Year : 2017

THE EFFECTS OF CLINAL POLYMORPHISMS ON DROSOPHILA LIFE HISTORY

Durmaz Mukaddes Esra

Durmaz Mukaddes Esra, 2017, THE EFFECTS OF CLINAL POLYMORPHISMS ON DROSOPHILA LIFE HISTORY

Originally published at : Thesis, University of Lausanne

Posted at the University of Lausanne Open Archive http://serval.unil.ch
Document URN : urn:nbn:ch:serval-BIB_28A5F9F485689

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Département d'Ecologie et Évolution

THE EFFECTS OF CLINAL POLYMORPHISMS ON *DROSOPHILA* LIFE HISTORY

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

Mukaddes Esra DURMAZ

Master de l'Université de Hacettepe, Turkey

Jury

Prof. Edward FARMER, président
Prof. Thomas FLATT, directeur de thèse
Prof. Tadeusz KAWECKI, co-directeur
Prof. Wolf BLANCKENHORN, expert
Prof. Fisun HAMARATOGLU, expert

Lausanne 2017

Table of Contents

Acknowledgements

First and foremost, I would like to thank my supervisor Prof. Thomas Flatt. I am truly grateful for his guidance and giving me the opportunity to work in this amazing scientific environment. I really appreciate all his support, amazingly quick responses for help and most importantly his trust on me. Thank you, Thomas.

I additionally want to thank Prof. Tadeusz Kawecki, my co-supervisor, members of Kawecki Lab for all the scientific discussion and advice.

Also, I would like to thank the members of my thesis committee Prof. Wolf Blanckernhorn, Prof. Fisun Hamaratoglu and Prof. Edward Farmer for encouragement and helpful comments on my thesis work.

Furthermore, I am very grateful to all past and present members of the Flatt Lab at the University of Lausanne, in particular to Dr. Martin Kapun, Dr. Kathrin Garschall, Clare Benson and Marisa Rodrigues. It was a pleasure working with you and without your help this thesis would not have been possible.

I would like to thank all my colleagues and friends at the University of Lausanne, especially to Arnaud, Caroline, Cindy, Guillaume, Helena, Jessica, Luis, Paul and Vera. Your friendship, support and help made my Ph.D. journey an amazing experience.

I would also like to thank my dearest friends Selen, Erdinc, Olcay, Eylul, Safinaz, Umut and Yigit, who were always there, whenever and wherever I needed them.

Finally, I extend my deepest thanks to my family for their support and patience. You are the true heroes of this Ph.D. work.

Abstract

Life-history traits are the most important determinants of Darwinian fitness and thus the prime phenotypic targets of selection. However, despite their importance for adaptation, the genetic basis of these fitness-related traits in natural populations remains poorly understood. The goal of my Ph.D. thesis is to study the phenotypic effects of genetic polymorphisms that exhibit putatively adaptive clinal differentiation along the North American east coast in *Drosophila melanogaster*. In Chapter 1, I provide a summary of life-history adaptation, spatially varying selection, phenotypic plasticity and *Drosophila* life-history. In Chapter 2, I examine the phenotypic effects of a strongly clinal variant, consisting of two single nucleotide polymorphisms (SNPs), in the insulin signaling transcription factor *foxo* under different thermal and dietary conditions. *foxo* has previously been implicated in life-history regulation using loss-of-function mutants and transgenes, but nothing is known about the effects of natural variants at this locus. My experiments show that the *foxo* polymorphism makes an important contribution to clinal variation in multiple fitness-related traits. Similarly, in Chapter 3, my colleagues and I investigate phenotypic clines along the North American east coast and the contribution of the 2-SNP *foxo* variant to patterns of clinal trait differentiation. In Chapter 4, I summarize our ongoing work on using the CRISPR/Cas9 genome editing technique to manipulate and functionally test *foxo* SNPs. In Chapter 5, we show that the inversion polymorphism *In(3R)Payne*, which exhibits adaptive clinal differentiation along the North American east coast, contributes to the well-known cline in body size. While *In(3R)P* is thought to be one of the main drivers of clinal adaptation, the phenotypic effects of this inversion are not well understood. In Chapter 6, I investigate whether *In(3R)P* contributes to variation in survival traits. I show that *In(3R)P* contributes to latitudinal clines in lifespan, starvation resistance and cold-shock survival. Finally, in Chapter 7, I provide a general discussion of my findings and an outlook for future work. Together, my dissertation work demonstrates that both polymorphisms examined here, are targets of spatially varying selection and that they have pleiotropic effects on several clinal life-history traits in *D. melanogaster*.

Résumé

Les traits d'histoire de vie sont les déterminants majeurs de la valeur adaptative (*fitness*) individuelle, au sens darwinien, et donc les principales cibles de la sélection naturelle. La base génétique de ces traits reste cependant peu comprise, malgré leur importance adaptative. Le but de ma thèse est l'étude des effets phénotypiques lié aux polymorphismes génétiques et leurs potentiels adaptatifs le long d'un gradient latitudinal, chez *Drosophila melanogaster*. Dans le premier chapitre, je propose une synthèse de nos connaissances sur les adaptations liées aux traits d'histoire de vie, les forces de sélection qui varient dans l'espace et la plasticité phénotypique chez la drosophile. Dans le chapitre 2, j'étudie les effets phénotypiques d'un variant clinal, consistant en deux polymorphismes nucléotidiques (SNPs) du facteur de transcription, *foxo*, associé à la voie de signalisation de l'insuline, sous différentes conditions de température et de régime alimentaire. Des études utilisant des transgènes et des mutants ayant perdu la fonction de *foxo*, ont montré que ce gène est impliqué dans la régulation de traits d'histoire de vie. Toutefois, à ce jour, les effets de mutation naturelles sur ce locus sont peu connues. Mes expériences montrent que le polymorphisme de *foxo* contribue fortement à la variation clinale de nombreux traits d'histoire de vie, le long de la côte nord-est américaine. Ce polymorphisme nucléotidique pourrait également contribuer à la variation phénotypique que l'on observe le long de la côte est américaine (Chapitre 3). Dans le chapitre 4, je résume notre expérience en cours, utilisant les techniques d'édition de génome CRISPR/Cas9 pour manipuler et tester ces mêmes variants génétiques de *foxo*. Dans le chapitre 5, nous montrons qu'un polymorphisme pour l'inversion *In(3R)Payne,* caractérisé par un gradient de différentiation adaptatif le long des côtes nord-est américaines, contribue à la variation de taille du corps des drosophiles, bien connue dans cette région. Alors que *In(3R)Payne* est supposé conditionner ce cline adaptatif, les effets phénotypiques de cette inversion sont peu connus à ce jour. Dans le chapitre 6, j'examine la contribution de *In(3R)Payne* aux variations des traits liés à la survie. Je montre que, non seulement *In(3R)Payne* joue un rôle dans la variation latitudinale de la survivance, mais aussi dans les variations de résistance à la famine et de survie aux chocs thermiques froids. Enfin, dans le chapitre 7, je propose une discussion générale de mes découvertes et propose des pistes pour poursuivre ces travaux. Globalement mon travail de thèse démontre que les deux polymorphismes étudiés sont les cibles de forces de sélection qui varient dans l'espace, et qu'ils ont des effets pléiotropiques sur un certain nombre de traits d'histoire de vie chez *Drosophila melanogaster*.

Chapter 1

Introduction to the Thesis

The major objective of my Ph.D. work is to examine the functional effects of potentially adaptive, clinally varying polymorphisms on *Drosophila* life-history in order to gain a better understanding of the genetics of adaptation.

Life history traits, such as age and size at maturity, fecundity, and lifespan, are the most important phenotypic components of Darwinian fitness and thus represent direct targets of natural selection (Stearns 1992; Flatt & Heyland 2011). Understanding the causes and consequences of life-history variation is thus of central importance for our understanding of adaptation (Barrett & Hoekstra 2011).

Despite a growing body of work on the molecular mechanisms that affect fitness-related traits and processes (e.g., growth and size, lifespan) – mainly from studies of large-effects mutants and transgenes in model organisms in the laboratory – we still know very little about the identity and the properties of naturally occurring loci and molecular polymorphisms that underpin adaptive variation in life-history traits (David *et al.* 1989; Mackay *et al.* 2009; Barrett & Hoekstra 2011; Le Corre & Kremer 2012). For example, quantitative trait locus (QTL) mapping, applied to populations and lines that are divergent for life-history traits, has been successfully employed to identify causative genomic regions of adaptive significance. However, the low resolution of QTL mapping (and of related mapping approaches) has typically made it difficult to identify the causative loci or quantitative trait nucleotides (QTNs) through fine-scale mapping (Mackay *et al.* 2009; Barrett & Hoekstra 2011; Le Corre & Kremer 2012; Pardo-Diaz *et al.* 2015). Thus, how naturally occurring polymorphisms contribute to adaptive life-history variation remains generally poorly understood.

One of the most powerful models for dissecting the genetic basis of life-history adaptation is the fruit fly *Drosophila melanogaster*, a cosmopolitan species of sub-Saharan African

origin, which has migrated out of Africa ~10,000 to 20,000 years ago, and colonized the New World and Australia during the 19th century (Fig. 1) (David & Bocquet 1975; David *et al.* 1989; De Jong & Bochdanovits 2003; Li & Stephan 2006; Hoffmann & Weeks 2007; Adrion *et al.* 2015). During this range expansion, this ancestrally tropical species has acquired major adaptations to novel temperate and seasonal habitats (David & Capy 1988; De Jong & Bochdanovits 2003; Paaby & Schmidt 2009). As a consequence of this evolutionary history, footprints of natural selection can be identified both at the phenotypic and genetic level by studying geographic patterns of life-history differentiation. A well-known example of this are clines, i.e gradual patterns of phenotypic and/or genetic change across environmental gradients, for example across latitude, presumably driven by gradients in temperature and/or seasonality. Indeed, a large body of work has identified numerous examples of clinal phenotypic and genetic differentiation among populations along the North American and Australian east coasts, gradients that span low-latitude (subtropical/tropical) to high-latitude (temperate habitats) (De Jong & Bochdanovits 2003; Schmidt *et al.* 2005a; b; Hoffmann & Weeks 2007; Paaby & Schmidt 2008; Kolaczkowski *et al.* 2011; Fabian *et al.* 2012; Cogni *et al.* 2013; 2014; Adrion *et al.* 2015; Kapun *et al.* 2016a).

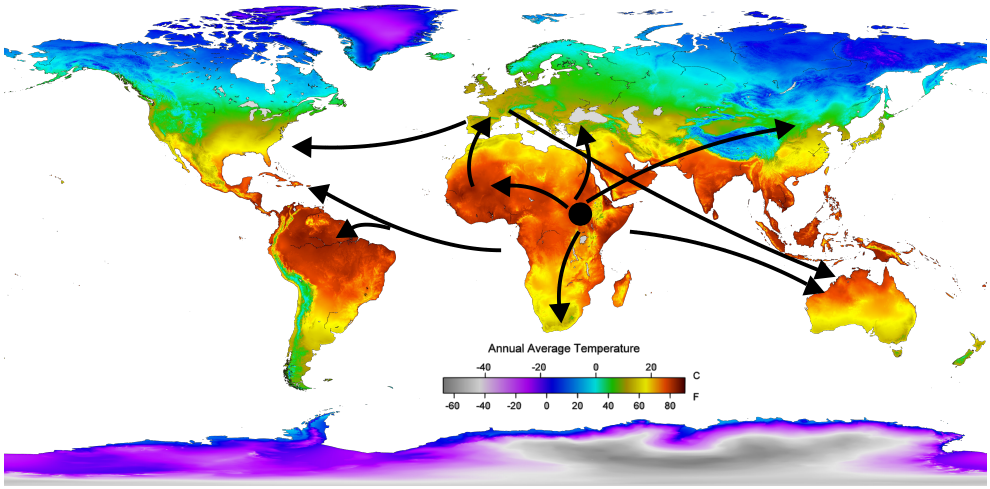


Fig 1. The colonization of Europe, Asia, the Americas and Australia by *Drosophila melanogaster*. The ancestral population in sub-Saharan Africa is shown with a black circle and the colonization routes are represented by arrows. Figure adapted from David & Capy (1988) (www.wikimedia.org.).

At the phenotypic level, major patterns of latitudinal trait differentiation have been observed for total body size and size-related traits, fecundity, stress resistance traits such as chill coma recovery, lifespan, and the propensity to undergo reproductive diapause or dormancy. Importantly, many of these clinal patterns have been found on multiple continents, thus strongly suggesting that these clines are adaptive and shaped by convergent spatially varying (clinal) selection (David & Capy 1988; David *et al.* 1989; Munjal *et al.* 1997; Schmidt *et al.* 2000; Agis & Schlötterer 2001; Hoffmann *et al.* 2001; Sezgin 2004; Schmidt *et al.* 2005a; Rako *et al.* 2007; Paaby *et al.* 2010; Kolaczkowski *et al.* 2011; Hoffmann *et al.* 2012; Hut *et al.* 2013; Campo *et al.* 2013; Paaby *et al.* 2014; Behrman *et al.* 2015; Mathur & Schmidt 2017). Flies from high-latitude populations are typically characterized by faster development, lower egg-to-adult survival (viability), larger body size, reduced wing loading, lower fecundity, higher propensity of reproductive diapause, increased resistance to starvation, cold and heat stress, and longer lifespan as compared to flies from low-latitude populations (Coyne & Beecham 1987; Karan *et al.* 1998; Azevedo *et al.* 1998; Land *et al.* 1999; Bochdanovits & de Jong 2003a; b; Hoffmann *et al.* 2005; Schmidt *et al.* 2005b; Schmidt & Paaby 2008; Folguera *et al.* 2008; Goenaga *et al.* 2013; Bhan *et al.* 2014; Mathur & Schmidt 2017).

Similarly, at the genetic level, numerous genotype frequency clines have been reported for allozymes, microsatellites, chromosomal inversion polymorphisms, and single nucleotide polymorphisms (SNPs), most recently on a genome-wide level for both the Australian and North American clines (Mettler *et al.* 1977; David *et al.* 1989; Bellen *et al.* 1992; Boussy *et al.* 1998; Agis & Schlötterer 2001; Weeks *et al.* 2002; Kennington *et al.* 2003; De Jong & Bochdanovits 2003; Hoffmann *et al.* 2004; Anderson *et al.* 2005; Kirkpatrick 2006; Rako *et al.* 2006; 2007; Kennington *et al.* 2007; Paaby *et al.* 2010; Kolaczkowski *et al.* 2011; Lee *et al.* 2011; Hoffmann *et al.* 2012; Fabian *et al.* 2012; Reinhardt *et al.* 2014; Bergland *et al.*

2014; Paaby *et al.* 2014; Bergland *et al.* 2016; Kapun *et al.* 2016). Several genomic studies of clinal differentiation have identified numerous clinally varying SNPs located in genes that have previously been characterized in molecular genetic and functional studies. Although many of these loci are known to play a major role in affecting the development and physiology of fitness-related traits, almost nothing is known yet about the fitness effects of naturally occurring SNPs located in or in proximity to these genes.

In a previous study from our group, Fabian *et al.* (2012) have performed the first genome-wide analysis of clinal differentiation along the North American east coast. They uncovered pervasive, genome-wide patterns of clinal genetic differentiation based on an F_{ST} outlier approach. Hundreds of clinally varying SNPs were found to reside in loci involved in the insulin/insulin-like growth factor signaling (IIS) / target of rapamycin (TOR), ecdysone, torso, EGFR, TGFβ/BMP, JAK/STAT, lipid metabolism, immunity and circadian rhythm pathways, pathways that are all involved in the molecular and physiological regulation of fitness-related traits, including growth, size, reproduction, stress resistance, somatic maintenance, and lifespan. In addition, many of the identified variants were found to exhibit parallel differentiation along the Australian cline, thus strengthening the case for these SNPs being subject to spatially varying (clinal) selection (Kolaczkowski *et al.* 2011; Fabian *et al.* 2012; Reinhardt *et al.* 2014; Kapun *et al.* 2016a). Notably, Fabian *et al*. (2012) found that the clinally varying SNPs are not homogeneously distributed along the genome. The majority of clinally varying SNPs (between >50-79%, depending on the analysis) is located on chromosomal arm *3R* in a region spanned by a large (approx. 8 Mb), cosmopolitan and clinally varying chromosomal inversion, *In(3R)Payne*.

As mentioned above, several molecular pathways that harbor clinally varying loci and SNPs in this dataset have previously been found in laboratory studies of large-effect mutants and transgenes to affect the molecular regulation of life history (Flatt & Heyland 2011; Fabian

et al. 2012). In particular, Fabian *et al.* (2012) identified many strongly clinally varying SNPs in multiple components of the IIS/TOR pathway, including SNPs located in *Drosophila insulin-like peptides 3 and 5* (*dilp3*, *dilp5*), *insulin-like receptor* (*InR*), *phosphatidyl-inositol-4,5-bis-phosphate 3-kinase* (*Pi3K*), *forkhead box-O transcription factor* (*foxo*), the *foxo* regulator *14-3-3ε*, *target of brain insulin* (*tobi*), *tuberous sclerosis complex 1* (*Tsc1*), *target of rapamycin* (*Tor*), and other loci involved in IIS (Fig. 2) (Fabian *et al.* 2012).

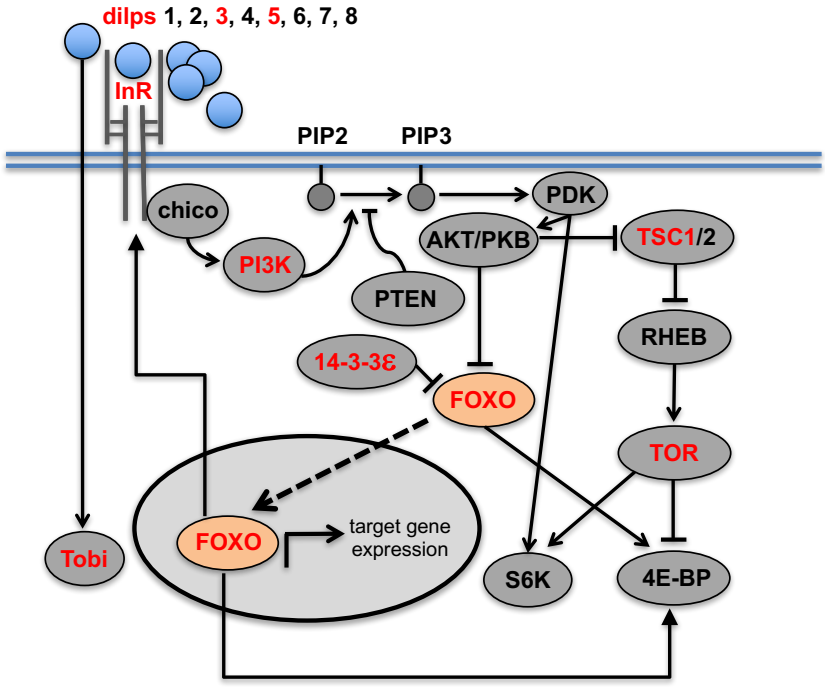


Figure 2. Clinal candidates in the insulin/TOR signaling pathway. Overview of the insulin/insulin-like growth factor signaling (IIS) / target of rapamycin (TOR) pathway in *D. melanogaster* (Oldham & Hafen 2003; Giannakou & Partridge 2007; Teleman 2010). Genes that harbor strongly clinally varying SNPs across latitude, identified by Fabian *et al.* (2012), are highlighted in red; arrows indicate activation and bar-ended lines represent inhibitory effects (see Chapter 2 for details).

Interestingly, loss-of-function mutations in the IIS/TOR pathway have been shown to have evolutionarily conserved effects on the regulation of growth, size, reproduction and lifespan in *Drosophila*, *C. elegans*, and the mouse (Tatar 2003; Murphy 2013; Papatheodorou *et al.* 2014). For example, it has been shown that reduced IIS, which leads to the activation of

FOXO, a central transcription factor in IIS, results in smaller body size, reduced fecundity and ovarian arrest. Loss-of-function mutations of *foxo*, for instance, cause prolonged developmental time, smaller body size, reduced fecundity, shorter lifespan and increased sensitivity to oxidative and starvation stress (Jünger *et al.* 2003; Kramer *et al.* 2003; Hwangbo *et al.* 2004; Giannakou *et al.* 2007; Kramer *et al.* 2008; Slack *et al.* 2011). Accordingly, it has been hypothesized that the IIS/TOR pathway might represent a major mediator of life-history variation and adaptation in *Drosophila* (De Jong & Bochdanovits 2003; Paaby *et al.* 2010; 2014). Yet, to date, we only have a small handful of examples of naturally occurring polymorphisms in the IIS and other functionally important pathways that have been shown to affect phenotypic variation in fitness components in natural populations.

One example is the *insulin-like receptor* (*InR*), which is well known from mutant studies to have pleiotropic effects on various fitness-related traits, including developmental time, body size, ovarian development, lifespan and stress resistance (Tatar *et al.* 2001; Okamoto *et al.* 2013; Liu *et al.* 2013). Interestingly, Paaby and collaborators (Paaby *et al.* 2010; 2014) have identified a clinally varying insertion-deletion (indel) polymorphism in *InR* which seems to be involved in climate adaptation and which confers pleiotropic effects on multiple life-history traits. Another example concerns the genetic factors underlying reproductive diapause or dormancy (Schmidt *et al.* 2008; Paaby *et al.* 2014), a plastic and pleiotropic trait syndrome that affects, in response to cold temperatures and shortened photoperiod, ovarian development, fecundity, stress resistance and lifespan, and which is thought to represent an overwintering adaptation of temperate populations (Saunders *et al.* 1989; Schmidt *et al.* 2005a; b; Schmidt & Conde 2006). Using QTL mapping, Schmidt *et al.* (2008) have identified a naturally occurring SNP in the gene *couch potato* (*cpo*) which underlies the clinal

variation in diapause propensity along the North American east coast (Schmidt *et al.* 2008; Paaby *et al.* 2014).

