbitol dehydrogenase. Proc Natl Acad Sci U S A. 95(7):3483–3488.

Johnsen JM, Teschke M, Pavlidis P, McGee BM, Tautz D, Ginsburg D, Baines JF. 2009. Selection on *cis*-Regulatory Variation at *B4galnt2* and Its Influence on von Willebrand Factor in House Mice. Mol Biol Evol. 26(3):567–578. doi:10.1093/molbev/msn284.

Juneja P, Quinn A, Jiggins FM. 2016. Latitudinal clines in gene expression and *cis*-regulatory element variation in *Drosophila melanogaster*. BMC Genomics. 17(1):981. doi:10.1186/s12864-016-3333-7.

Kalay G, Wittkopp PJ. 2010. Nomadic Enhancers: Tissue-Specific *cis*-Regulatory Elements of *yellow* Have Divergent Genomic Positions among *Drosophila* Species. PLOS Genet. 6(11):e1001222. doi:10.1371/journal.pgen.1001222.

Kapopoulou A, Pfeifer SP, Jensen JD, Laurent S. 2018. The Demographic History of African *Drosophila melanogaster*. Genome Biol Evol. 10(9):2338–2342. doi:10.1093/gbe/evy185.

Kapun M, Fabian DK, Goudet J, Flatt T. 2016. Genomic Evidence for Adaptive Inversion Clines in *Drosophila melanogaster*. Mol Biol Evol. 33(5):1317–1336. doi:10.1093/molbev/msw016.

Kavitha S, Chandra TS. 2014. Oxidative Stress Protection and Glutathione Metabolism in Response to Hydrogen Peroxide and Menadione in Riboflavinogenic Fungus *Ashbya gossypii*. Appl Biochem Biotechnol. 174(6):2307–2325. doi:10.1007/s12010-014-1188-4.

Kawecki TJ, Erkosar B, Dupuis C, Hollis B, Stillwell RC, Kapun M. 2021. The Genomic Architecture of Adaptation to Larval Malnutrition Points to a Trade-off with Adult Starvation Resistance in *Drosophila*. Mol Biol Evol. 38(7):2732–2749. doi:10.1093/molbev/msab061.

Keller A. 2007. *Drosophila melanogaster*'s history as a human commensal. Curr Biol. 17(3):R77–R81. doi:10.1016/j.cub.2006.12.031.

Kim I-S, Sohn H-Y, Jin I. 2011. Adaptive stress response to menadione-induced oxidative stress in *Saccharomyces cerevisiae* KNU5377. J Microbiol Seoul Korea. 49(5):816–823. doi:10.1007/s12275-011-1154-6.

King M-C, Wilson AC. 1975. Evolution at Two Levels in Humans and Chimpanzees. Science. 188(4184):107–116.

Kislev ME, Hartmann A, Bar-Yosef O. 2006. Early Domesticated Fig in the Jordan Valley. Science. 312(5778):1372–1374. doi:10.1126/science.1125910.

Lachaise D, Cariou M-L, David JR, Lemeunier F, Tsacas L, Ashburner M. 1988. Historical Biogeography of the *Drosophila melanogaster* Species Subgroup. In: Hecht MK, Wallace B, Prance GT, editors. Evolutionary Biology. Boston, MA: Springer US. (Evolutionary Biology). p. 159–225. [accessed 2021 Oct 28]. https://doi.org/10.1007/978-1-4613-0931-4_4.

Lai Y-T, Yeung CKL, Omland KE, Pang E-L, Hao Y, Liao B-Y, Cao H-F, Zhang B-W, Yeh C-F, Hung C-M, et al. 2019. Standing genetic variation as the predominant source for adaptation of a songbird. Proc Natl Acad Sci. 116(6):2152–2157.

Lalouette L, Williams CM, Hervant F, Sinclair BJ, Renault D. 2011. Metabolic rate and oxidative stress in insects exposed to low temperature thermal fluctuations. Comp Biochem Physiol A Mol Integr Physiol. 158(2):229–234. doi:10.1016/j.cbpa.2010.11.007.