Reproductive diapause represents a prominent example for adaptive phenotypic plasticity associated with environmental heterogeneity (Williams & Sokolowski 1993; Kubrak *et al.* 2014; Zhao *et al.* 2015); in stark contrast to flies from Maine, for example, the majority of genotypes in Florida is unable to enter diapause under diapause-inducing conditions (Schmidt *et al.* 2005a; b; Schmidt & Conde 2006, Schmidt *et al.* 2008). However, generally speaking, little is known about adaptive phenotypic plasticity in the context of clinal gradients. An attractive hypothesis is that genotypes from temperate, strongly seasonal high-latitude populations might be more plastic in response to environmental heterogeneity (e.g., temperature change, seasonality, food shortage) than those from tropical, low-latitude populations (Overgaard *et al.* 2011; Klepsatel *et al.* 2013; Mathur & Schmidt 2017). Yet, our understanding of plasticity and genotype-by-environment interactions across latitudinal gradients remains limited.

Interestingly, both *InR* and *cpo* are located in the genomic region spanned by the *In(3R)Payne* inversion (see above), which itself has been associated with climate adaptation. This large inversion polymorphism varies clinally along latitude on multiple continents, most prominently along the North American and Australian east coast; it exhibits intermediate frequency (~50%) at low latitudes but is rare or absent at high latitudes (Mettler *et al.* 1977; Stalker 1980; Inoue & Watanabe 1992; Anderson *et al.* 2005; Matzkin 2005; Fabian *et al.* 2012; Kapun *et al.* 2014; 2016a). Remarkably, the region spanned by *In(3R)Payne* actually contains the majority of clinally varying SNPs along the North American east coast (Fabian *et al*. 2012; Kapun *et al.* 2016a). Indeed, recent findings suggest that this inversion is adaptively maintained by spatially varying (clinal) selection, independent of admixture or population structure (Kapun *et al.* 2016a). It has thus been hypothesized that *In(3R)Payne* might

represent the major driver of clinality in North American and Australian *D. melanogaster* (De Jong & Bochdanovits 2003; Kennington *et al.* 2006; Kapun *et al.* 2016a). Perhaps in line with this notion, *In(3R)Payne* harbors, for example, several major genes of the IIS/TOR pathway (De Jong & Bochdanovits 2003; Paaby *et al.* 2010; Fabian *et al.* 2012; Paaby *et al.* 2014). Despite its potential importance in clinal adaptation, however, little is known about the effects of *In(3R)Payne* on clinally varying traits. A small number studies from Australia has found effects on body size (Weeks *et al.* 2002; Anderson *et al.* 2005; Rako *et al.* 2006; Kennington *et al.* 2007). In suppport of these previous findings, we have recently observed similar phenotypic effects for the North American cline: *In(3R)Payne* is strongly associated with body size (Kapun *et al.* 2016b). These parallel phenotypic effects across multiple clines clearly strengthen the case for spatially varying selection, but more data are needed to understand how *In(3R)Payne* contributes to clinal variation in fitness-related traits other than body size.

Thus, while genomic studies of clinal differentiation and adaptation have successfully identified many putatively adaptive polymorphisms, the hypothesized functional links between these candidate polymorphisms and variation in fitness-related traits (i.e., phenotypic targets of clinal selection) are not sufficiently well understood yet, for establishing such causative connections requires labor-intensive functional genetic studies of these natural variants (Barrett & Hoekstra 2011; Flatt 2016; Kapun *et al.* 2016a).

Here, in this Ph.D. thesis I provide functional evidence for the involvement of clinally varying polymorphisms in shaping patterns of adaptive clinal differentiation in life-history traits along the North American east coast. Based on our previous results (Fabian *et al.* 2012), I decided to prioritize two clinally varying candidate polymorphisms for functional experimentation: (1) a strongly clinal 2-SNP variant in the IIS transcription factor gene *foxo*,

and (2) the clinal chromosomal inversion polymorphism *In(3R)Payne*. **Examining the life-history effects of these polymorphisms defines the two central aims of my Ph.D. thesis.**

Ph.D. Objectives

The two major objectives of my Ph.D. dissertation research are defined follows:

Aim 1: To investigate the functional effects of a clinal polymorphism in the insulin signaling transcription factor gene *foxo* on *Drosophila* life history. Specifically, in this part of the thesis I investigated the functional links between a strongly clinal 2-SNP variant in the IIS transcription factor *foxo* and life-history phenotypes (**Chapters 2-4**). To examine the interplay between clinal variation and phenotypic plasticity, I examined the effects of this *foxo* variant under different thermal and dietary conditions (**Chapter 2**). To do so, my colleagues and I isolated this variant for experimental work by reconstituting outbred populations from individually sequenced lines of the *Drosophila* Genetic Reference Panel (DGRP) that are either fixed for the low-latitude or the high-latitude allelic state for this polymorphism. In addition, we examined clinal life-history variation among natural populations of *D. melanogaster* along the North American east coast in order to directly compare these traits clines to the phenotypic effects of the *foxo* polymorphism (**Chapter 3**). Finally, in ongoing work I am currently aiming to determine the causative effects of this variant using the CRISPR/Cas9 genome editing technique (**Chapter 4**).

Aim 2: To investigate life-history effects of the clinal inversion polymorphism *In(3R)Payne* in *D. melanogaster*. In this part of the thesis, I set out to examine the effects of a second clinally varying variant, the inversion polymorphism *In(3R)Payne*. To examine the impact of geography (clinality) and/or inversion karyotype (i.e., inverted vs. uninverted karyotypes) on clinal trait differentiation along the North American east coast, we established isochromosomal homokaryon lines isolated from populations approximating the end points of

the North American cline (Florida, Maine) and then assayed the lines for several survival traits known to vary clinally. Since temperature represents the presumably most important environmental factor covarying with latitude, I investigated the phenotypic effects of *In(3R)Payne* under different thermal conditions.

Together, my work provides novel insights into the genetic architecture of clinal adaptation and the interplay between clinality and phenotypic plasticity; in particular, my findings highlight the importance of natural variation in insulin signaling and of a major chromosomal inversion polymorphism in shaping life-history differentiation among natural populations of *D. melanogaster*.

References

Adrion JR, Hahn MW, Cooper BS (2015) Revisiting classic clines in *Drosophila melanogaster* in the age of genomics. *Trends in Genetics*, **31**, 434–444.

Agis M, Schlötterer C (2001) Microsatellite variation in natural *Drosophila melanogaster* populations from New South Wales (Australia) and Tasmania. *Molecular Ecology*, **10**, 1197–1205.

Anderson AR, Hoffmann AA, McKechnie SW, Umina PA, Weeks AR (2005) The latitudinal cline in the *In(3R)Payne* inversion polymorphism has shifted in the last 20 years in Australian *Drosophila melanogaster* populations. *Molecular Ecology*, **14**, 851–858.

Azevedo R, James AC, McCabe J, Partridge L (1998) Latitudinal variation of wing : thorax size ratio and wing-aspect ratio in *Drosophila melanogaster*. *Evolution*, **52**, 1353–1362.

Barrett RDH, Hoekstra HE (2011) Molecular spandrels: tests of adaptation at the genetic level. *Nature Reviews Genetics*, **12**, 767–780.

Behrman EL, Watson SS, O'Brien KR, Heschel MS, Schmidt PS (2015) Seasonal variation in life history traits in two *Drosophila* species. *Journal of Evolutionary Biology*, **28**, 1691–1704.

Bellen HJ, Vaessin H, Bier E *et al.* (1992) The *Drosophila* couch potato gene: an essential gene required for normal adult behavior. *Genetics*, **131**, 365–375.

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*. (D Bolnick, Ed,). *PLoS Genetics*, **10**, e1004775.

Bergland AO, Tobler R, Gonzalez J, Schmidt P, Petrov D (2016) Secondary contact and local adaptation contribute to genome-wide patterns of clinal variation in *Drosophila melanogaster*. *Molecular Ecology*, **25**, 1157–1174.

Bhan V, Parkash R, Aggarwal DD (2014) Effects of body-size variation on flight-related traits in latitudinal populations of *Drosophila melanogaster*. *Journal of Genetics*, **93**, 103–112.

Bochdanovits Z, de Jong G (2003a) Experimental evolution in *Drosophila melanogaster*: Interaction of temperature and food quality selection regimes. *Evolution*, **57**, 1829–1836.

Bochdanovits Z, de Jong G (2003b) Temperature dependence of fitness components in geographical populations of *Drosophila melanogaster*: changing the association between size and fitness. *Biological Journal of the Linnean Society*, **80**, 717–725.

Boussy IA, Itoh M, Itoh M, Rand D, Woodruff RC (1998) Origin and decay of the P element-associated latitudinal cline in Australian *Drosophila melanogaster*. *Genetica*, **104**, 45–57.

Campo D, Lehmann K, Fjeldsted C *et al.* (2013) Whole-genome sequencing of two North American *Drosophila melanogaster* populations reveals genetic differentiation and positive selection. *Molecular Ecology*, **22**, 5084–5097.

Cogni R, Kuczynski C, Koury S *et al.* (2013) The intensity of selection acting on the couch potato gene--spatial-temporal variation in a diapause cline. *Evolution*, **68**, 538–548.

Cogni R, Kuczynski K, Lavington E *et al.* (2014) Variation in *Drosophila melanogaster* central metabolic genes appears driven by natural selection both within and between populations. *Proceedings of the Royal Society B: Biological Sciences*, **282**, 20142688–20142688.

Coyne JA, Beecham E (1987) Heritability of two morphological characters within and among natural populations of *Drosophila melanogaster*. *Genetics*, **117**, 727–737.

David JR, Bocquet C (1975) Evolution in a cosmopolitan species: genetic latitudinal clines in *Drosophila melanogaster* wild populations. *Experientia*, **31**, 164–166.

David JR, Capy P (1988) Genetic variation of *Drosophila melanogaster* natural populations. *Trends in Genetics*, **4**, 106–111.

David JR, Alonso-Moraga A, Borai F *et al.* (1989) Latitudinal variation of Adh gene frequencies in *Drosophila melanogaster*: a Mediterranean instability. *Heredity*, **62**, 11–16.
De Jong G, Bochdanovits Z (2003) Latitudinal clines in *Drosophila melanogaster*: body size, allozyme frequencies, inversion frequencies, and the insulin-signalling pathway. *Journal of Genetics*, **82**, 207–223.
Endler JA (1977) Geographic variation, speciation, and clines. *Monographs in population biology*, **10**, 1–246.
Fabian DK, Kapun M, Nolte V *et al.* (2012) Genome-wide patterns of latitudinal differentiation among populations of *Drosophila melanogaster* from North America. *Molecular Ecology*, **21**, 4748–4769.
Flatt T (2016) Genomics of clinal variation in *Drosophila*: disentangling the interactions of selection and demography. *Molecular Ecology*, **25**, 1023–1026.
Flatt T, Heyland A (2011) *Mechanisms of Life History Evolution*. Oxford University Press.
Folguera G, Ceballos S, Spezzi L, Fanara JJ, Hasson E (2008) Clinal variation in developmental time and viability, and the response to thermal treatments in two species of *Drosophila*. *Biological Journal of the Linnean Society*, **95**, 233–245.
Giannakou ME, Partridge L (2007) Role of insulin-like signalling in *Drosophila* lifespan. *Trends in Biochemical Sciences*, **32**, 180–188.
Giannakou ME, Goss M, Jacobson J *et al.* (2007) Dynamics of the action of d*FOXO* on adult mortality in *Drosophila*. *Aging Cell*, **6**, 429–438.
Goenaga J, Fanara JJ, Hasson E (2013) Latitudinal variation in starvation resistance is explained by lipid content in natural populations of *Drosophila melanogaster*. *Evolutionary Biology*, **40**, 601–612.
Hoffmann AA, Weeks AR (2007) Climatic selection on genes and traits after a 100 year-old invasion: a critical look at the temperate-tropical clines in *Drosophila melanogaster* from eastern Australia. *Genetica*, **129**, 133–147.
Hoffmann AA, Blacket MJ, McKechnie SW *et al.* (2012) A proline repeat polymorphism of the Frost gene of *Drosophila melanogaster* showing clinal variation but not associated with cold resistance. *Insect Molecular Biology*, **21**, 437–445.
Hoffmann AA, Hallas R, Sinclair C, Mitrovski P (2001) Levels of variation in stress resistance in *Drosophila* among strains, local populations, and geographic regions: Patterns for desiccation, starvation, cold resistance, and associated traits. *Evolution*, **55**, 1621–1630.
Hoffmann AA, Shirriffs J, Scott M (2005) Relative importance of plastic vs genetic factors in adaptive differentiation: geographical variation for stress resistance in *Drosophila melanogaster* from from eastern Australia. *Functional Ecology*, **19**, 222–227.
Hoffmann A, Sgrò C, Weeks A (2004) Chromosomal inversion polymorphisms and adaptation. *Trends in Ecology & Evolution*, **19**, 482–488.
Hut RA, Paolucci S, Dor R, Kyriacou CP, Daan S (2013) Latitudinal clines: an evolutionary view on biological rhythms. *Proceedings of the Royal Society B: Biological Sciences*, **280**, 20130433–20130433.
Hwangbo DS, Gersham B, Tu M-P, Palmer M, Tatar M (2004) *Drosophila dFOXO* controls lifespan and regulates insulin signalling in brain and fat body. *Nature*, **429**, 562–566.
Inoue Y, Watanabe TK (1992) Chromosomal polymorphism in isofemale lines and cage populations of *Drosophila melanogaster*. *Evolution*, **46**, 797.
Jünger MA, Rintelen F, Stocker H *et al.* (2003) The *Drosophila* forkhead transcription factor *FOXO* mediates the reduction in cell number associated with reduced insulin signaling. *Journal of biology*, **2**, 20.
Kapun M, Fabian DK, Goudet J, Flatt T (2016a) Genomic evidence for adaptive inversion clines in *Drosophila melanogaster*. *Molecular Biology and Evolution*, **33**, 1317–1336.
Kapun M, Schalkwyk H, McAllister B, Flatt T, Schlötterer C (2014) Inference of

chromosomal inversion dynamics from Pool-Seq data in natural and laboratory populations of *Drosophila melanogaster*. *Molecular Ecology*, **23**, 1813–1827.
Kapun M, Schmidt C, Durmaz E, Schmidt PS, Flatt T (2016b) Parallel effects of the inversion *In(3R)Payne* on body size across the North American and Australian clines in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, **29**, 1059–1072.
Karan D, Dahiya N, Munjal AK *et al.* (1998) Desiccation and starvation tolerance of adult *Drosophila*: opposite latitudinal clines in natural populations of three different species. *Evolution*, **52**, 825.
Kennington WJ, Gockel J, Partridge L (2003) Testing for asymmetrical gene flow in a *Drosophila melanogaster* body-size cline. *Genetics*, **165**, 667–673.
Kennington WJ, Hoffmann AA, Partridge L (2007) Mapping regions within cosmopolitan inversion *In(3R)Payne* associated with natural variation in body size in *Drosophila melanogaster*. *Genetics*, **177**, 549–556.
Kennington WJ, Partridge L, Hoffmann AA (2006) Patterns of diversity and linkage disequilibrium within the cosmopolitan inversion *In(3R)Payne* in *Drosophila melanogaster* are indicative of coadaptation. *Genetics*, **172**, 1655–1663.
Kirkpatrick M (2006) Chromosome inversions, local adaptation and speciation. *Genetics*, **173**, 419–434.
Klepsatel P, Gáliková M, De Maio N *et al.* (2013) Variation in thermal performance and reaction norms among populations of *Drosophila melanogaster*. *Evolution*, **67**, 3573–3587.
Kolaczkowski B, Kern AD, Holloway AK, Begun DJ (2011) Genomic differentiation between temperate and tropical Australian populations of *Drosophila melanogaster*. *Genetics*, **187**, 245–260.
Kramer JM, Davidge JT, Lockyer JM, Staveley BE (2003) Expression of *Drosophila FOXO* regulates growth and can phenocopy starvation. *BMC developmental biology*, **3**, 5.
Kramer JM, Slade JD, Staveley BE (2008) *foxo* is required for resistance to amino acid starvation in *Drosophila*. *Genome*, **51**, 668–672.
Kubrak OI, Kučerová L, Theopold U, Nässel DR (2014) The sleeping beauty: how reproductive diapause affects hormone signaling, metabolism, immune response and somatic maintenance in *Drosophila melanogaster* (F Leulier, Ed,). *PLoS ONE*, **9**, e113051.
Land JVT, Putten PV, Zwaan, Kamping, Delden WV (1999) Latitudinal variation in wild populations of *Drosophila melanogaster*: heritabilities and reaction norms. *Journal of Evolutionary Biology*, **12**, 222–232.
Le Corre V, Kremer A (2012) The genetic differentiation at quantitative trait loci under local adaptation. *Molecular Ecology*, **21**, 1548–1566.
Lee SF, Sgrò CM, Shirriffs J *et al.* (2011) Polymorphism in the couch potato gene clines in eastern Australia but is not associated with ovarian dormancy in *Drosophila melanogaster*. *Molecular Ecology*, **20**, 2973–2984.
Li H, Stephan W (2006) Inferring the demographic history and rate of adaptive substitution in *Drosophila*. *PLoS Genetics*, **2**, 1580–1589.
Liu J, Visser-Grieve S, Boudreau J *et al.* (2013) Insulin activates the insulin receptor to downregulate the PTEN tumour suppressor. *Oncogene*, **33**, 3878–3885.
Mackay TFC, Stone EA, Ayroles JF (2009) The genetics of quantitative traits: challenges and prospects. *Nature Reviews Genetics*, **10**, 565–577.
Mathur V, Schmidt PS (2017) Adaptive patterns of phenotypic plasticity in laboratory and field environments in *Drosophila melanogaster*. *Evolution*, **71**, 465–474.
Matzkin LM (2005) The structure and population genetics of the breakpoints associated with the cosmopolitan chromosomal inversion *In(3R)Payne* in *Drosophila melanogaster*. *Genetics*, **170**, 1143–1152.
Mettler LE, Voelker RA, Mukai T (1977) Inversion clines in populations of *Drosophila*

melanogaster. *Genetics*, **87**, 169–176.
Munjal AK, Karan D, Gibert P *et al.* (1997) Thoracic trident pigmentation in *Drosophila melanogaster*: latitudinal and altitudinal clines in Indian populations. *Genetics Selection Evolution*, **29**, 601.
Murphy CT (2013) Insulin/insulin-like growth factor signaling in *C. elegans*. *WormBook*, 1–43.
Okamoto N, Nakamori R, Murai T *et al.* (2013) A secreted decoy of *InR* antagonizes insulin/IGF signaling to restrict body growth in *Drosophila*. *Genes & Development*, **27**, 87–97.
Oldham S, Hafen E (2003) Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control. *Trends in Cell Biology*, **13**, 79–85.
Overgaard J, Kristensen TN, Mitchell KA, Hoffmann AA (2011) Thermal tolerance in widespread and tropical *Drosophila* Species: Does phenotypic plasticity increase with latitude? *American Naturalist*, **178**, S80–S96.
Paaby AB, Schmidt PS (2008) Functional significance of allelic variation at methuselah, an aging gene in *Drosophila* (MW Hahn, Ed,). *PLoS ONE*, **3**, e1987.
Paaby AB, Schmidt PS (2009) Dissecting the genetics of longevity in*Drosophila melanogaster*. *Fly*, **3**, 29–38.
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**, 3395–3409.
Paaby AB, Blacket MJ, Hoffmann AA, Schmidt PS (2010) Identification of a candidate adaptive polymorphism for *Drosophila* life history by parallel independent clines on two continents. *Molecular Ecology*, **19**, 760–774.
Papatheodorou I, Petrovs R, Thornton JM (2014) Comparison of the mammalian insulin signalling pathway to invertebrates in the context of *FOXO*-mediated ageing. *Bioinformatics*, **30**, 2999–3003.
Pardo-Diaz C, Salazar C, Jiggins CD (2015) Towards the identification of the loci of adaptive evolution. *Methods in Ecology and Evolution*, **6**, 445–464.
Rako L, Anderson AR, Sgrò CM, Stocker AJ, Hoffmann AA (2006) The association between inversion *In(3R)Payne* and clinally varying traits in *Drosophila melanogaster*. *Genetica*, **128**, 373–384.
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. *Molecular Ecology*, **16**, 2948–2957.
Reinhardt JA, Kolaczkowski B, Jones CD, Begun DJ, Kern AD (2014) Parallel geographic variation in *Drosophila melanogaster*. *Genetics*, **197**, 361–373.
Saunders DS, Henrich VC, Gilbert LI (1989) Induction of diapause in *Drosophila melanogaster*: photoperiodic regulation and the impact of arrhythmic clock mutations on time measurement. *Proceedings of the National Academy of Sciences*, **86**, 3748–3752.
Schmidt PS, Conde DR (2006) Environmental heterogeneity and the maintenance of genetic variation for reproductive diapause in *Drosophila melanogaster*. *Evolution*, **60**, 1602–1611.
Schmidt PS, Paaby AB (2008) Reproductive diapause and life-history clines in North American populations of *Drosophila melanogaster*. *Evolution*, **62**, 1204–1215.
Schmidt PS, Duvernell DD, Eanes WF (2000) Adaptive evolution of a candidate gene for aging in *Drosophila*. *Proceedings of the National Academy of Sciences*, **97**, 10861–10865.
Schmidt PS, Matzkin L, Ippolito M, Eanes WF (2005a) Geographic variation in diapause incidence, life-history traits, and climatic adaptation in *Drosophila melanogaster*. *Evolution*, **59**, 1721–1732.

Schmidt PS, Paaby AB, Heschel MS (2005b) Genetic variance for diapause expression and associated life histories in *Drosophila melanogaster*. *Evolution*, **59**, 2616–2625.
Schmidt PS, Zhu C-T, Das J *et al.* (2008) An amino acid polymorphism in the couch potato gene forms the basis for climatic adaptation in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, **105**, 16207–16211.
Sezgin E (2004) Single-locus latitudinal clines and their relationship to temperate adaptation in metabolic genes and derived alleles in *Drosophila melanogaster*. *Genetics*, **168**, 923–931.
Slack C, Giannakou ME, Foley A, Goss M, Partridge L (2011) d*FOXO*-independent effects of reduced insulin-like signaling in *Drosophila*. *Aging Cell*, **10**, 735–748.
Stalker HD (1980) Chromosome-studies in wild populations of *Drosophila melanogaster*. 2. Relationship of inversion frequencies to latitude, season, wing-loading and flight activity. *Genetics*, **95**, 211–223.
Stearns SC (1992) *The Evolution of Life Histories*. Oxford University Press.
Tatar M (2003) The endocrine regulation of aging by insulin-like signals. *Science*, **299**, 1346–1351.
Tatar M, Kopelman A, Epstein D *et al.* (2001) A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function. *Science*, **292**, 107–110.
Teleman AA (2010) Molecular mechanisms of metabolic regulation by insulin in *Drosophila*. *The Biochemical journal*, **425**, 13–26.
Weeks AR, McKechnie SW, Hoffmann AA (2002) Dissecting adaptive clinal variation: markers, inversions and size/stress associations in *Drosophila melanogaster* from a central field population. *Ecology Letters*, **5**, 756–763.
Williams KD, Sokolowski MB (1993) Diapause in *Drosophila melanogaster* females: a genetic analysis. *Heredity*, **71**, 312–317.
Zhao X, Bergland AO, Behrman EL *et al.* (2015) Global transcriptional profiling of diapause and climatic adaptation in *Drosophila melanogaster*. *Molecular Biology and Evolution*, **33**, msv263–720.

Chapter 2

[Manuscript to be submitted to Molecular Ecology]

A clinal polymorphism in the insulin signaling transcription factor *foxo* contributes to life-history adaptation in *Drosophila*

Contributions by E. Durmaz: experimental design, assays, statistical analysis, interpretation of results, writing of the manuscript.

A clinal polymorphism in the insulin signaling transcription factor *foxo* contributes to life-history adaptation in *Drosophila*

ESRA DURMAZ,[1] SUBHASH RAJPUROHIT,[2] NICOLAS BETANCOURT,[2] DANIEL K. FABIAN,[3] MARTIN KAPUN,[1] PAUL SCHMIDT,[2*] and THOMAS FLATT[1*]

[1]*University of Lausanne, Department of Ecology and Evolution, Lausanne, Switzerland*

[2]*University of Pennsylvania, Department of Biology, Philadelphia, USA*

[3]*Department of Genetics, University of Cambridge, Cambridge, United Kingdom*

*Co-correspondence:

Thomas Flatt, Fax: +41 21 692 4165, E-mail: thomas.flatt@unil.ch

Paul Schmidt, Fax: +1 215 898 8780, E-mail: schmidtp@sas.upenn.edu

Keywords: cline, life history, adaptation, insulin signaling, pleiotropy, plasticity

Running title: CLINAL LIFE-HISTORY POLYMORPHISM

Abstract

A fundamental aim of adaptation genomics is to identify polymorphisms that underpin variation in fitness traits. In *Drosophila melanogaster* latitudinal life-history clines exist on multiple continents and thus make an excellent system for dissecting the genetics of adaptation. We have previously identified numerous clinal SNPs in the insulin signaling pathway, which is known from studies of laboratory mutants to affect fitness traits. With a few exceptions, however, effects of natural variants in this pathway have not been examined in *Drosophila*. Here we investigate how a strongly clinal 2-SNP variant in *foxo*, a transcriptional effector of insulin signaling, affects fitness-related traits (egg-to-adult survival, body size, starvation resistance, fat content). We isolated this polymorphism from the North American cline by reconstituting outbred populations, fixed for either the low- or high-latitude allele, from lines of the *Drosophila* Genetic Reference Panel (DGRP). Since both diet and temperature can modulate insulin signaling, we phenotyped both alleles at two temperatures (18°C, 25°C) and on two diets differing in their protein:carbohydrate ratio and sugar source. Consistent with clinal expectations, the high-latitude allele conferred larger size and reduced wing loading. Although starvation resistance is typically greater in high-latitude flies, the high-latitude allele was less resistant. The alleles also differed in the expression of a transcriptional target of FOXO. We observed few genotype-by-environment interactions; overall the reaction norms of the alleles were rather parallel. Together with previous work on the *insulin-like receptor*, our results demonstrate that natural variation in insulin signaling makes an important contribution to clinal life-history adaptation.

Introduction

Life-history traits are central to adaptation: because they affect survival and reproduction, they are the most important phenotypic determinants of fitness and organismal targets of selection (Stearns 1992). Surprisingly, however, despite their adaptive importance, little is known about their evolutionary genetic basis.

Although much has been learned about the genetics of fitness traits (e.g., size, lifespan), mainly from studies of large-effect mutants and transgenes in yeast, *C. elegans*, *Drosophila* and the mouse (Finch & Rose 1995; Oldham & Hafen 2003; Tatar *et al.* 2003; Fielenbach & Antebi 2008; Kenyon 2010), loci identified through functional analyses do not necessarily harbor segregating allelic variation that contributes to genetic variance for traits in natural populations (Flatt 2004; Flatt & Schmidt 2009; Vonesch *et al*. 2016; Birney 2016). In particular, the identity and presumably subtle effects of naturally occurring life-history polymorphisms are poorly known (Flatt & Schmidt 2009; Paaby & Schmidt 2009; Flatt & Heyland 2011). While adaptation genomics can in principle quite readily identify such candidate polymorphisms, a major – but rarely accomplished – objective is to experimentally validate these candidates as genic targets of selection (Barrett & Hoekstra 2011; Turner 2014; Flatt 2016). Thus, with a few exceptions, examples of causative life-history variants remain rare (Schmidt *et al*. 2008; McKechnie *et al*. 2010; Paaby *et al.* 2010; Jones *et al.* 2012; Johnston *et al*. 2013; Méndez-Vigo *et al.* 2013; Paaby *et al*. 2014; Barson *et al.* 2015; Catalán *et al.* 2016; reviewed in Mackay *et al*. 2009; Barrett & Hoekstra 2011).

Despite conceptual and methodological limitations of the so-called quantitative trait nucleotide (QTN) program (Rockman 2012), the identification of life-history polymorphisms allows addressing fundamental questions about the genetic basis of adaptation, including: (1) Which pathways and molecular functions underpin variation in fitness-related traits? (2) Are these mechanisms evolutionarily conserved? (3) What are the phenotypic effects of naturally

segregating life-history variants? (4) What is the molecular nature of life-history epistasis, pleiotropy and trade-offs? (5) Do life-history polymorphisms mediate plasticity and how? (6) Is the genetic basis of evolutionary changes in life history "predictable", i.e. relying on variation in the same pathways or genes? Or do life-history traits evolve unpredictably, i.e. via different pathways or loci, in different contexts?

A powerful model for dissecting the genetics of life-history adaptation is the vinegar fly *Drosophila melanogaster*, a species of sub-Saharan African origin, which has migrated out of Africa ~10,000 to 15,000 years ago and subsequently colonized the rest of the world (David & Bocquet 1975; David & Capy 1988; de Jong & Bochdanovits 2003; Hoffmann & Weeks 2007; Adrion *et al*. 2015). During the colonization of new climate zones, this ancestrally tropical insect has undergone a series of life-history adaptations to temperate, seasonal habitats (David & Capy 1988; de Jong & Bochdanovits 2003; Paaby & Schmidt 2009). This is particularly evident in the case of clines, i.e. directional patterns of phenotypic or genetic change across environmental gradients. Many studies have documented patterns of latitudinal differentiation among *D. melanogaster* populations that are presumably driven by spatially varying selection, for example along the North American and Australian east coasts, with the corresponding clines spanning subtropical/tropical and temperate habitats (de Jong & Bochdanovits 2003; Schmidt *et al*. 2005a, b; Hoffmann & Weeks 2007; Schmidt & Paaby 2008; Kolaczkowski *et al.* 2011; Fabian *et al.* 2012; Adrion *et al*. 2015; Cogni *et al*. 2017). Clinal trait differentiation has been found, for instance, for body size, fecundity, reproductive dormancy, stress resistance and lifespan, typically in a parallel fashion on multiple continents, suggesting that these patterns are adaptive (Coyne & Beecham 1987; Weeks *et al*. 2002; de Jong & Bochdanovits 2003; Schmidt *et al*. 2005a, b; Hoffmann & Weeks 2007; Schmidt & Paaby 2008; Adrion *et al*. 2015; Fabian *et al*. 2015; Kapun *et al.* 2016a).

To begin to identify the genetic basis of adaptive life-history clines in *D. melanogaster*, we have previously performed genome-wide analysis of latitudinal differentiation along the North American cline (Fabian *et al*. 2012 and Kapun *et al.* 2016b; also see Turner *et al.* 2008; Bergland *et al*. 2014; Reinhardt *et al.* 2014). Our analysis based on SNP F_{ST} outliers uncovered pervasive genome-wide patterns of clinality, with hundreds of clinally varying SNPs mapping to loci involved in the insulin/insulin-like growth factor signaling (IIS)/target of rapamycin (TOR), ecdysone, torso, EGFR, TGFβ/BMP, JAK/STAT, lipid metabolism, immunity and circadian rhythm pathways (Fabian *et al*. 2012). Many of the identified variants also exhibit parallel differentiation along the Australian cline (Fabian *et al.* 2012 and Kapun *et al*. 2016b; also cf. Kolaczkowski *et al.* 2011; Reinhardt *et al.* 2014; Machado *et al.* 2016), thereby strengthening the case for clinal adaptation. However, while many clinal variants might be shaped by selection, some of the observed differentiation might be due to non-adaptive factors, including population structure, demography, admixture or hitchhiking with causative sites (Endler 1977; Duchen *et al.* 2013; Kao *et al*. 2015; Bergland *et al*. 2016). Unambiguously identifying adaptive clinal variants thus requires comparing clinal patterns against neutral expectations and/or functional genetic testing (Barrett & Hoekstra 2011; Kapun *et al*. 2016b; Flatt 2016).

Interestingly, many of the pathways that harbor clinal loci are known from functional genetic studies to be implicated in the regulation of life-history physiology (Tatar *et al.* 2003; Fielenbach & Antebi 2008; Flatt & Heyland 2011; Flatt *et al*. 2013). In particular, we found strongly clinal SNPs in multiple components of the IIS/TOR pathway, including SNPs in *insulin-like peptide* genes *ilp 3* and *ilp5*, *insulin-like receptor* (*InR*), *phosphatidyl-inositol-4,5-bis-phosphate 3-kinase* (*Pi3K*), forkhead box-O transcription factor *foxo*, the *foxo* regulator *14-3-3ε, target of brain insulin* (*tobi*)*, tuberous sclerosis complex 1* (*Tsc1*), and *target of rapamycin* (*Tor*) (Fig. 1; Fabian *et al*. 2012; Kapun *et al.* 2016b). This pattern is compelling

since loss-of-function mutations in the IIS/TOR pathway have major, evolutionarily conserved effects on growth, size, reproduction, lifespan and stress resistance in *Drosophila*, *C. elegans*, and the mouse (Kenyon *et al*. 1993; Gems *et al*. 1998; Böhni *et al.* 1999; Brogiolo *et al.* 2001; Tatar *et al.* 2001; Clancy *et al.* 2001; Kenyon 2001; Oldham *et al*. 2002; Oldham & Hafen 2003; Holzenberger *et al*. 2003; Tatar *et al.* 2003; Partridge *et al*. 2005).

Since many fitness-related traits affected by IIS/TOR also exhibit phenotypic clines, it is tempting to hypothesize that natural variation in this pathway contributes to life-history clines, especially with regard to body size (de Jong & Bochdanovits 2003); yet, the evolutionary significance of natural variants in this pathway is poorly understood. An exception is an indel polymorphism in the *D. melanogaster InR* gene, which varies clinally along both the North American and Australian east coasts and which has multifarious life-history effects (Paaby *et al.* 2010, 2014). Consistent with the idea that IIS polymorphisms affect adaptation, natural variation in adult reproductive dormancy in *D. melanogaster* has been connected to the *Pi3K* gene (Williams *et al*. 2006), and work in *Caenorhabditis remanei* has identified a global selective sweep in the *Caenorhabditis* homolog of *Pi3K*, *age-1* (Jovelin *et al*. 2014). Multiple lines of evidence also indicate that insulin-like growth factor-1 (IGF-1) signaling mediates physiological life-history variation in vertebrate populations (Dantzer & Swanson 2011; Swanson & Dantzer 2014). Together, these findings suggest that allelic variation in IIS/TOR might profoundly affect life-history adaptation, but experimental evidence remains scarce.

Here we investigate the life-history effects of a clinal polymorphism in the forkhead box-O transcription factor gene *foxo* of *D. melanogaster* (Fig. 1), a major regulator of IIS that is homologous to *C. elegans daf-16* and mammalian *FOXO3A*. Molecular studies – mainly in the fly and nematode – have shown that FOXO plays a key role in regulating growth, lifespan and resistance to starvation and oxidative stress (Jünger *et al.* 2003; Puig *et al.* 2003; Libina *et*

al. 2003; Murphy *et al*. 2003; Kramer *et al*. 2003, 2008; Hwangbo *et al*. 2004; Puig & Tijan 2005; Fielenbach & Antebi 2008; Mattila *et al*. 2009; Slack *et al*. 2011). Moreover, genetic association studies in humans have linked polymorphisms in *FOXO3A* to longevity in centenarians (Flachsbart *et al*. 2008; Willcox *et al*. 2008). Natural *foxo* variants thus represent promising candidates for mediating life-history variation in natural populations.

From our genomic data (Fabian *et al*. 2012) we identified a strongly clinal 2-SNP variant in *foxo*, whose frequency changes across latitude from ~10% in Florida to ~70% in Maine (also see Betancourt *et al.*, submitted). To characterize the effects of this polymorphism we measured several fitness-related traits (egg-to-adult survival, proxies of size, starvation resistance, fat content) on replicate populations of the two alternative alleles. Since both diet and temperature can modulate IIS (Britton *et al.* 2002; Kramer *et al.* 2003; Puig & Tijan 2005; Giannakou *et al.* 2008; Teleman 2010; Puig & Mattila 2011; Li & Gong 2015; Zhang *et al.* 2015), we phenotyped both alleles at two temperatures (18°C, 25°C) and on two diets differing in their protein:carbohydrate (P:C) ratio and sugar source. Investigating phenotypic plasticity and genotype-by-environment interactions (G · E) for this variant is of interest since little is known about the relative importance of clinality versus plasticity and their interplay, with most previous work having focused on gene expression, not whole-organism traits (de Jong & Bochdanovits 2003; Hoffmann *et al*. 2005; Levine *et al*. 2011; Overgaard *et al*. 2011; Chen *et al*. 2012; Cooper *et al*. 2012; Zhao *et al.* 2015; Clemson *et al*. 2016; Mathur & Schmidt 2017). For example, *D. melanogaster* feeds and breeds on various kinds of rotting fruit, with P:C ratios exhibiting spatiotemporal variation (Lachaise *et al*. 1988; Hoffmann & McKechnie 1991; Markow *et al*. 1999; Keller 2007), but how dietary plasticity and G · E affect traits in a clinal context is not well understood. We give predictions for the expected phenotypic behavior of the *foxo* polymorphism in the Materials and Methods section below.

We find that the *foxo* polymorphism has pleiotropic effects on clinally varying life-history traits, thus confirming that it is a target of spatially varying selection. Both alternative alleles respond plastically to changes in temperature and diet, but there is little evidence for G · E interactions. In a companion paper we directly compare its effects in a constant laboratory environment to clinal expectations based on phenotypic data from six populations along the North American cline (Betancourt *et al*., submitted)

Materials and Methods

Predictions

Here we make qualitative predictions for the expected behavior of the *foxo* polymorphism with regard to (1) clinal phenotypic effects, (2) patterns of trait covariation determined by IIS, and (3) plasticity, G · E, and local adaptation (also see Betancourt *et al*., submitted). We compare our results to these predictions in the Results section.

(1) Latitudinal clinality. Traits expected to covary with high as compared to low latitude include faster development, lower egg-to-adult survival (viability), increased body size, reduced wing loading, reduced fecundity, prolonged lifespan, and increased resistance to starvation, cold and heat stress (Coyne & Beecham 1987; Azevedo *et al*. 1998; Bochdanovits & de Jong 2003a; de Jong & Bochdanovits 2003; Schmidt *et al*. 2005a, b; Folguera *et al*. 2008; Schmidt & Paaby 2008; Bhan *et al.* 2014; Mathur & Schmidt 2017; Betancourt *et al.*, submitted; for contrasting predictions for viability see Van't Land *et al.* 1999, and for starvation resistance cf. Karan *et al*. 1998, Robinson *et al*. 2002; Hoffmann *et al*. 2005, Goenaga *et al*. 2013). We expect the effects of the high- and low-latitude *foxo* alleles to agree with the overall phenotypic patterns along the cline, unless alleles exhibit countergradient effects on phenotype (Paaby *et al.* 2014).

(2) IIS. Traits expected to covary with reduced IIS include reduced body size, increased lifespan, resistance to starvation and cold, increased fat content, reduced fecundity, and activation of FOXO (Tatar *et al*. 2001, 2003; Oldham & Hafen 2003; Broughton *et al.* 2005; Teleman 2010). Loss-of-function mutants of *foxo* exhibit (depending on the allele) prolonged development, reduced weight, smaller wing size, reduced fecundity, shortened lifespan, and reduced survival upon oxidative and starvation stress (Jünger *et al.* 2003; Kramer *et al.* 2003, 2008; Hwangbo *et al.* 2004; Giannakou *et al*. 2004, 2008; Kramer *et al.* 2008; Slack *et al*. 2011); effects of IIS or *foxo* on viability are not well understood. Conversely, expression of *foxo* has opposite effects on most of these traits (e.g., lifespan, starvation resistance), yet causes decreased size (Kramer *et al.* 2003; Puig *et al.* 2003; Hwangbo *et al*. 2004; Kramer *et al.* 2008; Tang *et al.* 2011). We predict that the *foxo* alleles differ consistently along this IIS/*foxo* axis of trait covariation.

Notably, traits observed in flies from high versus low latitude resemble those of flies with low versus high IIS, respectively (de Jong & Bochdanovits 2003; Flatt *et al*. 2013; Paaby *et al*. 2014): lower fecundity, improved stress resistance, and longer lifespan observed in high-latitude flies are traits that are co-expressed in IIS mutants; however, flies from high-latitude populations are larger than low-latitude flies, yet reduced IIS causes smaller size.

(3) Plasticity, G · E, and local adaptation. With regard to thermal effects, we expect flies raised at lower temperature to exhibit prolonged development, reduced viability, larger size, reduced wing loading, lower fecundity, increased lifespan, and improved starvation resistance (David *et al.* 1994; Partridge *et al*. 1994a, b; Bochdanovits & de Jong 2003b; Trotta *et al*. 2006; Folguera *et al*. 2008; Klepsatel *et al.* 2013; Mathur & Schmidt 2017; cf. Hoffmann *et al*. 2005 for a contrasting prediction for starvation survival). With respect to dietary effects, higher P:C ratios are expected to cause increased viability, larger size but reduced starvation resistance (Lee & Jang 2014; Lihoreau *et al.* 2016; Reis 2016). In terms of G · E, genotypes

from temperate, seasonal high-latitude habitats might be more plastic than those from low-latitude habitats (Overgaard *et al.* 2011; Klepsatel *et al.* 2013); if so, patterns of differential plasticity between high- and low-latitude alleles might be consistent with patterns of local adaptation (Mathur & Schmidt 2017).