Landis G, Shen J, Tower J. 2012. Gene expression changes in response to aging compared to heat stress, oxidative stress and ionizing radiation in *Drosophila melanogaster*. Aging. 4(11):768–789.

Lavington E, Cogni R, Kuczynski C, Koury S, Behrman EL, O'Brien KR, Schmidt PS, Eanes WF. 2014. A Small System—High-Resolution Study of Metabolic Adaptation in the Central Metabolic Pathway to Temperate Climates in *Drosophila melanogaster*. Mol Biol Evol. 31(8):2032–2041. doi:10.1093/molbev/msu146.

Lazzaro BP, Flores HA, Lorigan JG, Yourth CP. 2008. Genotype-by-Environment Interactions and Adaptation to Local Temperature Affect Immunity and Fecundity in *Drosophila melanogaster*. PLOS Pathog. 4(3):e1000025. doi:10.1371/journal.ppat.1000025.

Leader DP, Krause SA, Pandit A, Davies SA, Dow JAT. 2018. FlyAtlas 2: a new version of the *Drosophila melanogaster* expression atlas with RNA-Seq, miRNA-Seq and sex-specific data. Nucleic Acids Res. 46(D1):D809–D815. doi:10.1093/nar/gkx976.

Lecheta MC, Awde DN, O'Leary TS, Unfried LN, Jacobs NA, Whitlock MH, McCabe E, Powers B, Bora K, Waters JS, et al. 2020. Integrating GWAS and Transcriptomics to Identify the Molecular Underpinnings of Thermal Stress Responses in *Drosophila melanogaster*. Front Genet. 11:658. doi:10.3389/fgene.2020.00658.

Li H, Stephan W. 2006. Inferring the Demographic History and Rate of Adaptive Substitution in *Drosophila*. PLOS Genet. 2(10):e166. doi:10.1371/journal.pgen.0020166.

Liu C, Rennie WA, Carmack CS, Kanoria S, Cheng J, Lu J, Ding Y. 2014. Effects of genetic variations on microRNA: target interactions. Nucleic Acids Res. 42(15):9543–9552. doi:10.1093/nar/gku675.

Liu XD, Thiele DJ. 1996. Oxidative stress induced heat shock factor phosphorylation and HSF-dependent activation of yeast *metallothionein* gene transcription. Genes Dev. 10(5):592–603. doi:10.1101/gad.10.5.592.

Loehlin DW, Ames JR, Vaccaro K, Carroll SB. 2019. A major role for noncoding regulatory mutations in the evolution of enzyme activity. Proc Natl Acad Sci. 116(25):12383–12389. doi:10.1073/pnas.1904071116.

Long E, Evans C, Chaston J, Udall JA. 2018. Genomic Structural Variations Within Five Continental Populations of *Drosophila melanogaster*. G3 GenesGenomesGenetics. 8(10):3247–3253. doi:10.1534/g3.118.200631.

Lucas K, Raikhel AS. 2013. Insect MicroRNAs: Biogenesis, expression profiling and biological functions. Insect Biochem Mol Biol. 43(1):24–38. doi:10.1016/j.ibmb.2012.10.009.

Luo M, Finet C, Cong H, Wei H, Chung H. 2020. The evolution of insect metallothioneins. Proc R Soc B Biol Sci. 287(1937):20202189. doi:10.1098/rspb.2020.2189.

Machado HE, Bergland AO, Taylor R, Tilk S, Behrman E, Dyer K, Fabian DK, Flatt T, González J, Karasov TL, et al. 2021. Broad geographic sampling reveals the shared basis and environmental correlates of seasonal adaptation in *Drosophila*. Nordborg M, Wittkopp PJ, Nordborg M, editors. eLife. 10:e67577. doi:10.7554/eLife.67577.

Mack KL, Ballinger MA, Phifer-Rixey M, Nachman MW. 2018. Gene regulation underlies environmental adaptation in house mice. Genome Res. 28(11):1636–1645. doi:10.1101/gr.238998.118.