Identification and isolation of the foxo polymorphism

We identified two strongly clinal SNPs in *foxo* in the data of Fabian *et al.* (2012) by using a F_{ST} outlier approach: an A/G polymorphism at position *3R*: 9892517 (F_{ST} = 0.48) and a T/G polymorphism at position *3R*: 9894559 (F_{ST} = 0.42) (Fig. S1A, Supporting Information; see Fabian *et al.* 2012 for details of outlier detection). The A/G polymorphism is a synonymous coding SNP, predicted to be located in the PEST region of the FOXO protein, which serves as a protein degradation signal (analysis with ExPASy [Artimo *et al*. 2012]; Fig. S2, Supporting Information). The T/G SNP is located in the first intron of *foxo*, with no biological function attributed to this position (Attrill *et al.* 2016). While our initial identification of these SNPs was based on only three populations (Florida, Pennsylvania, and Maine; Fabian *et al.* 2012), both SNPs are also strongly clinal in a more comprehensive dataset based on 10 populations along the cline (see Betancourt *et al*., submitted), collected by the *Drosophila* Real Time Evolution Consortium (Dros-RTEC; Bergland *et al*. 2014; Kapun *et al*. 2016b). Since the two SNPs are relatively close together (~2 kb apart; Fig. S1A, Supporting Information), we decided to study them experimentally in combination, as a 2-SNP genotype. The frequency of the high-latitude [HL] allele (A, T) for this 2-SNP variant ranges from ~10% in Florida to ~70% in Maine; conversely, the alternative low-latitude [LL] allele (G,G) is prevalent in Florida but at low frequency in Maine (Fig. S1A, Supporting Information; also see Betancourt *et al*., submitted).

To isolate this variant for experiments we used whole-genome sequenced inbred lines from the *Drosophila* Genetic Reference Panel (DGRP; Mackay *et al.* 2012) to reconstitute outbred populations either fixed for the LL (G,G) and the HL (A,T) alleles (see Betancourt *et al*., submitted). For each allele we used two independent sets of DGRP lines (sets A and B for HL; sets C and D for LL; each set consisting of 20 distinct lines) and two replicate population cages per set, giving a total of 8 cages (Fig. S3, Table S1, Supporting Information; see Betancourt *et al*., submitted). By analyzing the genomes of the DGRP lines used to set up experimental populations we confirmed that sets A and B versus sets C and D were fixed (F_{ST} = 1) for the HL and LL alleles, respectively; this also showed that there was no systematic differentiation, as measured by F_{ST}, in the genome-wide background of the focal alleles (see Betancourt *et al*., submitted). Figure S1B (Supporting Information) shows that the two focal SNPs are in perfect linkage disequilibrium (LD; r^2 = 1), without any significant LD in-between the two sites.

Population cages

Population cages were maintained at 25ºC, 12:12 h light:dark, 60% relative air humidity and controlled larval density. Larval density was kept constant via egg collections (200-300 eggs per bottle [6 oz. = 177 mL]; 10 bottles per cage), with eclosing adults being released into cages (17.5 x 17.5 x 17.5 cm; BugDorm®) at a density of ~2000-2500 adults per cage. Prior to assays cages were kept for 10 generations to allow for recombination among lines within each cage and to homogenize differences in genomic background between the alleles to be compared. Before setting up assays, we kept cages for 2 generations under common garden conditions (room temperature: ~22ºC, ~10:14 h light:dark, ~50% humidity).

Phenotype assays

The assays reported here were performed in Lausanne; independent assays were performed in Philadelphia (see Betancourt *et al*., submitted), allowing us to account for potential variation in life-history traits due to differences in laboratory assay conditions (Ackermann *et al.* 2001; see Betancourt *et al*., submitted).

In generation 13 we assayed flies for viability, size, starvation resistance and lipid content. Phenotypes were assayed under four environmental conditions, using a fully factorial 2-way design: 2 rearing temperatures (18°C, 25°C) by 2 diets differing in their P:C ratio and sugar source (sucrose [cornmeal-agar-yeast-sucrose] vs. molasses [cornmeal-agar-yeast-molasses] diet; P:C ~1:3.6 and ~1:12.3, respectively; see Table S2, Supporting Information, for details of nutrient content and media recipes). To initiate assays we collected ~6400 eggs from each cage, distributed them across 32 bottles (each with 200 eggs; 25 mL medium), and allocated 8 bottles to each of the 4 conditions (8 bottles · 8 cages · 4 conditions = 256 bottles). For all assays (except viability; see below), we collected eclosed adults in 48-h cohorts, allowed them to mate for 4 days under their respective thermal and dietary conditions, sexed them under light CO_2 anesthesia 4-6 days post-eclosion, and transferred them to fresh vials 24 h prior to assays. Flies used for size assays were stored at -20°C until measurement.

Viability (egg-to-adult survival) was calculated as the proportion of adult flies successfully developing from eggs by collecting 600 eggs per cage and placing them into vials containing 8 mL of medium, with 30 eggs per vial (5 vials · 8 cages · 4 conditions = 160 vials).

Body size was examined by measuring three proxies: wing area, thorax length and femur length (N = 26-30 wings, 10-15 thoraces, and 19-21 femurs per cage, treatment, and sex). Right wings and femurs were mounted on slides with CC/Mount™ tissue mounting medium (Sigma Aldrich) and slides sealed with cover slips. Thorax length was defined as the lateral distance between the upper tip of the thorax and the end of the scutellar plate (N = 10-15

individuals per cage, treatment, and sex). Images for morphometric measurements were taken with a digital camera (Leica DFC 290) attached to a stereo dissecting microscope (Leica MZ 125; Leica Microsystems GmbH, Wetzlar, Germany). We used ImageJ software (v.1.47) to measure femur and thorax length (mm) and to define landmarks for calculating wing area (mm^2). To measure wing area we defined 12 landmarks located at various vein intersections along the wing; the total area encompassed by these landmarks was estimated using a custom-made Python script (available upon request from MK). In brief, we split the polygon defined by the landmarks up into triangles and summed across their areas (Fig. S4, Supporting Information). Thorax and femur measurements were repeated three times per individual. From these data, we calculated the ratio of wing area:thorax length, which is inversely related to "wing loading" (Azevedo *et al*. 1998; Gilchrist *et al*. 2000).

Starvation resistance was measured by placing flies into vials containing 0.5% agar/water medium and scoring age at death (h) every 6 h until all flies had died (N = 5 vials · 10 flies per vial · 2 sexes · 8 cages · 4 conditions = 320 vials or 3200 flies). Since there is typically a positive correlation between starvation resistance and lipid content (Hoffmann & Harshman 1999), we also determined whole-body triacylglyceride (TAG) content (in μg per fly) using a serum triglyceride determination kit (Sigma Aldrich; Tennessen *et al.* 2014). For each cage and treatment, triglyceride content was estimated from 5-7-day-old females, either kept under fed or starved (24 h) conditions, by preparing 10 replicate homogenates, each made from 2 flies (8 cages · 4 conditions · 2 treatments · 10 replicates = 640 homogenates). To estimate fat loss upon starvation we calculated the difference between fat content under fed versus starved conditions, using treatment (fed vs. starved) means from each population cage (mean fat loss per fly, in μg).

qRT-PCR analysis of insulin signaling state

A well established transcriptional read-out of FOXO signaling is the insulin-like receptor InR: under conditions of high insulin (e.g., after a meal), InR synthesis is repressed by a feedback mechanism controlled by FOXO; conversely, under conditions of low insulin, activation of FOXO leads to upregulation of InR (Puig *et al*. 2003; Puig & Tjian 2005). To test whether the *foxo* alleles differ in IIS state we performed qRT-PCR, measuring InR mRNA abundance. For each cage and treatment, we extracted total RNA from 5-7-day-old snap-frozen females in triplicate, with each replicate prepared from 5 flies. RNA was extracted with the RNeasy kit (Qiagen) and reverse transcribed with the GoScript Reverse Transcription System (Promega). From each triplicate biological sample we prepared 3 technical replicates (8 cages · 4 conditions · 3 biological replicates · 3 technical replicates = 288 samples). Relative transcript abundance was normalized by using Actin as an endogenous control (Ponton *et al*. 2011). qRT-PCR was carried out using a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) and SYBR Green GoTaq qPCR Master Mix (Promega). Thermal cycling was conducted at 95°C for 2 min, followed by 42 cycles of amplification at 95°C for 15 s and 60°C for 1 min, and using the following melting curve: 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Quantification of relative abundance for each sample was based on the Δ*CT* method. We used the following primer sequences (Casas-Tinto *et al.* 2007; Ponton *et al*. 2011): *Actin forward*, 5'-GCGTCGGTCAATTCAATCTT-3'; *Actin reverse*, 5'-AAGCTGCAACCTCTTCGTCA-3'; *InR forward*, 5'-CACAAGCTGGAAAGAAAGTGC-3'; and *InR reverse*, 5'- CAAACACGTTTCGATAATATTTTTCT-3'.

Statistical analysis

Analyses were performed with JMP (SAS, Raleigh, NC, USA; v.11.1.1). Data were analyzed with analysis of variance (ANOVA), testing the fixed effects of allele (*A*; HL vs. LL),

temperature (*T*; 18°C vs. 25°C), diet (*D*; sucrose vs. molasses), set (*S*; independent blocks of DGRP lines) nested within *A*, replicate cage (*C*) nested within the combination of *A* and *S*, and all 2- and 3-way interactions: $y = A + T + D + A \cdot T + A \cdot D + T \cdot D + A \cdot T \cdot D + S(A) + C(A,S)$, where *y* denotes the response variable (trait). For simplicity, the sexes were analyzed separately (i.e., to reduce the number of higher-order interactions). Whenever measuring multiple individuals from vials, we estimated the random effect of vial (*V*), nested within the combination of *A*, *S* and *C*, using restricted maximum likelihood (REML); since the estimates of this variance component are not of primary biological interest we do not report them. Viability data were arcsine square-root transformed prior to analysis. Analysis of thorax and femur length was performed on means across 3 measures per individual; because wings and thoraces were measured on separate individuals, analysis of wing:thorax ratio was done on population (cage) means. For fat content, we included the fixed effect of starvation treatment (*Tr*; fed vs. starved); interactions involving *A* and *Tr* (i.e., $A \cdot Tr$; $A \cdot D \cdot Tr$) test for allelic differences in fat loss upon starvation. We performed this analysis separately for the two rearing temperatures.

Results

foxo polymorphism affects viability

We find that the naturally occurring *foxo* variant affects viability, with the LL allele exhibiting higher egg-to-adult survival than the HL allele (Fig. 2; Table 1), consistent with observations suggesting that viability might be higher at low latitudes (Folguera *et al*. 2008; but see Van't Land *et al.* 1999). Diet – but not temperature – also affected viability, with egg-to-adult survival being higher on sucrose diet than on carbohydrate-rich molasses diet (Fig. 2; Table 1), in agreement with a recent study (Lihoreau *et al.* 2016). We did not find any evidence for G × E interactions with regard to viability.

Clinal foxo alleles vary in body size

Since both latitude and IIS affect size (de Jong & Bochdanovits 2003), we next examined three proxies of body size (wing area, thorax and femur length). The HL allele conferred larger femur length (Fig. 3; Table 1; in females but not males), wing area (Fig. S5; Table S3, Supporting Information), and wing:thorax ratio than the LL allele (Fig. 4; Table 1; for thorax data see Fig. S6; Table S3, Supporting Information). These results are consistent with the positive size cline in North America (Coyne & Beecham 1987; Betancourt *et al.*, submitted) and with reduced wing loading at high latitude (Azevedo *et al*. 1998; Bhan *et al.* 2014). Since *foxo* overexpression can reduce overall size (Jünger *et al*. 2003), it is possible that the LL allele exhibits increased FOXO function as compared to the HL allele. For all size traits, females were larger than males (Fig. 4; Fig. 5; Table 1; Fig. S5; Fig. S6; Table S3, Supporting Information). With regard to the plastic effects of temperature, femur length, thorax length and wing area were larger at 18°C than at 25°C (Fig. 3; Fig. S5, Fig. S6, Supporting Information; Table 1; Table S3, Supporting Information), as expected based on previous work (David *et al.* 1994; Partridge *et al*. 1994a). In terms of dietary plasticity, femur and thorax length were larger on sucrose than on molasses diet (Fig. 3; Table 1; Fig. S6; Table S3, Supporting Information), in line with the observation that carbohydrate-rich diet causes smaller size (Reis 2016); however, wing area and wing:thorax ratio were larger on molasses than on sucrose diet (Fig. S5; Table S3, Supporting Information; and Fig. 4; Table 1). Although we found a few G × E interactions for size traits (Fig. 4; Fig. 5; Table 1; Fig. S5; Fig. S6; Table S3, Supporting Information), the allelic reaction norms were remarkably parallel across environmental conditions.

Natural variation at foxo influences starvation resistance and fat catabolism

The *foxo* alleles differed in their effects on female starvation resistance (Fig. 5; Table 1), as might be expected based on the observation that *foxo* mutants are more starvation sensitive than wildtype (Jünger *et al.* 2003; Kramer *et al*. 2003, 2008). However, contrary to clinal predictions (Schmidt & Paaby 2008; Mathur & Schmidt 2017; Betancourt *et al*., submitted), LL females were more starvation resistant than HL females (Fig. 5; Table 1), suggesting a countergradient effect; in males, there were no allelic differences in resistance (Fig. S7; Table S3, Supporting Information). Overall females were more resistant than males (Fig. 5; Table 1; Fig. S7; Table S3, Supporting Information), consistent with some but not other studies (Goenaga *et al.* 2010; but see Matzkin *et al*. 2009). For both females and males, starvation resistance was higher at 18°C than at 25°C (Fig. 5; Table 1; Fig. S7; Table S3, Supporting Information), as previously reported (Mathur & Schmidt 2017). Flies raised on molasses diet were more resistant than those raised on sucrose diet (Fig. 5; Table 1; Fig. S7; Table S3, Supporting Information), supporting the finding that lower P:C ratios favor higher resistance (Chippindale *et al.* 1993; Lee & Jang 2014). We also found evidence for an allele by diet interaction: allelic differences in resistance were more pronounced on molasses than sucrose diet (Fig. 5; Table 1; Fig. S7; Table S3, Supporting Information).

To further examine the physiological basis of the starvation response we quantified how much fat female flies mobilize upon starvation (Fig 6; Table 2; males were not examined since they did not show allelic differences in resistance). Paralleling our result that LL females are more resistant than HL females, we found that the amount of fat catabolized under starvation was greater in LL than in HL females, under almost all conditions (except for females raised on sucrose diet at 25°C; see Fig. 6 and Table 2: significant allele by diet by starvation treatment interaction at 25°C but not at 18°C). Fat loss upon starvation was greater

for flies raised on molasses than on sucrose diet (Fig 6; Table 2), again matching the results for starvation resistance itself.

foxo alleles differ in their transcriptional feedback control of InR

From the above patterns we predicted that the LL allele would exhibit decreased IIS and increased FOXO activity: the LL allele has smaller size but higher starvation resistance, i.e. traits that co-occur in IIS mutants or flies with increased FOXO activity. To test this hypothesis we performed qRT-PCR analysis of a major transcriptional target of FOXO, InR: when IIS is low, FOXO becomes active and upregulates InR transcription, while under high IIS FOXO is inactive and represses InR (Puig *et al*. 2003; Puig & Tjian 2005). In support of this hypothesis we found that the LL allele had a ~12% higher level of InR transcript than the HL allele (Fig. S8; Table S4, Supporting Information). Dietary conditions also affected InR levels, with flies raised on molasses producing more InR than flies raised on sucrose diet (Fig. S8; Table S4, Supporting Information).

Discussion

Connecting adaptive phenotypes to genotypes

Understanding how organisms adapt to heterogeneous environments, and unraveling the genotype-phenotype map underlying such adaptation, is a central problem of evolutionary genetics (Levins 1968; Lewontin 1974; Endler 1977, 1986; Barrett & Hoekstra 2011).

In *D. melanogaster*, an ancestrally tropical insect, seasonality and cold winters at high latitude select for genotypes that are stress resistant and able to overwinter, whereas subtropical/tropical low-latitude conditions select for rapid development and high fecundity; traits correlated with these features (e.g., size, lifespan) evolve as correlated responses to selection, leading to trade-offs across geography (James & Partridge 1995; Schmidt *et al.*

2005a, b; Schmidt & Paaby 2008; Paaby & Schmidt 2009; Flatt *et al.* 2013; Paaby *et al*. 2014; Fabian *et al.* 2015). This makes *D. melanogaster* a powerful system for dissecting the genetic basis of adaptation. However, little is known about the polymorphisms that underpin life-history adaptation in this or other species (Finch & Rose 1995; Flatt & Schmidt 2009; Paaby & Schmidt 2009; Barrett & Hoekstra 2011; Flatt & Heyland 2011; Flatt *et al.* 2013; Paaby *et al.* 2014).

Several lines of evidence suggest that genes of the IIS/TOR pathway might be promising candidates underlying life-history adaptation in *D. melanogaster* (de Jong & Bochdanovits 2003): (1) laboratory mutants in this pathway often mirror life-history traits and trade-offs observed in natural populations (de Jong & Bochdanovits 2003; Clancy *et al*. 2001; Tatar *et al*. 2001; Tatar and Yin 2001; Tatar *et al*. 2003; Paaby *et al*. 2010; Flatt *et al.* 2013; Paaby *et al.* 2014); (2) reproductive dormancy in response to cool temperature and short photoperiod, a genetically variable and clinal trait (Williams & Sokolowski 1993; Schmidt *et al*. 2005a, b; Schmidt & Paaby 2008), is physiologically regulated by IIS (Williams *et al.* 2006; Flatt *et al.* 2013; Kubrak *et al*. 2014; Schiesari *et al*. 2016; Zhao *et al*. 2016); (3) genomic analyses of clinal differentiation has identified many clinal SNPs in the IIS/TOR pathway presumably shaped by spatially varying selection (Fig. 1; Kolaczkowksi *et al.* 2011; Fabian *et al.* 2012; Kapun *et al*. 2016b); and (4) genome-wide analyses of variation in size traits have identified novel regulators of growth, several of which interact with the IIS/TOR pathway (Vonesch *et al.* 2016; Strassburger *et al.* 2017).

Testing targets of selection in a genomic context requires experiments to identify the adaptive effects of individual alleles (Barrett & Hoekstra 2011; Turner 2014; Flatt 2016). In support of the idea that variation in IIS contributes to adaptation in *D. melanogaster* (de Jong & Bochdanovits 2003), Paaby and colleagues have identified a clinal indel polymorphism in *InR* with pleiotropic effects on development, size, fecundity, lifespan, oxidative stress

resistance, chill coma recovery, and insulin signaling (Paaby *et al*. 2010, 2014). Here we have studied the life-history effects of a clinal polymorphism in another IIS gene, *foxo*, a variant that we have identified from our genomic analysis of the North American cline (Fabian *et al.* 2012). Our results complement those of Paaby *et al*. (2010, 2014) and give further credence to the hypothesis of de Jong & Bochdanovits (2003).

The effects of natural versus null alleles at the foxo locus

Previous work with loss-of-function mutants and transgenes has uncovered a major role of *foxo* in the regulation of growth, lifespan and resistance to starvation and oxidative stress (Jünger *et al.* 2003; Puig *et al.* 2003; Kramer *et al*. 2003; Giannakou *et al.* 2004; Hwangbo *et al*. 2004; Kramer *et al*. 2008; Slack *et al*. 2011), but nothing is known about the effects of natural alleles at this locus. An important distinction in this context is that null mutants, by definition, reveal the complete set of functions and phenotypes of a given gene and may thus be highly pleiotropic, whereas "evolutionarily relevant" mutations or alleles might have much more subtle effects, with little or no pleiotropy (Stern 2000). Based on our knowledge of the traits affected by *foxo* in null mutants and transgenes (Jünger *et al.* 2003; Kramer *et al*. 2003, 2008; Slack *et al*. 2011), we measured how the clinal 2-SNP variant affects size traits and starvation resistance.

Although we could neither predict the directionality nor the degree of pleiotropy of the allelic effects *a priori*, we found that the *foxo* polymorphism differentially affects size-related traits and starvation resistance. With regard to growth and size, our findings from a natural variant agree well with functional genetic studies showing that *foxo* affects body size and wing area (Jünger *et al.* 2003; Slack *et al*. 2011; Tang *et al*. 2011). Similarly, our observation that variation at *foxo* affects survival and fat content upon starvation is consistent with the fact that *foxo* mutants display reduced starvation resistance (Jünger *et al.* 2003; Kramer *et al*.

2003, 2008). In contrast, although *foxo* null mutants produce viable adults (Jünger *et al.* 2003; Slack *et al*. 2011), whether distinct *foxo* alleles vary in viability has not been examined; here we find that the two natural alleles differ in egg-to-adult survival. We also asked whether the alleles differentially affect mRNA abundance of InR, a transcriptional target of FOXO (Puig *et al.* 2003; Puig & Tjian 2005). Indeed, the LL allele had higher InR levels, consistent with the LL genotype exhibiting reduced IIS and higher FOXO activity. For most traits measured, both alleles reacted plastically to changes in diet and temperature in the direction predicted from previous work (Partridge *et al*. 1994a, b; Lee & Jang 2014; Lihoreau *et al.* 2016; Mathur & Schmidt 2017), yet we found little evidence for allele by environment interactions.

While our experimental design does not allow us to disentangle the contribution of the 2 individual SNPs to the total effects seen in the 2-SNP haplotype, it is noteworthy that a natural polymorphism defined by variation at only two (albeit linked) nucleotide positions has strongly pleiotropic effects on viability, several proxies of size and starvation resistance. This supports the idea that the architecture of life-history traits, connected via multiple trade-offs, is inherently pleiotropic (Williams 1957; Finch & Rose 1995; Flatt *et al*. 2005; Flatt & Promislow 2007; Flatt & Schmidt 2009; Flatt *et al*. 2013; Paaby *et al*. 2014) – and provides a contrast to the model from evo-devo which posits that most evolutionarily relevant mutations exhibit little or no pleiotropy (Stern 2011). The pleiotropic effects of the *foxo* variant might also explain why it is being maintained as polymorphic in natural populations along the cline.

Insulin signaling, clinality, and countergradient variation

How does the *foxo* variant contribute to the phenotypic cline observed across latitude? High-latitude flies tend to be characterized by rapid development, reduced viability, larger size, decreased fecundity, longer lifespan and improved stress resistance as compared to low-latitude flies, and this differentiation is genetically based (Coyne & Beecham 1987; Azevedo

et al. 1998; Schmidt *et al*. 2005a,b; Folguera *et al*. 2008; Schmidt & Paaby 2008; Mathur & Schmidt 2017; Betancourt *et al.*, submitted). Do the allelic effects go in the same direction as the latitudinal gradient, representing cogradient variation, or do certain allelic effects run counter to the cline, representing countergradient variation (Levins 1968; Conover & Schultz 1995)? Cogradient variation occurs when diversifying selection favors different traits in different environments, as expected from selection along a cline, whereas countergradient variation occurs when stabilizing selection favors similar traits in different environments (Conover & Schultz 1995; Marcil *et al*. 2006).

Consistent with clinal expectation, the HL allele confers larger size (Coyne & Beecham 1987; de Jong & Bochdanovits 2003); increased wing:thorax ratio, which corresponds to reduced "wing loading", a trait hypothesized to be adaptive for flight at cold temperature (Stalker 1980; David *et al.* 1994; Azevedo *et al*. 1998; Bhan *et al.* 2014); and reduced viability (Folguera *et al*. 2008). Conversely, the LL allele exhibits smaller size, increased wing loading, and higher viability. Thus, these results demonstrate that the *foxo* variant contributes to the observed phenotypic cline in the predicted direction (gradient or cogradient variation) and that it is maintained by spatially varying selection. (For a remarkable example where size is subject to countergradient – not cogradient – variation along an altitudinal gradient in Puerto Rican *D. melanogaster* see Levins 1968, 1969).

For starvation resistance, we found – against clinal predictions – that the HL allele is less resistant than the LL allele, suggesting countergradient variation. Interestingly, a similar countergradient effect, on size, has been observed for the polymorphism in *InR* mentioned above: the high-latitude InR^{short} allele confers smaller size, even though high-latitude flies are normally bigger (Paaby *et al.* 2014). Likewise, for a clinal variant of *neurofibromin 1* (*Nf1*) the high-latitude haplotype has smaller wing size, an effect that runs counter to the cline (Lee *et al.* 2013).

For IIS itself, de Jong & Bochdanovits (2003) predicted that temperate fly populations might be characterized by 'thrifty' genotypes with high IIS, whereas tropical populations might have a higher frequency of 'spendthrift' genotypes with low IIS. Our finding that the low-latitude *foxo* allele likely exhibits increased FOXO activity and lower IIS seems to support this, yet Paaby *et al.* (2014) found that IIS was lower for the high-latitude *InR* allele. The directionality of IIS effects along the cline thus remains difficult to predict (de Jong & Bochdanovits 2003).

As noted by Lee *et al*. (2013) and Paaby *et al.* (2014), clinal variants subject to countergradient effects might interact epistatically with other loci affecting the trait, or they might be affected by antagonistic selection pressures (Schluter *et al.* 1991). Conflicting selection pressures on clinal variants might be particularly acute when they exhibit pleiotropic effects on multiple traits, as is the case for the polymorphisms at *Nf1, InR*, and *foxo*. These examples illustrate the complexity of dissecting the dynamics of clinal selection and the genotype-phenotype map underlying clinal adaptation (Lee *et al*. 2013; Paaby *et al*. 2014; Adrion *et al*. 2015; Flatt 2016).

Limits to our reductionist understanding of adaptation?

The above considerations make clear the limitations of using a reductionist approach to establish adaptive effects of individual alleles (Rockman 2012). In his famous 1974 book *The Genetic Basis of Evolutionary Change* Richard Lewontin writes: "Even if it were possible to randomize the alleles at a single locus with respect to the rest of the genome and then to measure the marginal fitnesses of the alternative genotypes at that locus to an arbitrary level of accuracy, it would be a useless occupation. Genes in populations do not exist in random combinations with other genes. The alleles at a locus are segregating in a context that includes

a great deal of correlation with the segregation of other genes at nearby loci... Context and interaction are of the essence." (Lewontin 1974, p. 318).

This is an incisive critique of the kind of experimental approach we have used here, and to a large extent we agree with Lewontin. However, adopting the alternative approach, i.e. using a macroscopic, quantitative genetics description, also comes at a cost, namely treating the genetic architecture of adaptive traits as a phenomenological, mechanistic black box (Houle 2001; Stern & Orgogozo 2008; Barret & Hoekstra 2011; Flatt & Heyland 2011; Rockman 2012; Flatt *et al*. 2013; Nunes *et al*. 2013).

The problem is that – in the absence of functional analysis of candidate loci or alleles – neither population nor quantitative genetics can provide an explicit understanding of how causative polymorphisms map to evolutionarily relevant traits (Nunes *et al*. 2013). For instance, quantitative trait locus (QTL) mapping has rarely been able to identify causative loci or nucleotide variants of functional relevance (Rockman 2012; Nunes *et al*. 2013). In fact, as Lewontin has argued himself (1974, 2000), if an adequate description of evolution is to be given, evolutionary genetics must tackle the problem of mapping genotypes into phenotypes: "Much of the past and the present problems of population genetics can be understood only as an attempt to finesse the unsolved problem of an adequate description of development." (Lewontin 2000, p. 7).

Growing evidence for a major role of IIS in life-history variation

The IIS pathway might serve a good example of how mechanistic and evolutionary insights can be combined to gain a more complete understanding of life-history evolution (Houle 2001; Flatt & Heyland 2011). Since the 1990s, a great deal has been learned about the genetic, developmental and physiological effects of this pathway in model organisms. This work has shown that IIS mutants affect major fitness-related traits, and this in turn has

illuminated our understanding of the molecular underpinnings of growth, size, lifespan and trade-offs (Partridge & Gems 2002; Tatar *et al*. 2003; Flatt *et al.* 2005; Flatt & Heyland 2011). In particular, these studies have revealed that IIS plays an evolutionarily conserved role in the physiological regulation of longevity (Partridge & Gems 2002; Tatar *et al*. 2003); they have also given us some of the clearest examples of alleles exhibiting antagonistic pleiotropy (Williams 1957; Flatt & Promislow 2007). The functional characterization of this pathway thus promised an opportunity for evolutionary geneticists to identify natural variants involved in life-history evolution (de Jong & Bochdanovits 2003). On the other hand, "life-history loci" identified via functional genetic analysis must not necessarily contribute to standing variation for these traits in the wild (Flatt 2004; Flatt & Schmidt 2009).

For some time, it thus remained unclear whether natural variation in this pathway impacts variation in fitness-related traits in natural populations (cf. Reznick 2005). This situation has changed quite substantially in recent years: to date, we have growing evidence that IIS contributes to life-history variation and adaptation in flies and other insects, worms, fish, reptiles and mammals, including effects on longevity in humans (de Jong & Bochdanovits 2003; Williams *et al*. 2006; O'Neill *et al.* 2007; Flachsbart *et al.* 2008; Suh *et al.* 2008; Willcox *et al*. 2008; Alvarez-Ponce *et al*. 2009; Sparkman *et al*. 2009, 2010; Paaby *et al*. 2010; Stuart & Page 2010; Dantzer & Swanson 2012; Jovelin *et al*. 2014; Paaby *et al.* 2014; Swanson & Dantzer 2014; McGaugh *et al*. 2015; Schwartz & Bronikowski 2016; Zhao *et al*. 2016). This work thus illustrates how, by studying a candidate pathway from multiple angles, one might be able to connect genotypes to molecular mechanisms to environments and to adaptive phenotypes (cf. Houle 2001; Flatt *et al.* 2005; Flatt *et al*. 2013).

Conclusions

Here we have found that a clinal polymorphism in the insulin signaling transcription factor gene *foxo* pleiotropically affects fitness-related traits that are themselves known to be clinally varying, including egg-to-adult survival, several size traits, starvation resistance and fat content. The directionality of most of these effects matches the observed phenotypic cline (Schmidt *et al.* 2005a, b; Schmidt & Paaby 2008; Betancourt *et al.*, submitted), thus confirming previous genomic data suggesting that this variant is shaped by selection (Fabian *et al.* 2012). Our results are also in good agreement with functional studies of the *foxo* locus (Jünger *et al*. 2003, 2008; Kramer *et al.* 2008; Slack *et al*. 2011). Together with the results on *InR* (Paaby *et al*. 2010, 2014), our study demonstrates that variation in IIS makes an important and – at least partly – predictable contribution to clinal life-history adaptation in *Drosophila*.

Acknowledgements

We thank the members of the Schmidt and Flatt labs for assistance in the lab, and Tad Kawecki, Wolf Blanckenhorn and Marc Tatar for helpful discussions. Our research was funded by the Swiss National Science Foundation (SNSF grants PP00P3_133641; PP00P3_165836; 310030E-164207 to TF), the Austrian Science Foundation (FWF P21498-B11 to TF), the National Institutes of Health (NIH 5R01GM100366 to PS), the National Science Foundation (NSF DEB 0921307 to PSS), and the Department of Ecology and Evolution at the University of Lausanne.

References

Ackermann M, Bijlsma R, James AC, *et al.* (2001) Effects of assay conditions in life history experiments with *Drosophila melanogaster*. *Journal of Evolutionary Biology*, **14**, 199-209.

Adrion JR, Hahn MW, Cooper BS. 2015. Revisiting classic clines in *Drosophila melanogaster* in the age of genomics. *Trends in Genetics*, **31**, 434-444.

Alvarez-Ponce D, Aguadé M, Rozas J (2009) Network-level molecular evolutionary analysis of the insulin/TOR signal transduction pathway across 12 *Drosophila* genomes. *Genome Research*, **19**, 234-242.

Artimo P, Jonnalagedda M, Arnold K *et al.* (2012) ExPASy: SIB bioinformatics resource

portal. *Nucleic Acids Research*, **40**, W597-W603.

Attrill H, Falls K, Goodman JL *et al.* (2016) FlyBase: establishing a Gene Group resource for *Drosophila melanogaster*. *Nucleic Acids Research*, **44**, D786-D792.

Azevedo R, James AC, McCabe J, Partridge L (1998) Latitudinal variation of wing : thorax size ratio and wing-aspect ratio in *Drosophila melanogaster*. *Evolution*, **52**, 1353-1362.

Barrett RDH, Hoekstra HE (2011) Molecular spandrels: tests of adaptation at the genetic level. *Nature Reviews Genetics*, **12**, 767-780.

Barson NJ, Aykanat T, Hindar K, *et al.* (2015) Sex-dependent dominance at a single locus maintains variation in age at maturity in salmon. *Nature*, **528**, 405-408.

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 Genetics*, **10**, e1004775.

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. Molecular Ecology*, **25**, 1157-1174.

Betancourt NJ, Rajpuorhit S, Durmaz E, Kapun M, Flatt T, Schmidt PS (2017) Allelic polymorphism at *foxo* contributes to adaptive patterns of life history differentiation in natural populations of *Drosophila melanogaster*, submitted manuscript.

Bhan V, Parkash R, Aggarwal DD (2014) Effects of body-size variation on flight-related traits in latitudinal populations of *Drosophila melanogaster*. *Journal of Genetics*, **93**, 103-112.

Birney E (2016) The Mighty Fruit Fly Moves into Outbred Genetics. *PLoS* Genetics, **12**, e1006388.

Bochdanovits Z, de Jong G (2003a) Temperature dependence of fitness components in geographical populations of *Drosophila melanogaster*: changing the association between size and fitness. *Biological Journal of the Linnean Society*, **80**, 717-725.

Bochdanovits Z, de Jong G (2003b) Experimental evolution in *Drosophila melanogaster*: interaction of temperature and food quality selection regimes. *Evolution*, **57**,1829-1836.

Böhni R, Riesgo-Escovar J, Oldham S, *et al.* (1999) Autonomous control of cell and organ size by CHICO, a *Drosophila* homolog of vertebrate IRS1-4. *Cell*, **97**, 865-875.

Britton J, Lockwood W, Li L, Cohen S, Edgar B (2002) *Drosophila*'s insulin/PI3-kinase pathway coordinates cellular metabolism with nutritional conditions. *Developmental Cell*, **2**, 239-249.

Brogiolo W, Stocker H, Ikeya T, *et al.* (2001) An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. *Current Biology*, 11, 213-221.

Broughton SJ, Piper MDW, Ikeya T, *et al*. (2005) Longer lifespan, altered metabolism, and stress resistance in *Drosophila* from ablation of cells making insulin-like ligands. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 3105-3110.

Casas-Tinto S, Marr MT 2nd, Andreu P, Puig O (2007) Characterization of the *Drosophila* insulin receptor promoter. *Biochimica Biophysica Acta*, **1769**, 236-243.

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 Genetics*, **12**, e1005987.

Chen Y, Lee SF, Blanc E, *et al.* (2012) Genome-wide transcription analysis of clinal genetic variation in *Drosophila*. *PLoS ONE*, **7**, e34620.

Chippindale AK, Leroi AM, Kim SB, Rose MR (1993) Phenotypic plasticity and selection in *Drosophila* life-history evolution. I. Nutrition and the cost of reproduction. *Journal of Evolutionary Biology*, **6**, 171-193.

Clancy DJ, Gems D, Harshman LG, *et al.* (2001) Extension of life-span by loss of CHICO, a *Drosophila* insulin receptor substrate protein. *Science*, **292**, 104-106.

Clemson AS, Sgro CM, Telonis-Scott M. (2016) Thermal plasticity in *Drosophila melanogaster* populations from eastern Australia: quantitative traits to transcripts. *Journal of Evolutionary Biology*, **29**, 2447-2463.

Cogni R, Kuczynski K, Koury S, *et al.* (2017) On the Long-term Stability of Clines in Some Metabolic Genes in *Drosophila melanogaster*. *Scientific Reports*,**7**, 42766.

Conover DO, Schultz ET (1995) Phenotypic similarity and the evolutionary significance of countergradient variation. *Trends in Ecology and Evolution*, **10**, 248-252.

Cooper BS, Tharp II JM, Jernberg II, Angilletta Jr MJ (2012) Developmental plasticity of thermal tolerances in temperate and subtropical populations of *Drosophila melanogaster*. *Journal of Thermal Biology*, **37**, 211-216.

Coyne JA, Beecham E (1987) Heritability of Two Morphological Characters Within and Among Natural Populations of *Drosophila melanogaster*. *Genetics*, **117**, 727-737.

Dantzer B, Swanson EM (2011) Mediation of vertebrate life histories via insulin-like growth factor-1. *Biological Reviews*, **87**, 414-429.

David JR, Bocquet C (1975) Evolution in a cosmopolitan species: genetic latitudinal clines in *Drosophila melanogaster* wild populations. *Experientia*, **31**, 164-166.

David JR, Capy P (1988) Genetic variation of *Drosophila melanogaster* natural populations. *Trends in Genetics*, **4**, 106-111.

David JR, Moreteau B, Gauthier JP *et al.* (1994) Reaction Norms of Size Characters in Relation to Growth Temperature in *Drosophila melanogaster* - an Isofemale Lines Analysis. *Genetics Selection Evolution*, **26**, 229-251.

de Jong G, Bochdanovits Z (2003) Latitudinal clines in *Drosophila melanogaster*: body size, allozyme frequencies, inversion frequencies, and the insulin-signalling pathway. *Journal of Genetics*, **82**, 207-223.

Duchen P, Zivkovic D, Hutter S, Stephan W, Laurent S (2013) Demographic inference reveals African and European admixture in the North American *Drosophila melanogaster* population. *Genetics*, **193**, 291-301.

Endler JA (1977) *Geographic Variation, Speciation and Clines.* Princeton University Press, Princeton, NJ.

Endler JA (1986) *Natural Selection in the Wild.* Princeton University Press, Princeton, NJ.

Fabian DK, Kapun M, Nolte V *et al.* (2012) Genome-wide patterns of latitudinal differentiation among populations of *Drosophila melanogaster* from North America. *Molecular Ecology*, **21**, 4748-4769.

Fabian DK, Lack JB, Mathur V, *et al.* (2015) Spatially varying selection shapes life history clines among populations of *Drosophila melanogaster* from sub-Saharan Africa. *Journal of Evolutionary Biology*, **28**, 826-840.

Fielenbach N, Antebi A (2008) *C. elegans* dauer formation and the molecular basis of plasticity. *Genes & Development*, **22**, 2149-2165.

Finch CE, Rose MR (1995) Hormones and the physiological architecture of life history evolution. *Quarterly Review of Biology*, **70**, 1-52.

Flachsbart F, Caliebe A, Kleindorp R, *et al.* (2009) Association of FOXO3A variation with human longevity confirmed in German centenarians. *Proceedings of the National Academy*

of Sciences of the United States of America, **106**, 2700-2705.

Flatt T (2004) Assessing natural variation in genes affecting *Drosophila* lifespan. *Mechanisms of Ageing and Development*, **125**, 155-159.

Flatt T (2016) Genomics of clinal variation in *Drosophila*: disentangling the interactions of selection and demography. *Molecular Ecology*, **25**, 1023-1026.

Flatt T, Heyland A (2011) *Mechanisms of Life History Evolution*. Oxford University Press, Oxford.

Flatt T, Promislow DE (2007) Physiology. Still pondering an age-old question. *Science*, **318**, 1255-1256.

Flatt T, Schmidt PS (2009) Integrating evolutionary and molecular genetics of aging. *Biochimica et Biophysica Acta*, **1790**, 951-962.

Flatt T, Tu M-P, Tatar M (2005) Hormonal pleiotropy and the juvenile hormone regulation of *Drosophila* development and life history. *BioEssays*, **27**, 999-1010.

Flatt T, Amdam GV, Kirkwood TBL, Omholt SW (2013) Life-History Evolution and the Polyphenic Regulation of Somatic Maintenance and Survival. *Quarterly Review of Biology*, **88**, 185-218.

Folguera G, Ceballos S, Spezzi L, Fanara JJ, Hasson E (2008) Clinal variation in developmental time and viability, and the response to thermal treatments in two species of *Drosophila. Biological Journal of the Linnean Society*, **95**, 233-245.

Gems D, Sutton A, Sundermeyer M, *et al*. (1998) Two pleiotropic classes of *daf-2* mutation affect larval arrest, adult behavior, reproduction and longevity in *Caenorhabditis elegans*. *Genetics*, **150**, 129-155.

Giannakou ME, Partridge L (2007) Role of insulin-like signalling in *Drosophila* lifespan. *Trends in Biochemical Sciences*, **32**, 180-188.

Giannakou ME, Goss M, Junger MA, *et al.* (2004) Long-lived *Drosophila* with overexpressed dFOXO in adult fat body. *Science*, **305**, 361.

Giannakou ME, Goss M, Partridge L (2008) Role of dFOXO in lifespan extension by dietary restriction in *Drosophila melanogaster*: not required, but its activity modulates the response. *Aging Cell*, **7**, 187-198.

Gilchrist AS, Azevedo RBR, Partridge L, O'Higgins P (2000) Adaptation and constraint in the evolution of *Drosophila melanogaster* wing shape. *Evolution & Development*, **2**, 114-124.

Goenaga J, Fanara JJ, Hasson E (2010) A quantitative genetic study of starvation resistance at different geographic scales in natural populations of *Drosophila melanogaster*. Genetical Research, **92**, 253-259.

Goenaga J, Fanara JJ, Hasson E (2013) Latitudinal Variation in Starvation Resistance is Explained by Lipid Content in Natural Populations of *Drosophila melanogaster*. *Evolutionary Biology*, **40**, 601-612.

Hoffmann AA, Harshman LG (1999) Desiccation and starvation resistance in *Drosophila*: patterns of variation at the species, population and intrapopulation levels. *Heredity*, **83**, 637-643.

Hoffmann AA, McKechnie SW (1991) Heritable variation in resource utilization and response in a winery population of *Drosophila melanogaster*. *Evolution*, **45**, 1000-1015.

Hoffmann AA, Weeks AR (2007) Climatic selection on genes and traits after a 100 year-old invasion: a critical look at the temperate-tropical clines in *Drosophila melanogaster* from eastern Australia. *Genetica*, **129**, 133-147.

Hoffmann AA, Shirriffs J, Scott M (2005) Relative importance of plastic vs genetic factors in adaptive differentiation: geographical variation for stress resistance in *Drosophila*

melanogaster from eastern Australia. *Functional Ecology*, **19**, 222-227.

Holzenberger M, Dupont J, Ducos B, *et al*. (2003) IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature*, **421**, 182-187.

Houle D (2001) Characters as the Units of Evolutionary Change In: The Character Concept in Evolutionary Biology (ed. Wagner GP), pp. 109-140. Academic Press: San Diego.

Hwangbo DS, Gersham B, Tu MP, Palmer M, Tatar M (2004) *Drosophila* dFOXO controls lifespan and regulates insulin signalling in brain and fat body. *Nature*, **429**, 562-566.

James AC, Partridge L (1995) Thermal evolution of rate of larval development in *Drosophila melanogaster* in laboratory and field populations. *Journal of Evolutionary Biology*, **8**, 315-330.