Mackay TFC, Richards S, Stone EA, Barbadilla A, Ayroles JF, Zhu D, Casillas S, Han Y, Magwire MM, Cridland JM, et al. 2012. The *Drosophila melanogaster* Genetic Reference Panel. Nature. 482(7384):173–178. doi:10.1038/nature10811.

MacMillan HA, Andersen JL, Davies SA, Overgaard J. 2015. The capacity to maintain ion and water homeostasis underlies interspecific variation in *Drosophila* cold tolerance. Sci Rep. 5:18607. doi:10.1038/srep18607.

MacMillan HA, Andersen JL, Loeschcke V, Overgaard J. 2015. Sodium distribution predicts the chill tolerance of *Drosophila melanogaster* raised in different thermal conditions. Am J Physiol-Regul Integr Comp Physiol. 308(10):R823–R831. doi:10.1152/ajpregu.00465.2014.

MacMillan HA, Knee JM, Dennis AB, Udaka H, Marshall KE, Merritt TJS, Sinclair BJ. 2016. Cold acclimation wholly reorganizes the *Drosophila melanogaster* transcriptome and metabolome. Sci Rep. 6(1):28999. doi:10.1038/srep28999.

Mallard F, Nolte V, Tobler R, Kapun M, Schlötterer C. 2018. A simple genetic basis of adaptation to a novel thermal environment results in complex metabolic rewiring in *Drosophila*. Genome Biol. 19(1):119. doi:10.1186/s13059-018-1503-4.

Marden JH, Rogina B, Montooth KL, Helfand SL. 2003. Conditional tradeoffs between aging and organismal performance of Indy long-lived mutant flies. Proc Natl Acad Sci U S A. 100(6):3369–3373. doi:10.1073/pnas.0634985100.

Maret W. 1994. Oxidative metal release from metallothionein via zinc-thiol/disulfide interchange. Proc Natl Acad Sci U S A. 91(1):237–241.

Mateo L, Rech GE, González J. 2018. Genome-wide patterns of local adaptation in Western European *Drosophila melanogaster* natural populations. Sci Rep. 8(1):16143. doi:10.1038/s41598-018-34267-0.

McClung JM, Judge AR, Powers SK, Yan Z. 2010. p38 MAPK links oxidative stress to autophagy-related gene expression in cachectic muscle wasting. Am J Physiol-Cell Physiol. 298(3):C542–C549. doi:10.1152/ajpcell.00192.2009.

McVean G. 2007. The Structure of Linkage Disequilibrium Around a Selective Sweep. Genetics. 175(3):1395–1406. doi:10.1534/genetics.106.062828.

Mensch J, Lavagnino N, Carreira VP, Massaldi A, Hasson E, Fanara JJ. 2008. Identifying candidate genes affecting developmental time in *Drosophila melanogaster*: pervasive pleiotropy and gene-by-environment interaction. BMC Dev Biol. 8(1):78. doi:10.1186/1471-213X-8-78.

Mills RE, Luttig CT, Larkins CE, Beauchamp A, Tsui C, Pittard WS, Devine SE. 2006. An initial map of insertion and deletion (INDEL) variation in the human genome. Genome Res. 16(9):1182–1190. doi:10.1101/gr.4565806.

Milo R, Jorgensen P, Moran U, Weber G, Springer M. 2010. BioNumbers—the database of key numbers in molecular and cell biology. Nucleic Acids Res. 38(Database issue):D750–D753. doi:10.1093/nar/gkp889.

Mitchell KA, Hoffmann AA. 2010. Thermal ramping rate influences evolutionary potential and species differences for upper thermal limits in *Drosophila*. Funct Ecol. 24(3):694–700. doi:10.1111/j.1365-2435.2009.01666.x.

Mockett RJ, Sohal RS. 2006. Temperature-dependent trade-offs between longevity and fertility in the *Drosophila* mutant, *methuselah*. Exp Gerontol. 41(6):566–573. doi:10.1016/j.exger.2006.03.015.

Moszyńska A, Gebert M, Collawn JF, Bartoszewski R. 2017. SNPs in microRNA target sites and their potential role in human disease. Open Biol. 7(4):170019. doi:10.1098/rsob.170019.