Johnston SE, Gratten J, Berenos C, *et al.* (2013) Life history trade-offs at a single locus maintain sexually selected genetic variation. *Nature*, **502**, 93-95.

Jones FC, Grabherr MG, Chan YF *et al.* (2012) The genomic basis of adaptive evolution in threespine sticklebacks. *Nature*, **484**, 55-61.

Jovelin R, Comstock JS, Cutter AD, Phillips PC (2014) A Recent Global Selective Sweep on the *age-1* Phosphatidylinositol 3-OH Kinase Regulator of the Insulin-Like Signaling Pathway Within *Caenorhabditis remanei*. *G3: Genes|Genomes|Genetics*, **4**, 1123-1133.

Jünger MA, Rintelen F, Stocker H *et al.* (2003) The *Drosophila* forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. *Journal of Biology*, **2**, 20.

Kao JY, Zubair A, Salomon MP, Nuzhdin SV, Campo D (2015) Population genomic analysis uncovers African and European admixture in *Drosophila melanogaster* populations from the south-eastern United States and Caribbean Islands. *Molecular Ecology*, **24**, 1499-1509.

Kapun M, Schmidt C, Durmaz E, Schmidt PS, Flatt T (2016a) Parallel effects of the inversion In(3R)Payne on body size across the North American and Australian clines in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, **29**, 1059-1072.

Kapun M, Fabian DK, Goudet J, Flatt T (2016b) Genomic Evidence for Adaptive Inversion Clines in *Drosophila melanogaster*. *Molecular Biology and Evolution*, **33**, 1317-1336.

Karan D, Dahiya N, Munjal AK *et al.* (1998) Desiccation and Starvation Tolerance of Adult *Drosophila*: Opposite Latitudinal Clines in Natural Populations of Three Different Species. *Evolution*, **52**, 825-831.

Keller A (2007) *Drosophila melanogaster*'s history as a human commensal. *Current Biology*, **17**, R77-R81.

Kenyon C (2001) A conserved regulatory system for aging. *Cell*, **105**, 165-168.

Kenyon CJ (2010) The genetics of ageing. *Nature*, **464**, 504-512.

Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R (1993) A *C. elegans* mutant that lives twice as long as wild type. *Nature*, **366**, 461-464.

Klepsatel P, Gáliková M, De Maio N *et al.* (2013) Variation in Thermal Performance and Reaction Norms Among Populations of *Drosophila melanogaster*. *Evolution*, **67**, 3573-3587.

Klepsatel P, Gáliková M, Huber CD, Flatt T (2014) Similarities and differences in altitudinal versus latitudinal variation for morphological traits in *Drosophila melanogaster*. *Evolution*, **68**, 1385-1398.

Kolaczkowski B, Kern AD, Holloway AK, Begun DJ (2011) Genomic differentiation between temperate and tropical Australian populations of *Drosophila melanogaster*. *Genetics*, **187**, 245–260.

Kramer JM, Davidge JT, Lockyer JM, Staveley BE (2003) Expression of *Drosophila* FOXO

regulates growth and can phenocopy starvation. *BMC Developmental Biology*, **3**, 5.

Kramer JM, Slade JD, Staveley BE (2008) *foxo* is required for resistance to amino acid starvation in Drosophila. *Genome*, **51**, 668-672.

Kubrak OI, Kučerová L, Theopold U, Nässel DR (2014) The Sleeping Beauty: How Reproductive Diapause Affects Hormone Signaling, Metabolism, Immune Response and Somatic Maintenance in *Drosophila melanogaster*. *PLoS ONE*, **9**, e113051.

Lachaise D, Cariou M-L, David JR, *et al.* (1988) Historical biogeography of the *Drosophila melanogaster* species subgroup. *Evolutionary Biology*, **22**, 159-225.

Lee KP, Jang T (2014) Exploring the nutritional basis of starvation resistance in *Drosophila melanogaster. Functional Ecology*, **28**, 1144-1155.

Lee SF, Eyre-Walker YC, Rane RV, *et al*. (2013) Polymorphism in the *neurofibromin* gene, *Nf1*, is associated with antagonistic selection on wing size and development time in *Drosophila melanogaster*. *Molecular Ecology*, **22**, 2716-2725.

Levine MT, Eckert ML, Begun DJ (2011) Whole-Genome Expression Plasticity across Tropical and Temperate *Drosophila melanogaster* Populations from Eastern Australia. *Molecular Biology and Evolution*, **28**, 249-256.

Levins, R (1968) *Evolution in Changing in Environments.* Princeton University Press, Princeton.

Levins R (1969) Thermal Acclimation and Heat Resistance in *Drosophila* Species. *American Naturalist*, **103**, 483-499.

Lewontin RC (1974) *The Genetic Basis of Evolutionary Change.* Columbia University Press, New York.

Lewontin RC (2000) The Problem of Population Genetics. In: Evolutionary Genetics – From Molecules to Morphology (eds Singh RS, Krimbas CB), pp. 5-23. Cambridge University Press, Cambridge.

Li Q, Gong Z (2015) Cold-sensing regulates *Drosophila* growth through insulin-producing cells. *Nature Communications*, **6**, 10083.

Libina N, Berman J, Kenyon C (2003) Tissue-specific activities of *C. elegans* DAF-16 in the regulation of lifespan. *Cell*, **115**, 489-502.

Lihoreau M, Poissonnier L-A, Isabel G, Dussutour A (2016) *Drosophila* females trade off good nutrition with high-quality oviposition sites when choosing foods. *Journal of Experimental Biology*, **219**, 2514-2524.

Machado HE, Bergland AO, O'Brien KR, *et al.* (2016) Comparative population genomics of latitudinal variation in *Drosophila simulans* and *Drosophila melanogaster*. *Molecular Ecology*, **25**, 723-740.

Mackay TF, Stone EA, Ayroles JF (2009) The genetics of quantitative traits: challenges and prospects. *Nature Reviews Genetics*, **10**, 565-577.

Mackay TFC, Richards S, Stone EA *et al.* (2012) The *Drosophila melanogaster* Genetic Reference Panel. *Nature*, **482**, 173-178.

Marcil J, Swain DP, Hutchings JA (2006) Countergradient variation in body shape between two populations of Atlantic cod (*Gadus morhua*). *Proceedings of the Royal Society of London B*, **273**, 217-223.

Markow TA, Raphael B, Dobberfuhl D, *et al.* (1999) Elemental stoichiometry of *Drosophila* and their hosts. *Functional Ecology*, **13**, 78-84.

Mathur V, Schmidt PS (2017) Adaptive patterns of phenotypic plasticity in laboratory and field environments in *Drosophila melanogaster*. *Evolution*, **71**, 465-474.

Mattila J, Bremer A, Ahonen L, Kostiainen R, Puig O (2009) *Drosophila* FoxO Regulates

Organism Size and Stress Resistance through an Adenylate Cyclase. *Molecular and Cellular Biology*, **29**, 5357-5365.

Matzkin LM, Watts TD, Markow TA (2009) Evolution of stress resistance in *Drosophila*: interspecific variation in tolerance to desiccation and starvation. *Functional Ecology*, **23**, 521-527.

McGaugh SE, Bronikowski AM, Kuo C-H, *et al.* (2015) Rapid molecular evolution across amniotes of the IIS/TOR network. *Proceedings of the National Academy of Sciences of the United States of America*, **112**, 7055-7060.

McKechnie SW, Blacket MJ, Song SV *et al.* (2010) A clinally varying promoter polymorphism associated with adaptive variation in wing size in *Drosophila*. *Molecular Ecology*, **19**, 775-784.

Méndez-Vigo B, Martínez-Zapater JM, Alonso-Blanco C (2013) The Flowering Repressor *SVP* Underlies a Novel *Arabidopsis thaliana* QTL Interacting with the Genetic Background. *PLoS Genetics*, **9**, e1003289.

Murphy CT, McCarroll SA, Bargmann CI, *et al.* (2003) Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*. *Nature*, **424**, 277-283.

Nunes MDS, Arif S, Schlötterer C, McGregor AP (2013) A Perspective on Micro-Evo-Devo: Progress and Potential. *Genetics*, **195**, 625-634.

Oldham S, Hafen E (2003) Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control. *Trends in Cell Biology*, **13**, 79-85.

Oldham S, Stocker H, Laffargue M, *et al.* (2002) The *Drosophila* insulin/IGF receptor controls growth and size by modulating PtdIns P3 levels. *Development*, **129**, 4103-4109.

Overgaard J, Kristensen TN, Mitchell KA, Hoffmann AA (2011) Thermal Tolerance in Widespread and Tropical *Drosophila* Species: Does Phenotypic Plasticity Increase with Latitude? *American Naturalist*, **178**, S80-S96.

Paaby AB, Schmidt PS (2009) Dissecting the genetics of longevity in *Drosophila melanogaster*. *Fly*, **3**, 1-10.

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**, 3395–3409.

Paaby AB, Blacket MJ, Hoffmann AA, Schmidt PS (2010) Identification of a candidate adaptive polymorphism for *Drosophila* life history by parallel independent clines on two continents. *Molecular Ecology*, **19**, 760-774.

Partridge L, Gems D (2002) Mechanisms of ageing: public or private? *Nature Reviews Genetics*, **3**, 165-175.

Partridge L, Barrie B, Fowler K, French V (1994a) Evolution and development of body size and cell size in *Drosophila melanogaster* in response to temperature. *Evolution*, **48**, 1269-1276.

Partridge L, Barrie B, Fowler K, French V (1994b) Thermal Evolution of Pre-Adult Life-History Traits in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, **7**, 645-663.

Partridge L, Gems D, Withers DJ (2005) Sex and Death: What Is the Connection? *Cell*, **120**, 461-472.

Ponton F, Chapuis M-P, Pernice M, Sword GA, Simpson SJ (2011) Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in *Drosophila melanogaster*. *Journal of Insect Physiology*, **57**, 840-850

Puig O, Mattila J (2011) Understanding Forkhead Box Class O Function: Lessons from *Drosophila melanogaster*. *Antioxidants & Redox Signaling*, **14**, 635-647.

Puig O, Tjian R (2005) Transcriptional feedback control of insulin receptor by dFOXO / FOXO1. *Genes & Development*, **19**, 2435-2446.

Puig O, Marr MT, Ruhf ML, Tjian R (2003) Control of cell number by *Drosophila* FOXO: downstream and feedback regulation of the insulin receptor pathway. *Genes & Development*, **17**, 2006–2020.

Reinhardt JA, Kolaczkowski B, Jones CD, Begun DJ, Kern AD (2014) Parallel Geographic Variation in *Drosophila melanogaster*. *Genetics*, **197**, 361-373.

Reis T (2016) Effects of Synthetic Diets Enriched in Specific Nutrients on *Drosophila* Development, Body Fat, and Lifespan. *PLoS ONE*, **11**, e0146758.

Reznick DN (2005) The genetic basis of aging: an evolutionary biologist's perspective. *Science Aging Knowledge Environment*, **11**, pe7.

Robinson SJW, Zwaan B, Partridge L (2000) Starvation Resistance and Adult Body Composition in a Latitudinal Cline of *Drosophila melanogaster*. *Evolution*, **54**, 1819-1824.

Rockman MV (2012) The QTN program and the alleles that matter for evolution: all that's gold does not glitter. *Evolution*, **66**, 1-17.

Schiesari L, Andreatta G, Kyriacou CP, O'Connor MB, Costa R (2016) The Insulin-Like Proteins dILPs-2/5 Determine Diapause Inducibility in *Drosophila. PLoS ONE*, **11**, e0163680.

Schluter D, Price TD, Rowe L (1991) Conflicting selection pressures and life history trade-offs. *Proceedings of the Royal Society of London B*, **246**,11-17.

Schmidt PS, Conde DR (2006) Environmental Heterogeneity and the Maintenance of Genetic Variation for Reproductive Diapause in *Drosophila melanogaster*. *Evolution*, **60**, 1602.

Schmidt PS, Paaby AB (2008) Reproductive Diapause and Life-History Clines in North American Populations of *Drosophila melanogaster*. *Evolution*, **62**, 1204-1215.

Schmidt PS, Duvernell DD, Eanes WF (2000) Adaptive evolution of a candidate gene for aging in *Drosophila*. *Proceedings of the National Academy of Sciences*, **97**, 10861–10865.

Schmidt PS, Matzkin L, Ippolito M, Eanes WF (2005a) Geographic Variation in Diapause Incidence, Life-History Traits, and Climatic Adaptation in *Drosophila melanogaster*. *Evolution*, **59**, 1721-1732.

Schmidt PS, Paaby AB, Heschel MS (2005b) Genetic variance for diapause expression and associated life histories in *Drosophila melanogaster*. *Evolution*, **59**, 2616-2625.

Schmidt P, Zhu C, Das J, Batavia M, Yang L, Eanes M (2008). An amino acid polymorphism in the *couch potato* gene forms the basis for climatic adaptation in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences USA*, **105**, 16207-16211.

Schwartz TS, Bronikowski AM (2016) Evolution and Function of the Insulin and Insulin-like Signaling Network in Ectothermic Reptiles: Some Answers and More Questions. *Integrative and Comparative Biology*, **56**, 171-184.

Slack C, Giannakou ME, Foley A, Goss M, Partridge L (2011) dFOXO-independent effects of reduced insulin-like signaling in *Drosophila*. *Aging Cell*, **10**, 735-748.

Sparkman AM, Vleck CM, Bronikowski AM (2009) Evolutionary ecology of endocrine-mediated life-history variation in the garter snake *Thamnophis elegans*. *Ecology*, **90**, 720-728.

Sparkman AM, Byars D, Ford NB, Bronikowski AM (2010) The role of insulin-like growth factor-1 (IGF-1) in growth and reproduction in female brown house snakes (*Lamprophis fuliginosus*). *General and Comparative Endocrinology*, **168**, 408-414.

Strassburger K, Zoeller T, Sandmann T, *et al.* 2017. Sorting & Sequencing Flies By Size: Identification Of Novel TOR Regulators And Parameters For Successful Sorting. Preprint,

bioRxiv, doi: https://doi.org/10.1101/119719.

Stalker HD (1980) Chromosome-Studies in Wild Populations of *Drosophila melanogaster* .2. Relationship of Inversion Frequencies to Latitude, Season, Wing-Loading and Flight Activity. *Genetics*, **95**, 211-223.

Stearns SC (1992) *The Evolution of Life Histories*. Oxford University Press, New York.

Stern DL (2000) Perspective: evolutionary developmental biology and the problem of variation. *Evolution*, **54,** 1079-1091.

Stern DL, Orgogozo V (2008) The loci of evolution: how predictable is genetic evolution? *Evolution*, **62**, 2155-2177.

Stuart JA, Page MM (2010) Plasma IGF-1 is negatively correlated with body mass in a comparison of 36 mammalian species. *Mechanisms of Ageing and Development*, **131**, 591-598.

Suh Y, Atzmon G, Cho MO, *et al.* (2008) Functionally significant insulin-like growth factor I receptor mutations in centenarians. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 3438-3442.

Swanson EM, Dantzer B (2014) Insulin-like growth factor-1 is associated with life-history variation across Mammalia. *Proceedings of the Royal Society of London B*, **281**, 20132458.

Tang HY, Smith-Caldas MS, Driscoll MV, Salhadar S, Shingleton AW (2011) FOXO regulates organ-specific phenotypic plasticity in *Drosophila*. *PLoS Genetics*, **7(11)**, e1002373.

Tatar M, Yin C-M (2001) Slow aging during insect reproductive diapause: why butterflies, grasshoppers and flies are like forms. *Experimental Gerontology*, **36**, 723-738.

Tatar M, Bartke A, Antebi A (2003) The Endocrine Regulation of Aging by Insulin-like Signals. *Science*, **299**, 1346-1351.

Tatar M, Kopelman A, Epstein D *et al.* (2001) A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function. *Science*, **292**, 107-110.

Teleman AA (2010) Molecular mechanisms of metabolic regulation by insulin in *Drosophila.* *Biochemical Journal*, **425**, 13-26.

Tennessen JM, Barry WE, Cox J, Thummel CS (2014) Methods for studying metabolism in *Drosophila*. *Methods*, **68**, 105-115.

Trotta V, Calboli FC, Ziosi M, *et al.* (2006) Thermal plasticity in *Drosophila melanogaster*: A comparison of geographic populations. *BMC Evolutionary Biology*, **6**, 67.

Turner TL (2014) Fine-mapping natural alleles: quantitative complementation to the rescue. *Molecular Ecology*, **23**, 2377–2382.

Turner TL, Levine MT, Eckert ML, Begun DJ. (2008) Genomic Analysis of Adaptive Differentiation in *Drosophila melanogaster*. *Genetics*, **179**, 455-473.

Van't Land J, van Putten P, Zwaan B, *et al.* (1999) Latitudinal variation in wild populations of *Drosophila melanogaster*: heritabilities and reaction norms. *Journal of Evolutionary Biology*, **12**, 222-232.

Vonesch SC, Lamparter D, Mackay TFC, Bergmann S, Hafen E (2016) Genome-Wide Analysis Reveals Novel Regulators of Growth in *Drosophila melanogaster*. *PLoS Genetics*, **12**, e1005616.

Weeks AR, McKechnie SW, Hoffmann AA (2002) Dissecting adaptive clinal variation: markers, inversions and size/stress associations in *Drosophila melanogaster* from a central field population. *Ecology Letters*, **5**, 756–763.

Willcox BJ, Donlon TA, He Q, *et al.* (2008) FOXO3A genotype is strongly associated with human longevity. *Proceedings of the National Academy of Sciences of the United States of*

America, **105**, 13987-1399.

Williams GC (1957) Pleiotropy, natural selection, and the evolution of senescence. *Evolution*, **11**, 398-411.

Williams K, Busto M, Suster M, *et al*. (2006) Natural variation in *Drosophila melanogaster* diapause due to the insulin-regulated PI3-kinase. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 15911-15915.

Williams KD, Sokolowski MB (1993) Diapause in *Drosophila melanogaster* females: a genetic analysis. *Heredity*, **71**, 312-317.

Zhang B, Xiao R, Ronan Elizabeth A, *et al.* (2015) Environmental Temperature Differentially Modulates *C. elegans* Longevity through a Thermosensitive TRP Channel. *Cell Reports*, **11**, 1414-1424.

Zhao L, Wit J, Svetec N, Begun DJ (2015) Parallel Gene Expression Differences between Low and High Latitude Populations of *Drosophila melanogaster* and *D. simulans*. *PLoS Genetics*, **11**, e1005184.

Zhao X, Bergland AO, Behrman EL, *et al.* (2016) Global Transcriptional Profiling of Diapause and Climatic Adaptation in *Drosophila melanogaster*. *Molecular Biology and Evolution*, **33**, 707-720.

Author Contributions

PS and TF conceived the study and designed research; SR and NB established populations; ED, SR, NB, DF and MK performed research and analyzed data; and ED, PS and TF wrote the paper.

Data Accessibility

Data deposited at Dryad. doi link to be added upon publication.

Table 1. Summary of ANOVA results. ANOVA results for egg-to-adult survival, femur length, the ratio of wing area:thorax length, and female starvation resistance. White and grey cells show the results for females and males, respectively. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. See Results for details.

Factor	Egg-to-adult survival	Femur Length	Wing Area: Thorax Length Ratio	Starvation Resistance
Allele	$F_{1,32}=20.65^{***}$	$F_{1,32}=16.662^{***}$	$F_{1,4}=46.6377^{***}$	$F_{1,32}=23.86^{***}$
		$F_{1,32}=0.1573$	$F_{1,4}=82.1679^{***}$	
Temperature	$F_{1,114}=3.24$	$F_{1,1923}=1617.795^{***}$	$F_{1,18}=477.4462^{***}$	$F_{1,1547}=732.08^{***}$
		$F_{1,1923}=443.6041^{***}$	$F_{1,18}=1366.872^{***}$	
Diet	$F_{1,114}=8.43^{**}$	$F_{1,1923}=144.7179^{***}$	$F_{1,18}=50.348^{***}$	$F_{1,1547}=129.99^{***}$
		$F_{1,1923}=68.2378^{***}$	$F_{1,18}=127.7711^{***}$	
Allele x Temperature	$F_{1,114}=2.25$	$F_{1,1923}=0.3556$	$F_{1,18}=0.144$	$F_{1,1547}=3.43$
		$F_{1,1923}=1.4012$	$F_{1,18}=0.3154$	
Temperature x Diet	$F_{1,114}=1.85$	$F_{1,1923}=13.2584^{***}$	$F_{1,18}=16.6361^{***}$	$F_{1,1547}=14.81^{***}$
		$F_{1,1923}=4.6497$	$F_{1,18}=56.3609^{***}$	
Allele x Diet	$F_{1,114}=1.71$	$F_{1,1923}=3.2833$	$F_{1,18}=0.2063$	$F_{1,1547}=16.22^{***}$
		$F_{1,1923}=4.0377^{*}$	$F_{1,18}=2.5286$	
Allele x Temperature x Diet	$F_{1,114}=0.39$	$F_{1,1923}=6.4056^{*}$	$F_{1,18}=0$	$F_{1,1547}=1.63$
		$F_{1,1923}=0.9495$	$F_{1,18}=8.341^{**}$	
Set(Allele)	$F_{2,32}=2.50$	$F_{2,32}=5.8853^{**}$	$F_{2,4}=6.8604^{**}$	$F_{2,32}=45.24^{***}$
		$F_{2,32}=0.7511$	$F_{2,4}=3.7987^{*}$	
Cage(Set, Allele)	$F_{4,32}=61.25^{***}$	$F_{4,32}=37.4303^{***}$	*NA*	$F_{4,32}=11.17^{***}$
		$F_{4,32}=415.6616^{***}$	*NA*	

Table 2. ANOVA results for female fat loss upon starvation. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. See Results for details.

	Fat content	
Factor	**18°C**	**25°C**
Allele	$F_{1,32}$=0.0224	$F_{1,32}$=1.8986
Diet	$F_{1,301}$=70.9711***	$F_{1,300}$=310.8217***
Treatment	$F_{1,301}$=223.4784***	$F_{1,300}$=130.68***
Allele x Diet	$F_{1,301}$=20.5823***	$F_{1,300}$=6.9304**
Diet x Treatment	$F_{1,301}$=25.4602***	$F_{1,300}$=21.3097***
Allele x Treatment	$F_{1,301}$=7.0094**	$F_{1,300}$=1.242
Allele x Diet x Treatment	$F_{1,301}$=0	$F_{1,300}$=7.0267**
Set(Allele)	$F_{2,32}$=13.1143***	$F_{2,32}$=4.2374*
Cage(Set, Allele)	$F_{4,32}$=9.4591***	$F_{4,32}$=1.4424

Figure Legends

Fig. 1. Clinal candidates in the insulin/TOR signaling pathway. Overview of the insulin/insulin-like growth factor signaling (IIS)/target of rapamycin (TOR) pathway in *Drosophila melanogaster* (Oldham & Hafen 2003; Giannakou & Partridge 2007; Teleman 2010). Genes that harbor strongly clinally varying SNPs across latitude, identified by Fabian *et al.* (2012), are highlighted in red; arrows indicate activation and bar-ended lines represent inhibitory effects. In response to nutrients, IIS is activated by binding of ligands, called insulin-like peptides (ilps 1-8), to the insulin-like receptor (InR) at the cell membrane. Inside the cell, signaling is transduced by an insulin receptor substrate (IRS) protein called chico. This activates phosphoinositide-3-kinase (PI3K) which converts phosphatidylinositol (3,4)-bisphosphate (PIP2) into phosphatidylinositol (3,4,5)-trisphosphate (PIP3). In turn, PIP3 stimulates pyruvate dehydrogenase kinase (PDK) and activates protein kinase B (AKT/PKB). The action of PI3K is antagonized by phosphatase and tensin homologue (PTEN) which converts PIP3 back to PIP2. AKT/PKB suppresses the forkhead (FKH) box O transcription factor FOXO by phosphorylating it; upon reduced IIS, FOXO becomes dephosphorylated and moves into the nucleus where it regulates the expression of hundreds of target genes. Target genes of FOXO include InR, controlled via a transcriptional feedback loop, and initiation factor 4E-binding protein (4E-BP); another target gene of IIS is target of brain insulin (Tobi), which encodes a glucosidase, but the details of its regulation remain poorly understood. FOXO is antagonized by 14-3-3ε. AKT/PKB antagonizes the activity of the tuberous sclerosis complex 1/2 (TSC1/TSC2); TSC1/2 in turn inactivates RAS homologue enriched in brain (RHEB). The inactivation of RHEB deinhibits, i.e. activates, target of rapamycin (TOR). TOR then activates the effector gene S6 kinase (S6K) and inhibits the negative regulator 4E-BP. The phenotypic effects of naturally occuring alleles of the genes in the IIS/TOR pathway remain poorly understood, but clinal polymorphisms in *InR* (Paaby *et al.* 2010, 2013) and *foxo* (this study) have pleiotropic effects on life history in *Drosophila*.

Fig. 2. Egg-to-adult survival. Effects of the clinal *foxo* variant on the proportion egg-to-adult survival (viability). (A) Dietary reaction norms at 18°C. (B) Dietary reaction norms at 25°C. (C) Thermal reaction norms measured on sucrose diet. (D) Thermal reaction norms measured on molasses diet. Data in (A, B) are the same as those shown in (C, D). Shown are means and standard errors. Red lines: low-latitude (LL) allele, blue lines: high-latitude (HL) allele.

Fig. 3. Femur length. Effects of the *foxo* polymorphism on femur length (mm) in females and males. (A) Dietary reaction norms at 18°C. (B) Dietary reaction norms at 25°C. (C) Thermal reaction norms measured on sucrose diet. (D) Thermal reaction norms measured on molasses diet. Data in (A, B) are the same as those shown in (C, D). Shown are means and standard errors. Red lines: low-latitude (LL) allele, blue lines: high-latitude (HL) allele.

Fig. 4. Wing:thorax ratio. Effects of the *foxo* variant on the ratio of wing area:thorax length (mm) in females and males. (A) Dietary reaction norms at 18°C. (B) Dietary reaction norms at 25°C. (C) Thermal reaction norms measured on sucrose diet. (D) Thermal reaction norms measured on molasses diet. Data in (A, B) are the same as those shown in (C, D). Shown are means and (propagated) standard errors. Red lines: low-latitude (LL) allele, blue lines: high-latitude (HL) allele.

Fig. 5. Starvation resistance. Effects of the clinal *foxo* polymorphism on age at death upon starvation in females. (A) Dietary reaction norms at 18°C. (B) Dietary reaction norms at 25°C. (C) Thermal reaction norms measured on sucrose diet. (D) Thermal reaction norms

measured on molasses diet. Data in (A, B) are the same as those shown in (C, D). Shown are means and standard errors. Red lines: low-latitude (LL) allele, blue lines: high-latitude (HL) allele.

Fig. 6. Fat loss upon starvation. Effects of the clinal *foxo* variant on female triglyceride loss upon starvation (μg/fly). (A) Dietary reaction norms at 18°C. (B) Dietary reaction norms at 25°C. (C) Thermal reaction norms measured on sucrose diet. (D) Thermal reaction norms measured on molasses diet. Data in (A, B) are the same as those shown in (C, D). Shown are means and (propagated) standard errors. Red lines: low-latitude (LL) allele, blue lines: high-latitude (HL) allele.

Figure 1

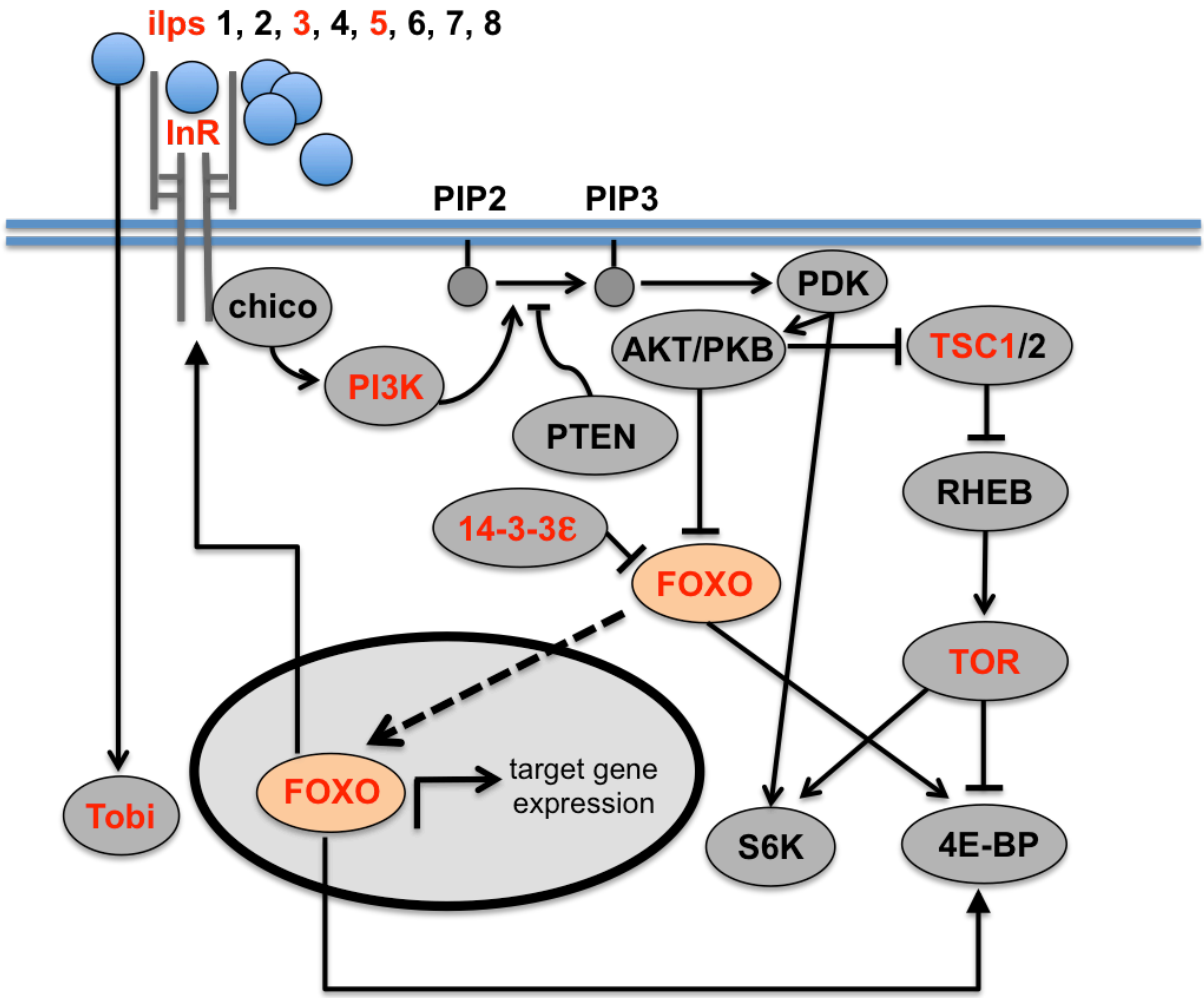

ilps 1, 2, 3, 4, 5, 6, 7, 8
InR
PIP2
PIP3
chico
PDK
AKT/PKB
TSC1/2
PI3K
PTEN
RHEB
14-3-3ε
FOXO
TOR
FOXO
target gene
expression
Tobi
S6K
4E-BP

Figure 2

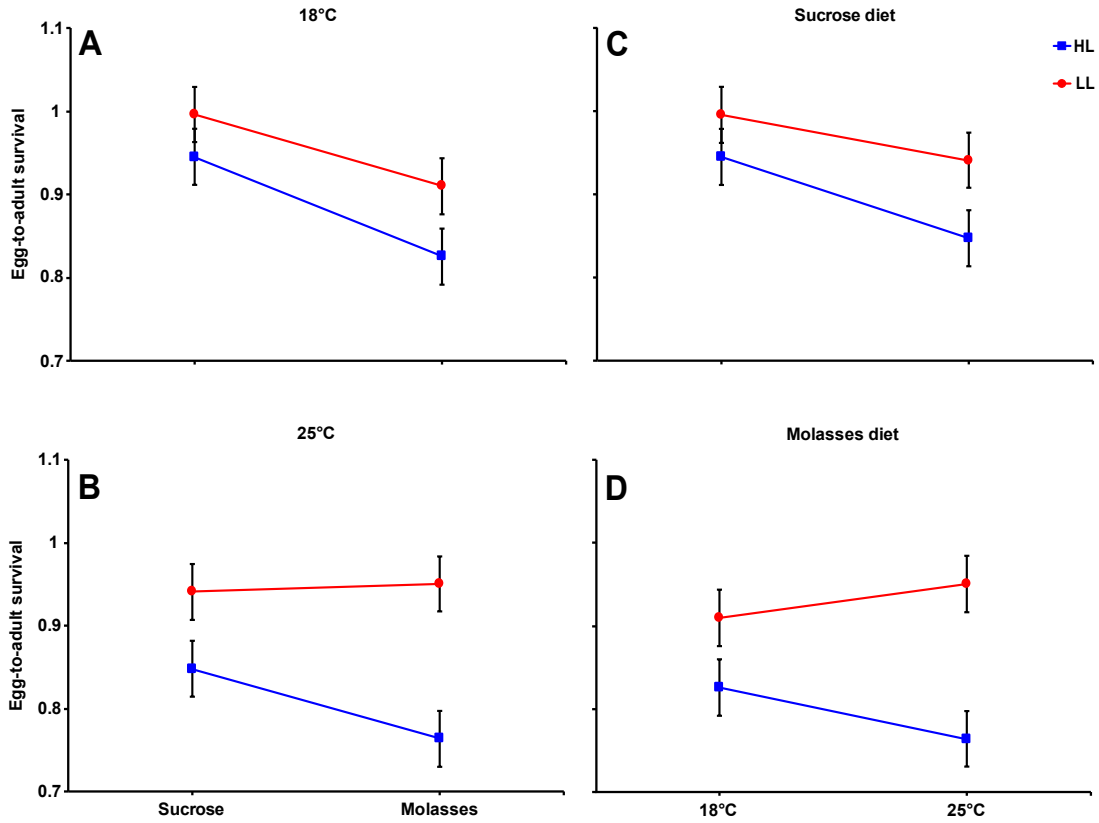

18°C
A
Sucrose diet
C
HL
LL
Egg-to-adult survival
1.1
1
0.9
0.8
0.7
25°C
B
Molasses diet
D
Sucrose
Molasses
18°C
25°C

Figure 3

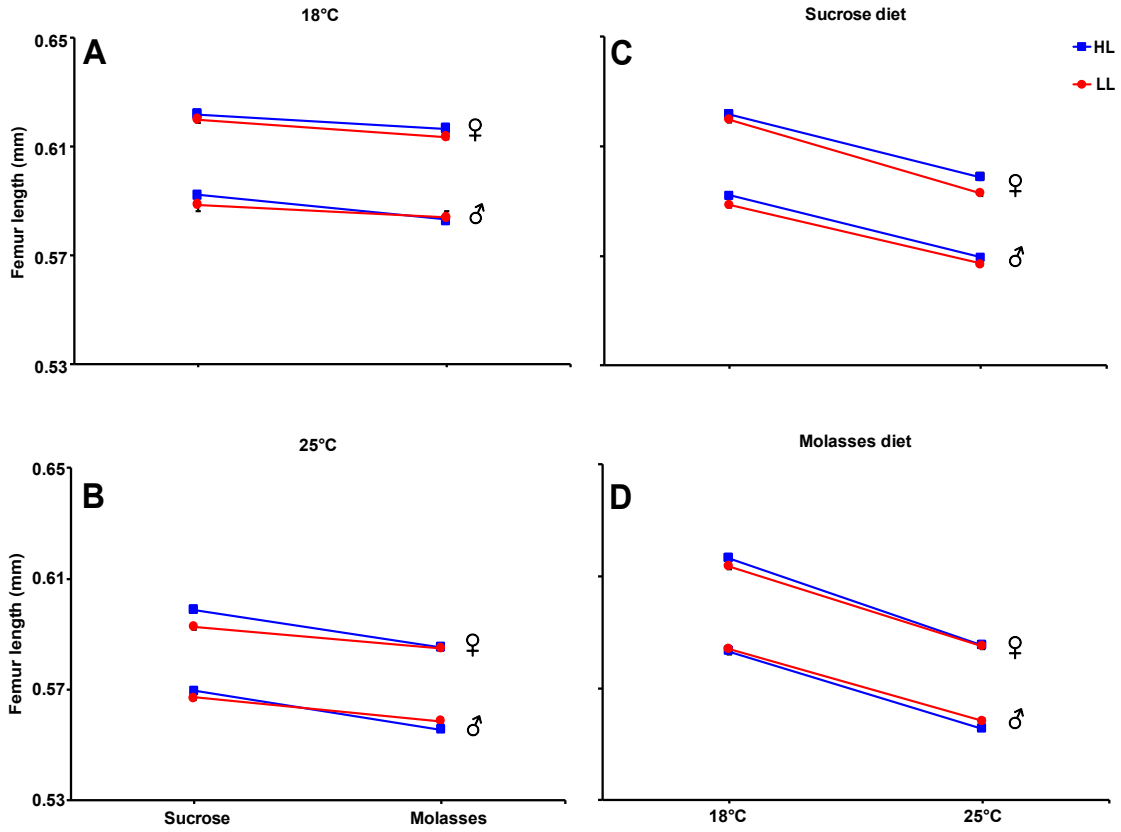

18°C
Sucrose diet
25°C
Molasses diet
A
B
C
D
HL
LL
♀
♂
Femur length (mm)
0.65
0.61
0.57
0.53
Sucrose
Molasses
18°C
25°C

Figure 4

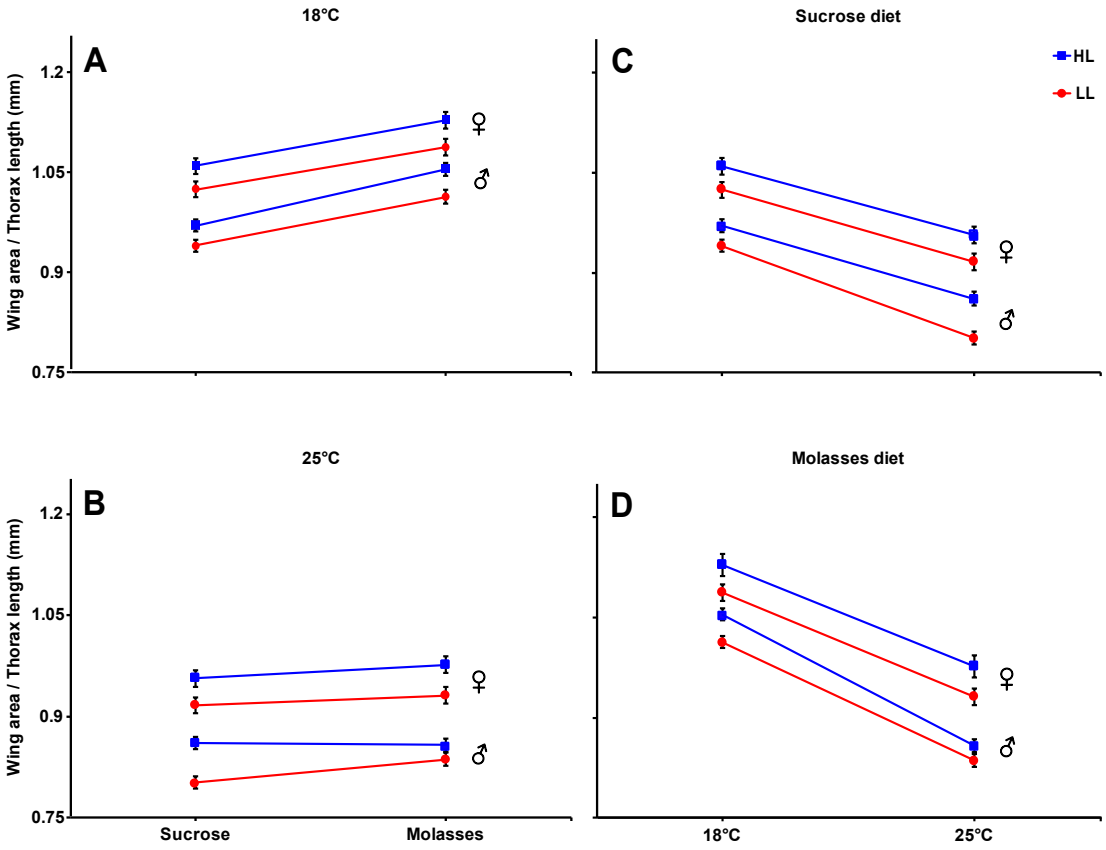

18°C
Sucrose diet
A
C
B
D
HL
LL
♀
♂
Wing area / Thorax length (mm)
1.2
1.05
0.9
0.75
25°C
Molasses diet
Sucrose
Molasses
18°C
25°C