Murphy D, Dan*cis* B, Brown JR. 2008. The evolution of core proteins involved in microRNA biogenesis. BMC Evol Biol. 8(1):92. doi:10.1186/1471-2148-8-92.

Newell NR, New FN, Dalton JE, McIntyre LM, Arbeitman MN. 2016. Neurons That Underlie *Drosophila melanogaster* Reproductive Behaviors: Detection of a Large Male-Bias in Gene Expression in *fruitless*-Expressing Neurons. G3 GenesGenomesGenetics. 6(8):2455–2465. doi:10.1534/g3.115.019265.

Ng CT, Yong LQ, Hande MP, Ong CN, Yu LE, Bay BH, Baeg GH. 2017. Zinc oxide nanoparticles exhibit cytotoxicity and genotoxicity through oxidative stress responses in human lung fibroblasts and *Drosophila melanogaster*. Int J Nanomedicine. 12:1621–1637. doi:10.2147/IJN.S124403.

Nghe P, Kogenaru M, Tans SJ. 2018. Sign epistasis caused by hierarchy within signalling cascades. Nat Commun. 9(1):1451. doi:10.1038/s41467-018-03644-8.

O'Donnell DR, Parigi A, Fish JA, Dworkin I, Wagner AP. 2014. The Roles of Standing Genetic Variation and Evolutionary History in Determining the Evolvability of Anti-Predator Strategies. PLOS ONE. 9(6):e100163. doi:10.1371/journal.pone.0100163.

Ometto L, Glinka S, De Lorenzo D, Stephan W. 2005. Inferring the Effects of Demography and Selection on *Drosophila melanogaster* Populations from a Chromosome-Wide Scan of DNA Variation. Mol Biol Evol. 22(10):2119–2130. doi:10.1093/molbev/msi207.

Orr HA, Betancourt AJ. 2001. Haldane's Sieve and Adaptation From the Standing Genetic Variation. Genetics. 157(2):875–884. doi:10.1093/genetics/157.2.875.

Overgaard J, Kristensen TN, Mitchell KA, Hoffmann AA. 2011. Thermal tolerance in widespread and tropical *Drosophila* species: does phenotypic plasticity increase with latitude? Am Nat. 178 Suppl 1:S80-96. doi:10.1086/661780.

Owusu-Ansah E, Banerjee U. 2009. Reactive oxygen species prime *Drosophila* haematopoietic progenitors for differentiation. Nature. 461(7263):537–541. doi:10.1038/nature08313.

Paaby AB, Bergland AO, Behrman EL, Schmidt PS. 2014. A highly pleiotropic amino acid polymorphism in the *Drosophila* insulin receptor contributes to life-history adaptation. Evolution. 68(12):3395–3409. doi:10.1111/evo.12546.

Pathak A, Munjal A, Parkash R. 2018. Cold acclimation conditions constrain plastic responses for resistance to cold and starvation in *Drosophila immigrans*. Biol Open. 7(6):bio034447. doi:10.1242/bio.034447.

Patten MM. 2019. The X chromosome favors males under sexually antagonistic selection. Evolution. 73(1):84–91. doi:10.1111/evo.13646.

Pickering AM, Staab TA, Tower J, Sieburth D, Davies KJA. 2013. A conserved role for the 20S proteasome and Nrf2 transcription factor in oxidative stress adaptation in mammals, *Caenorhabditis elegans* and *Drosophila melanogaster*. J Exp Biol. 216(4):543–553. doi:10.1242/jeb.074757.

Pinal N, Martín M, Medina I, Morata G. 2018. Short-term activation of the Jun N-terminal kinase pathway in apoptosis-deficient cells of *Drosophila* induces tumorigenesis. Nat Commun. 9(1):1541. doi:10.1038/s41467-018-04000-6.

Pitchers W, Pool JE, Dworkin I. 2013. Altitudinal Clinal Variation in Wing Size and Shape in African *Drosophila melanogaster*: One Cline or Many? Evolution. 67(2):438–452. doi:10.1111/j.1558-5646.2012.01774.x.

Plaisier CL, Lo F-Y, Ashworth J, Brooks AN, Beer KD, Kaur A, Pan M, Reiss DJ, Facciotti MT, Baliga NS. 2014. Evolution of context dependent regulation by expansion of feast/famine regulatory proteins. BMC Syst Biol. 8(1):122. doi:10.1186/s12918-014-0122-2.

Pool JE, Corbett-Detig RB, Sugino RP, Stevens KA, Cardeno CM, Crepeau MW, Duchen P, Emerson JJ, Saelao P, Begun DJ, et al. 2012. Population Genomics of Sub-Saharan *Drosophila melanogaster*: African Diversity and Non-African Admixture. PLOS Genet. 8(12):e1003080. doi:10.1371/journal.pgen.1003080.

Prud'homme B, Gompel N, Carroll SB. 2007. Emerging principles of regulatory evolution. Proc Natl Acad Sci. 104(suppl 1):8605–8612. doi:10.1073/pnas.0700488104.

Rajpurohit S, Gefen E, Bergland AO, Petrov DA, Gibbs AG, Schmidt PS. 2018. Spatiotemporal dynamics and genome-wide association analysis of desiccation tolerance in *Drosophila melanogaster*. Mol Ecol. 27(17):3525–3540. doi:10.1111/mec.14814.

Rako L, Blacket MJ, McKechnie SW, Hoffmann AA. 2007. Candidate genes and thermal phenotypes: identifying ecologically important genetic variation for thermotolerance in the Australian *Drosophila melanogaster* cline. Mol Ecol. 16(14):2948–2957. doi:10.1111/j.1365-294X.2007.03332.x.

Ramirez-Corona BA, Fruth S, Ofoegbu O, Wunderlich Z. 2021. The mode of expression divergence in *Drosophila* fat body is infection-specific. Genome Res. 31(6):1024–1034. doi:10.1101/gr.269597.120.

Reinhardt JA, Kolaczkowski B, Jones CD, Begun DJ, Kern AD. 2014. Parallel Geographic Variation in *Drosophila melanogaster*. Genetics. 197(1):361–373. doi:10.1534/genetics.114.161463.

Renganaath K, Cheung R, Day L, Kosuri S, Kruglyak L, Albert FW. 2020. Systematic identification of *cis*-regulatory variants that cause gene expression differences in a yeast cross. eLife. 9:e62669. doi:10.7554/eLife.62669.

Reynolds-Peterson C, Xu J, Zhao N, Cruse C, Yonel B, Trasorras C, Toyoda H, Kinoshita-Toyoda A, Dobson J, Schultheis N, et al. 2020. Heparan Sulfate Structure Affects Autophagy, Lifespan, Responses to Oxidative Stress, and Cell Degeneration in *Drosophila parkin* Mutants. G3 GenesGenomesGenetics. 10(1):129–141. doi:10.1534/g3.119.400730.

Rivera-Barahona A, Pérez B, Richard E, Desviat LR. 2017. Role of miRNAs in human disease and inborn errors of metabolism. J Inherit Metab Dis. 40(4):471–480. doi:10.1007/s10545-017-0018-6.

Robinson SJW, Partridge L. 2001. Temperature and clinal variation in larval growth efficiency in *Drosophila melanogaster*. J Evol Biol. 14(1):14–21. doi:10.1046/j.1420-9101.2001.00259.x.

Ronald J, Akey JM. 2005. Genome-wide scans for loci under selection in humans. Hum Genomics. 2(2):113. doi:10.1186/1479-7364-2-2-113.

Rousselle M, Simion P, Tilak M-K, Figuet E, Nabholz B, Galtier N. 2020. Is adaptation limited by mutation? A timescale-dependent effect of genetic diversity on the adaptive substitution rate in animals. PLOS Genet. 16(4):e1008668. doi:10.1371/journal.pgen.1008668.

Ruttkay-Nedecky B, Nejdl L, Gumulec J, Zitka O, Masarik M, Eckschlager T, Stiborova M, Adam V, Kizek R. 2013. The Role of Metallothionein in Oxidative Stress. Int J Mol Sci. 14(3):6044–6066. doi:10.3390/ijms14036044.

Samani P, Bell G. 2010. Adaptation of experimental yeast populations to stressful conditions in relation to population size. J Evol Biol. 23(4):791–796. doi:10.1111/j.1420-9101.2010.01945.x.

Saminadin-Peter SS, Kemkemer C, Pavlidis P, Parsch J. 2012. Selective Sweep of a *cis*-Regulatory Sequence in a Non-African Population of *Drosophila melanogaster*. Mol Biol Evol. 29(4):1167–1174. doi:10.1093/molbev/msr284.

Santos ME, Braasch I, Boileau N, Meyer BS, Sauteur L, Böhne A, Belting H-G, Affolter M, Salzburger W. 2014. The evolution of cichlid fish egg-spots is linked with a *cis*-regulatory change. Nat Commun. 5(1):5149. doi:10.1038/ncomms6149.

Sato M, Bremner I. 1993. Oxygen free radicals and metallothionein. Free Radic Biol Med. 14(3):325–337. doi:10.1016/0891-5849(93)90029-T.

Saunders MA, Liang H, Li W-H. 2007. Human polymorphism at microRNAs and microRNA target sites. Proc Natl Acad Sci. 104(9):3300–3305. doi:10.1073/pnas.0611347104.

Schmidt PS, Zhu C-T, Das J, Batavia M, Yang L, Eanes WF. 2008. An amino acid polymorphism in the *couch potato* gene forms the basis for climatic adaptation in *Drosophila melanogaster*. Proc Natl Acad Sci. 105(42):16207–16211. doi:10.1073/pnas.0805485105.

Schratt GM, Tuebing F, Nigh EA, Kane CG, Sabatini ME, Kiebler M, Greenberg ME. 2006. A brain-specific microRNA regulates dendritic spine development. Nature. 439(7074):283–289. doi:10.1038/nature04367.

Selbach M, Schwanhäusser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N. 2008. Widespread changes in protein synthesis induced by microRNAs. Nature. 455(7209):58–63. doi:10.1038/nature07228.

Sellis D, Callahan BJ, Petrov DA, Messer PW. 2011. Heterozygote advantage as a natural consequence of adaptation in diploids. Proc Natl Acad Sci. 108(51):20666–20671. doi:10.1073/pnas.1114573108.

Simonsen A, Cumming RC, Brech A, Isakson P, Schubert DR, Finley KD. 2008. Promoting basal levels of autophagy in the nervous system enhances longevity and oxidant resistance in adult *Drosophila*. Autophagy. 4(2):176–184. doi:10.4161/auto.5269.

Smith JM, Haigh J. 1974. The hitch-hiking effect of a favourable gene. Genet Res. 23(1):23–35. doi:10.1017/S0016672300014634.

Sørensen JG, Dahlgaard J, Loeschcke V. 2001. Genetic variation in thermal tolerance among natural populations of *Drosophila buzzatii*: down regulation of *Hsp70* expression and variation in heat stress resistance traits. Funct Ecol. 15(3):289–296. doi:10.1046/j.1365-2435.2001.00525.x.

Sørensen JG, Loeschcke V. 2001. Larval crowding in *Drosophila melanogaster* induces *Hsp70* expression, and leads to increased adult longevity and adult thermal stress resistance. J Insect Physiol. 47(11):1301–1307. doi:10.1016/S0022-1910(01)00119-6.

Sørensen JG, Schou MF, Kristensen TN, Loeschcke V. 2016. Thermal fluctuations affect the transcriptome through mechanisms independent of average temperature. Sci Rep. 6(1):30975. doi:10.1038/srep30975.

Sprengelmeyer QD, Mansourian S, Lange JD, Matute DR, Cooper BS, Jirle EV, Stensmyr MC, Pool JE. 2020. Recurrent Collection of *Drosophila melanogaster* from Wild African Environments and Genomic Insights into Species History. Mol Biol Evol. 37(3):627–638. doi:10.1093/molbev/msz271.

Steige KA, Laenen B, Reimegård J, Scofield DG, Slotte T. 2017. Genomic analysis reveals major determinants of *cis*-regulatory variation in *Capsella grandiflora*. Proc Natl Acad Sci. 114(5):1087–1092. doi:10.1073/pnas.1612561114.

Stern DL. 2000. Perspective: Evolutionary Developmental Biology and the Problem of Variation. Evolution. 54(4):1079–1091. doi:10.1111/j.0014-3820.2000.tb00544.x.

Straub T, Zabel A, Gilfillan GD, Feller C, Becker PB. 2013. Different chromatin interfaces of the *Drosophila* dosage compensation complex revealed by high-shear ChIP-seq. Genome Res. 23(3):473–485. doi:10.1101/gr.146407.112.

Takeuchi H, Rigden DJ, Ebrahimi B, Turner PC, Rees HH. 2005. Regulation of ecdysteroid signalling during *Drosophila* development: identification, characterization and modelling of ecdysone oxidase, an enzyme involved in control of ligand concentration. Biochem J. 389(3):637–645. doi:10.1042/BJ20050498.

Tamura K, Subramanian S, Kumar S. 2004. Temporal Patterns of Fruit Fly (*Drosophila*) Evolution Revealed by Mutation Clocks. Mol Biol Evol. 21(1):36–44. doi:10.1093/molbev/msg236.

Teets NM, Hahn DA. 2018. Genetic variation in the shape of cold-survival curves in a single fly population suggests potential for selection from climate variability. J Evol Biol. 31(4):543–555. doi:https://doi.org/10.1111/jeb.13244.

Thomas NO, Shay KP, Kelley AR, Butler JA, Hagen TM. 2016. Glutathione maintenance mitigates age-related susceptibility to redox cycling agents. Redox Biol. 10:45–52. doi:10.1016/j.redox.2016.09.010.

Tobler R, Hermisson J, Schlötterer C. 2015. Parallel trait adaptation across opposing thermal environments in experimental *Drosophila melanogaster* populations. Evolution. 69(7):1745–1759. doi:10.1111/evo.12705.

Verta J-P, Jones FC. 2018. Predominance of *cis*-regulatory changes in parallel expression divergence of sticklebacks. [accessed 2021 Aug 19]. https://www.biorxiv.org/content/10.1101/412932v1.

Vieira C, Pasyukova EG, Zeng Z-B, Hackett JB, Lyman RF, Mackay TFC. 2000. Genotype-Environment Interaction for Quantitative Trait Loci Affecting Life Span in *Drosophila melanogaster*. Genetics. 154(1):213–227. doi:10.1093/genetics/154.1.213.

Wang MC, Bohmann D, Jasper H. 2003. JNK signaling confers tolerance to oxidative stress and extends lifespan in *Drosophila*. Dev Cell. 5(5):811–816. doi:10.1016/s1534-5807(03)00323-x.

Waskar M, Landis GN, Shen J, Curtis C, Tozer K, Abdueva D, Skvortsov D, Tavaré S, Tower J. 2009. *Drosophila melanogaster* p53 has developmental stage-specific and sex-specific effects on adult life span indicative of sexual antagonistic pleiotropy. Aging. 1(11):903–936.

Wayne ML, Soundararajan U, Harshman LG. 2006. Environmental stress and reproduction in *Drosophila melanogaster*: starvation resistance, ovariole numbers and early age egg production. BMC Evol Biol. 6(1):57. doi:10.1186/1471-2148-6-57.

Weasner BM, Weasner BP, Neuman SD, Bashirullah A, Kumar JP. 2016. Retinal Expression of the *Drosophila eyes absent* Gene Is Controlled by Several Cooperatively Acting *Cis*-regulatory Elements. PLOS Genet. 12(12):e1006462. doi:10.1371/journal.pgen.1006462.

Weinreich DM, Watson RA, Chao L. 2005. Perspective: Sign Epistasis and Genetic Costraint on Evolutionary Trajectories. Evolution. 59(6):1165–1174. doi:10.1111/j.0014-3820.2005.tb01768.x.

Williams CM, Watanabe M, Guarracino MR, Ferraro MB, Edison AS, Morgan TJ, Boroujerdi AFB, Hahn DA. 2014. Cold adaptation shapes the robustness of metabolic networks in *Drosophila melanogaster*. Evolution. 68(12):3505–3523. doi:10.1111/evo.12541.

Wittkopp PJ, Haerum BK, Clark AG. 2004. Evolutionary changes in *cis* and *trans* gene regulation. Nature. 430(6995):85–88. doi:10.1038/nature02698.

Wittkopp PJ, Haerum BK, Clark AG. 2008. Regulatory changes underlying expression differences within and between *Drosophila* species. Nat Genet. 40(3):346–350. doi:10.1038/ng.77.

Wittkopp PJ, Kalay G. 2012. *Cis*-regulatory elements: molecular mechanisms and evolutionary processes underlying divergence. Nat Rev Genet. 13(1):59–69. doi:10.1038/nrg3095.

Wittkopp PJ, Vaccaro K, Carroll SB. 2002. Evolution of *yellow* Gene Regulation and Pigmentation in *Drosophila*. Curr Biol. 12(18):1547–1556. doi:10.1016/S0960-9822(02)01113-2.

Wray GA. 2007. The evolutionary significance of *cis*-regulatory mutations. Nat Rev Genet. 8(3):206–216. doi:10.1038/nrg2063.

Wu S-C, Liao C-W, Pan R-L, Juang J-L. 2012. Infection-Induced Intestinal Oxidative Stress Triggers Organ-to-Organ Immunological Communication in *Drosophila*. Cell Host Microbe. 11(4):410–417. doi:10.1016/j.chom.2012.03.004.

Yi S-X, Moore CW, Lee RE. 2007. Rapid cold-hardening protects *Drosophila melanogaster* from cold-induced apoptosis. Apoptosis. 12(7):1183–1193. doi:10.1007/s10495-006-0048-2.

Zhang P, Wu W, Chen Q, Chen M. 2019. Non-Coding RNAs and their Integrated Networks. J Integr Bioinforma. 16(3):20190027. doi:10.1515/jib-2019-0027.

Zhao Y, He S, Liu C, Ru S, Zhao H, Yang Z, Yang P, Yuan X, Sun S, Bu D, et al. 2008. MicroRNA regulation of messenger-like noncoding RNAs: a network of mutual microRNA control. Trends Genet. 24(7):323–327. doi:10.1016/j.tig.2008.04.004.

Acknowledgements

I would like to thank my supervisor, John Parsch, for all his efforts, guidance and mentorship throughout my research pursuits. I would also like to thank all of the technical and administrative staff Gisela Brinkmann, Gabriele Kumpfmüller, Christine Rottenberger, Anica Vrljic, Ingrid Kroiß, Andrea Ibrahim and Bianca Hölldobler for their support, and especially Hildegard Lainer, for being a reliable authority on troubleshooting and for her extraordinary assistance in the lab. To my students over the years I thank you all for your hard work and I look forward to seeing all the amazing things you accomplish in the future.

For all the breakthrough realizations and sanity checks during coffee breaks, I thank my fellow doctoral students in the LSM program and in particular, my comrade and officemate Aleksei Belyi who was always ready to help. In terms of role models I could not ask for better than Eliza Argyridou and Amanda Glaser-Schmitt who freely offered their hard-won experience, outstanding knowledge and friendship. I am grateful to all past and present members of the Evolutionary Biology department, all of whom it has been a pleasure to know and work alongside, with special thanks to Annabella Königer, Sonja Grath, Ana Catalán and Vera Warmuth for their insight and influence on my professional development.

For who I am today, I thank my parents who gave their all and sacrificed so much to help me on my journey, and my sisters, Esther and Annalisa, who are my best friends and greatest sources of motivation and courage. To Fabian, I thank you for all your empathy and patience through the work days that stretched into work nights, where I needed "just another hour".