Figure 5

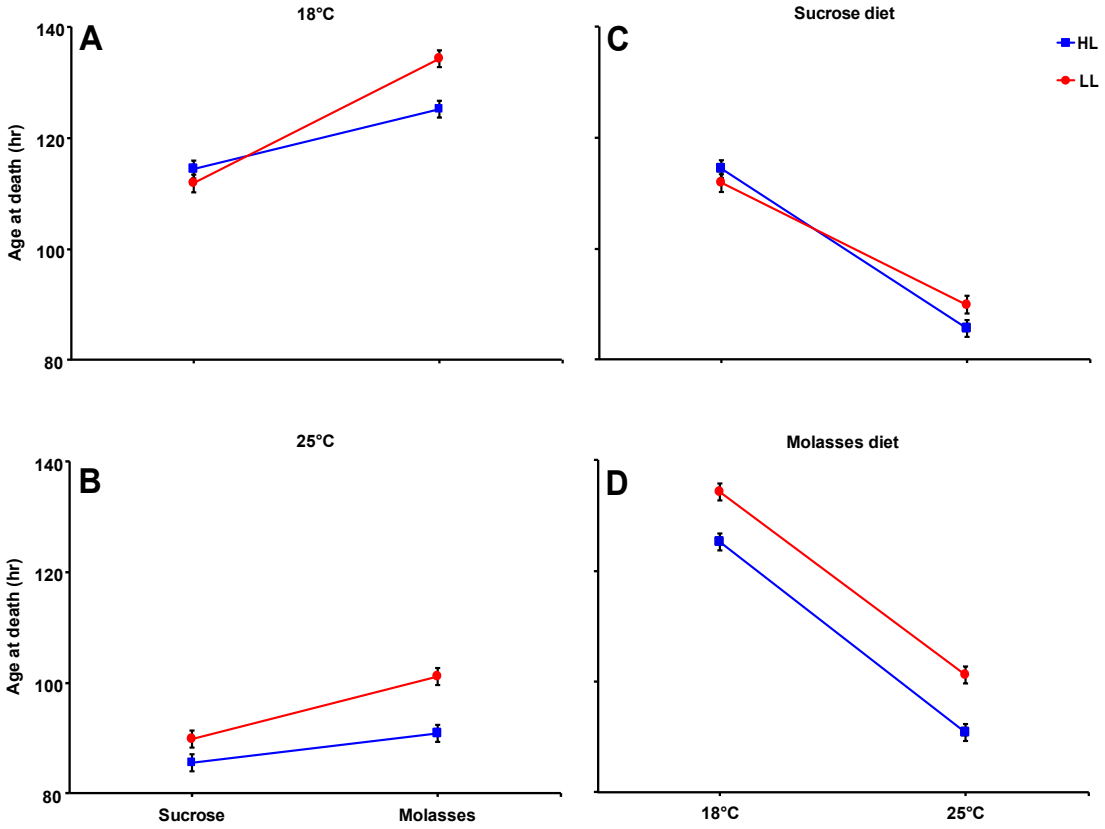
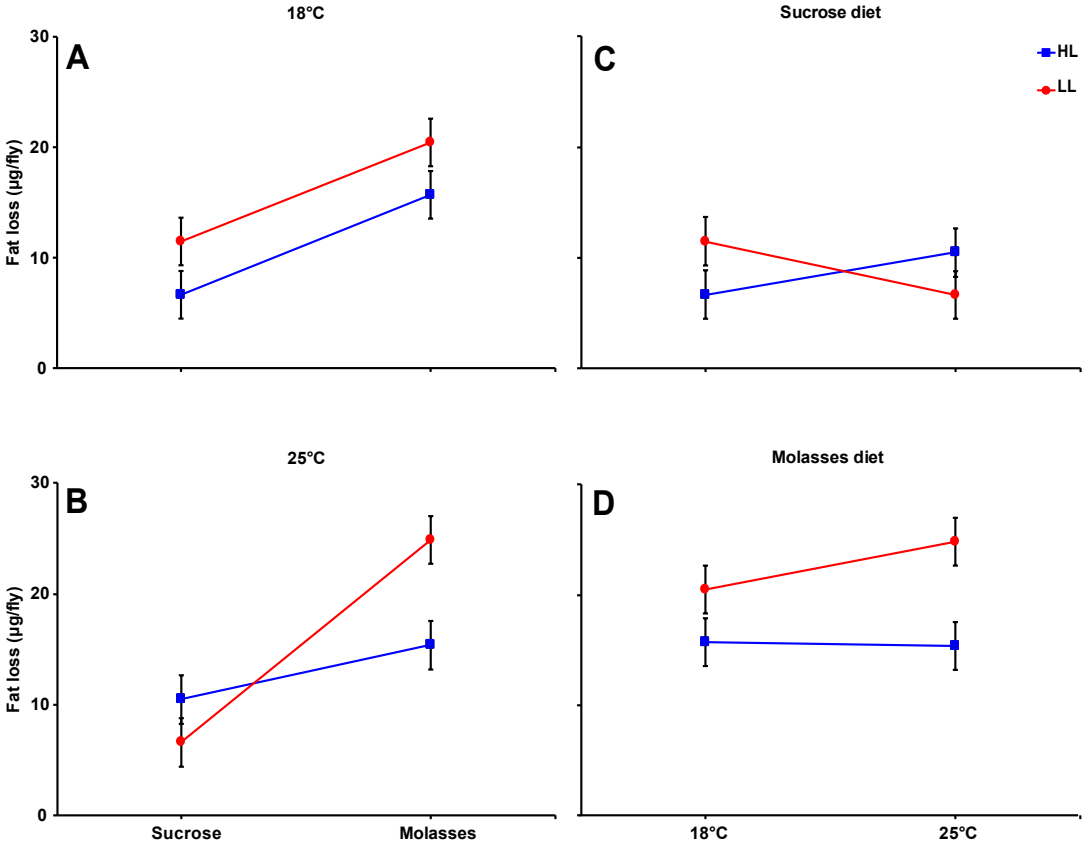

18°C
A
140
120
100
80
Age at death (hr)
Sucrose diet
C
HL
LL
25°C
B
Sucrose
Molasses
Molasses diet
D
18°C
25°C

Figure 6


18°C
Sucrose diet
A
C
HL
LL
Fat loss (µg/fly)
30
20
10
0
25°C
Molasses diet
B
D
Sucrose
Molasses
18°C
25°C

Supporting Information for Durmaz *et al.*, A clinal polymorphism in the insulin signaling transcription factor *foxo* contributes to life-history adaptation in *Drosophila*

Supporting Information Figure Legends

Fig. S1. **Clinal *foxo* candidate SNPs.** (A) Allele frequencies of clinal *foxo* SNPs in Florida (red), Pennsylvania (green) and Maine (blue), identified by Fabian *et al.* 2012 and conditioned to raise in frequency from Florida to Maine. The two strongly clinal *foxo* SNPs studied here are marked with star symbols; the x-axis shows the genomic position of the SNPs on chromosome *3R* in million base pairs (mbp). The plot underneath the x-axis shows the gene model for *foxo*. (B) Linkage disequilibrium (LD; as measured by pairwise r^2) among all polymorphic *foxo* SNPs (minor allele frequency ≥ 0.1) in the DGRP lines used to set up experimental populations (see Materials and Methods section). The two focal SNPs are in perfect LD in the experimental populations (r^2 =1), but there is no significant LD among other, non-focal sites. Also see Fig. S3.

Fig. S2. **PEST motif prediction for FOXO**. The T/G polymorphism in *foxo* at position the *3R*: 9894559, is predicted to be located in the PEST region of the FOXO protein (analysis of *foxo* sequence using ExPASy [Artimo *et al*. 2012]); PEST motifs serve as protein degradation signals (Artimo *et al*. 2012). The potential PEST motif (RPENFVEPTDELDSTK) between amino acid positions 49 and 64 (shown in green) encompasses the *foxo* SNP at position 51 (E).

Fig. S3. **Experimental design for reconstituted outbred *foxo* populations**. We isolated the 2-SNP *foxo* variant by reconstituting outbred populations, fixed for either the low- or high-latitude allele, from lines of the *Drosophila* Genetic Reference Panel (DGRP). Each *foxo* allele was represented by two independent sets of distinct DGRP lines, with two replicate cages per set. See Materials and Methods section for details; also see Fig. S1B.

Fig. S4. **Coordinates of landmarks used to estimate wing area**. We calculated the total wing area encompassed by 12 landmarks (in yellow) by splitting the polygon up into triangles (shown in different colors) and by summing across the areas defined by these triangles. See Materials and Methods section for details.

Fig. S5. **Effects of the *foxo* variant on total wing area**. Effects of the clinal *foxo* variant on wing area (mm^2) in females and males. (A) Dietary reaction norms at 18°C. (B) Dietary reaction norms at 25°C. (C) Thermal reaction norms on sucrose diet. (D) Thermal reaction norms on molasses diet. Shown are means and standard errors. Red lines: low-latitude (LL) allele, blue lines: high-latitude (HL) allele. See Results section for details.

Fig. S6. **Effects of the *foxo* variant on thorax length**. Effects of the clinal *foxo* variant on thorax length (mm) in females and males. (A) Dietary reaction norms at 18°C. (B) Dietary reaction norms at 25°C. (C) Thermal reaction norms on sucrose diet. (D) Thermal reaction

norms on molasses diet. Shown are means and standard errors. Red lines: low-latitude (LL) allele, blue lines: high-latitude (HL) allele. See Results section for details.

Fig. S7. **Effects of the *foxo* variant on male survival upon starvation**. Effects of the clinal *foxo* variant on age at death upon starvation in males. (A) Dietary reaction norms at 18°C. (B) Dietary reaction norms at 25°C. (C) Thermal reaction norms on sucrose diet. (D) Thermal reaction norms on molasses diet. Shown are means and standard errors. Red lines: low-latitude (LL) allele, blue lines: high-latitude (HL) allele. See Results section for details.

Fig. S8. **Effects of the *foxo* variant on relative abundance of insulin-like receptor (InR) transcription levels**. (A) Low-latitude (LL) allele has higher level of InR transcription than the high-latitude (HL) allele. (B) Carbohydrate-rich molasses diet resulted in more InR transcripts than the sucrose diet. Shown are means and standard errors. See Results section for details.

Figure S1

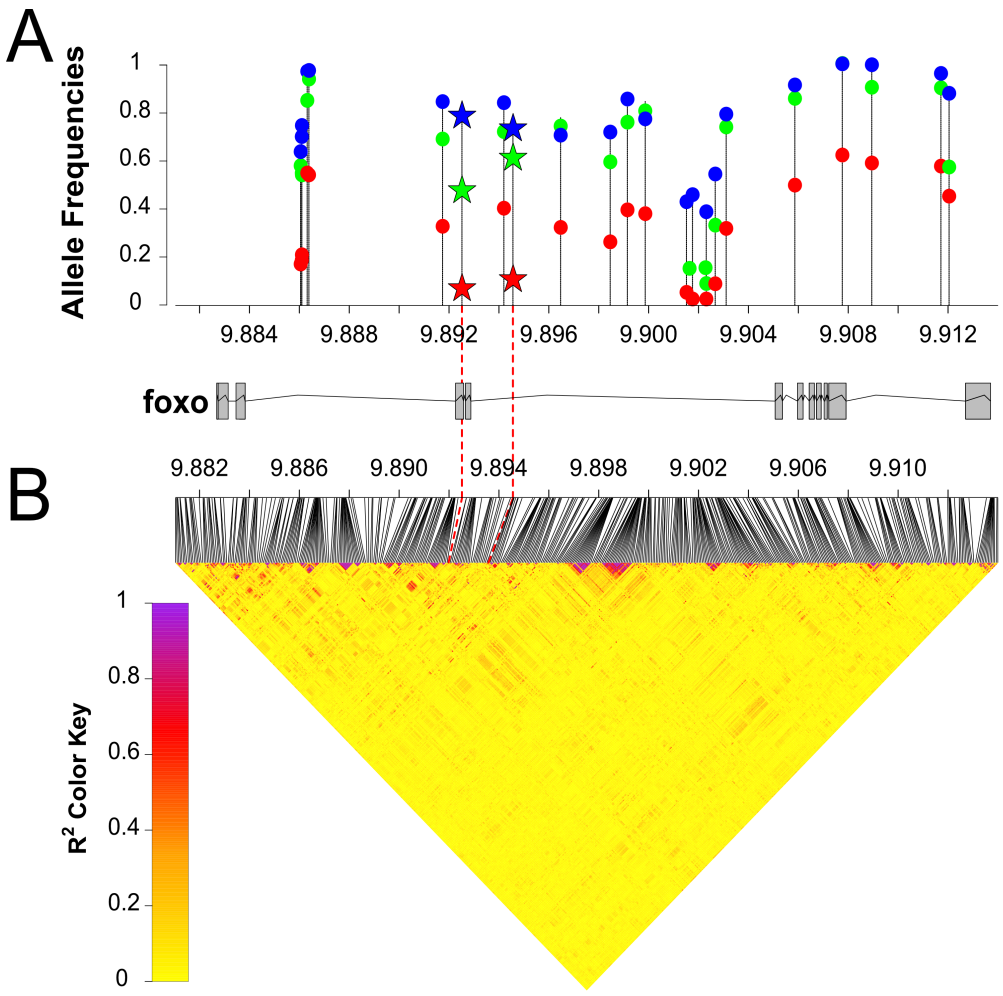

A
Allele Frequencies
1
0.8
0.6
0.4
0.2
0
9.884
9.888
9.892
9.896
9.900
9.904
9.908
9.912
foxo
B
9.882
9.886
9.890
9.894
9.898
9.902
9.906
9.910
R2 Color Key
1
0.8
0.6
0.4
0.2
0

Figure S2

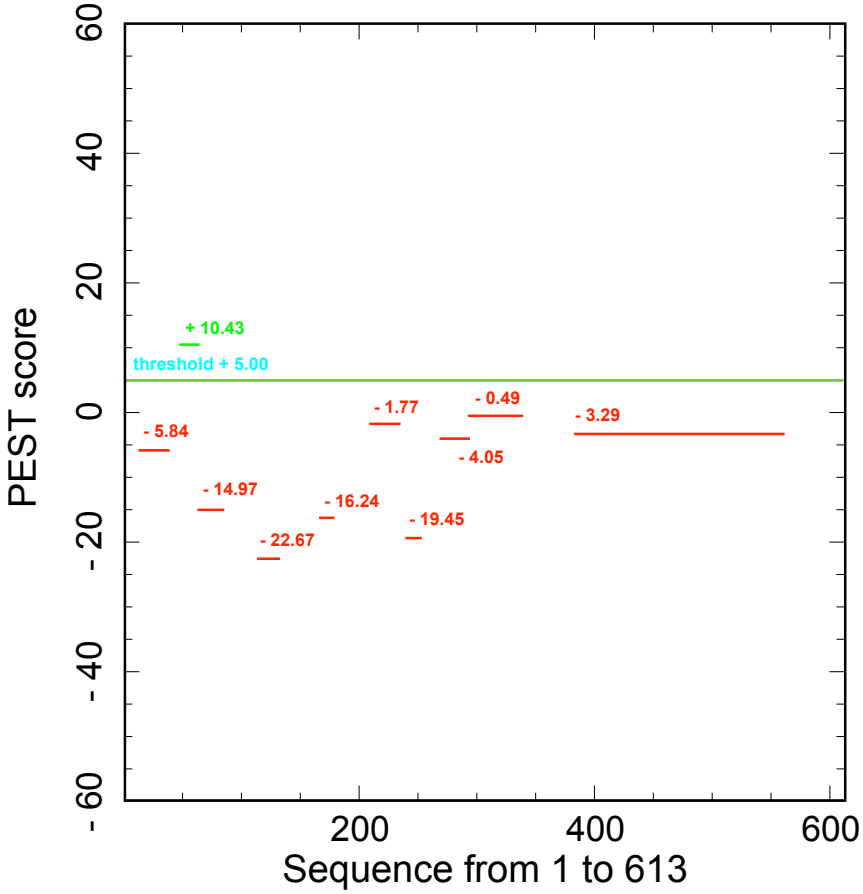

PEST score
60
40
20
0
- 20
- 40
- 60
200
400
600
Sequence from 1 to 613
+ 10.43
threshold + 5.00
- 5.84
- 14.97
- 22.67
- 16.24
- 19.45
- 1.77
- 0.49
- 4.05
- 3.29

Figure S3

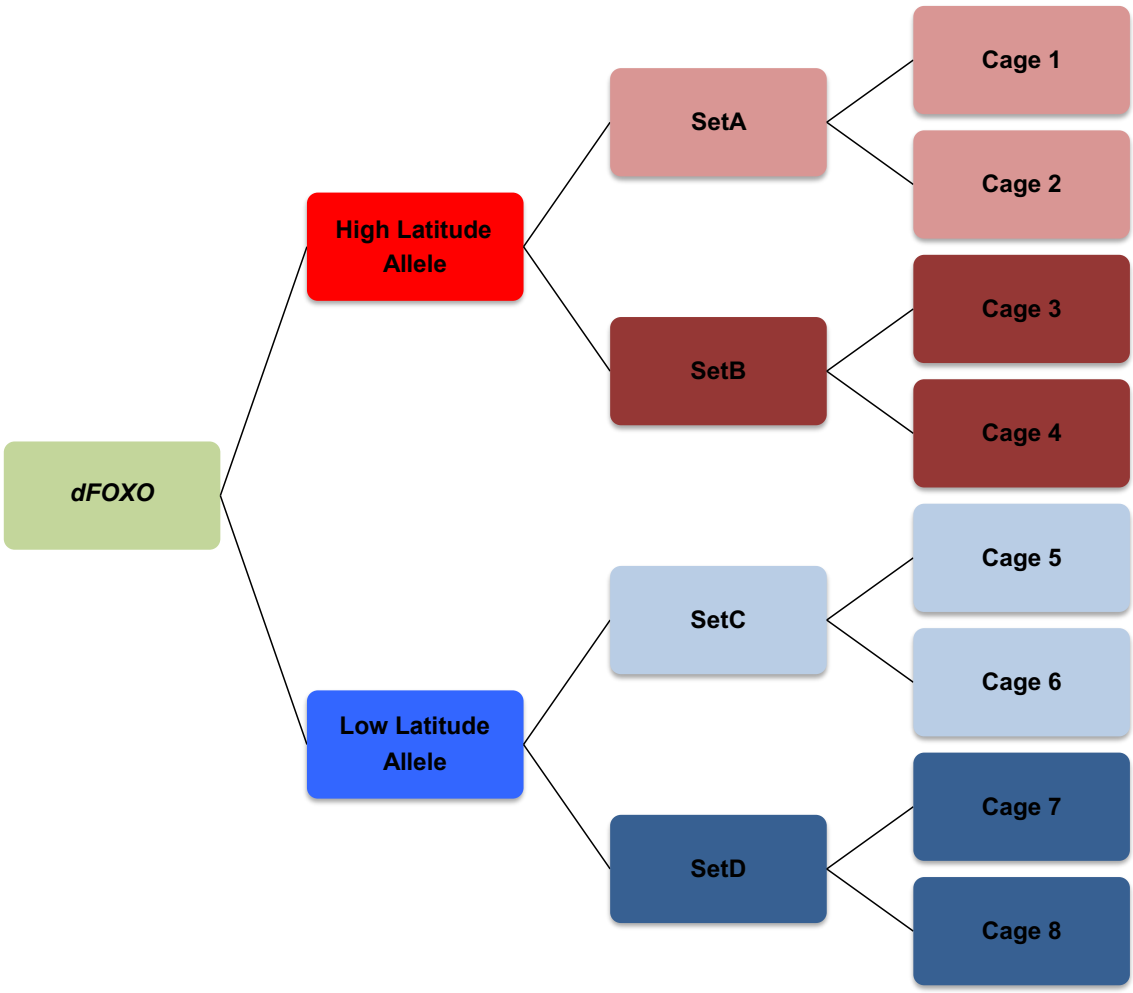

dFOXO
High Latitude Allele
Low Latitude Allele
SetA
SetB
SetC
SetD
Cage 1
Cage 2
Cage 3
Cage 4
Cage 5
Cage 6
Cage 7
Cage 8

Figure S4

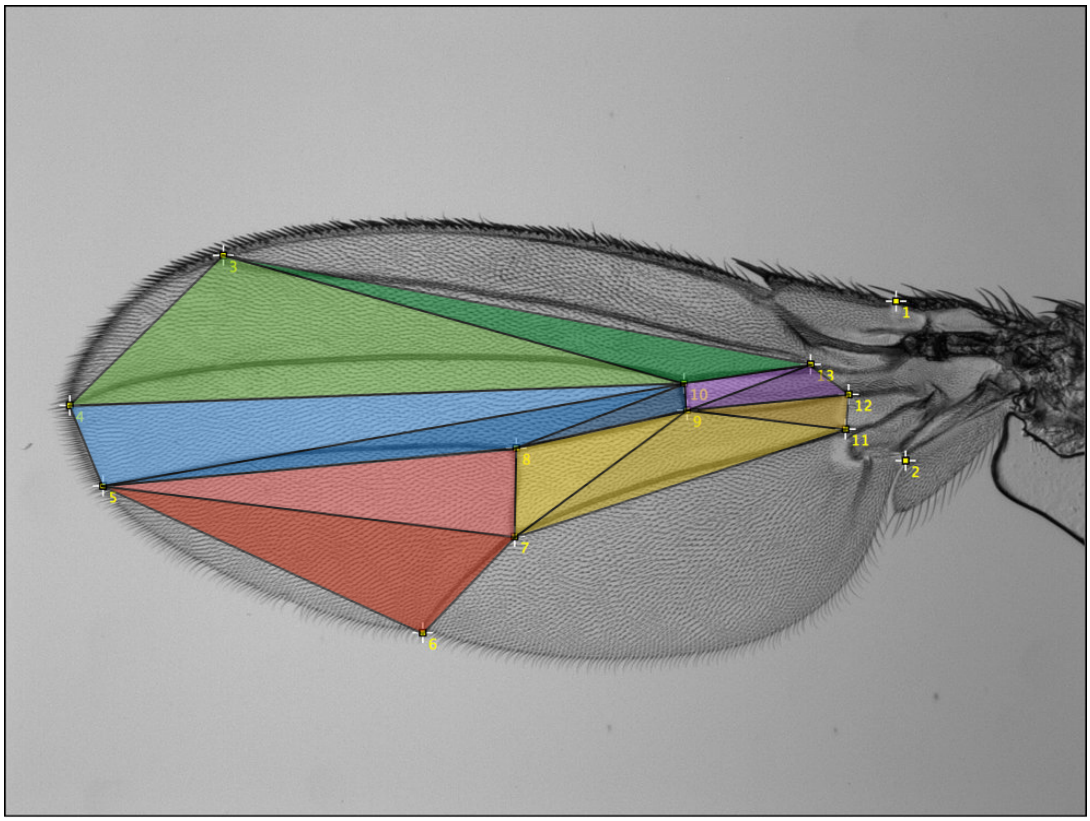


Figure S5

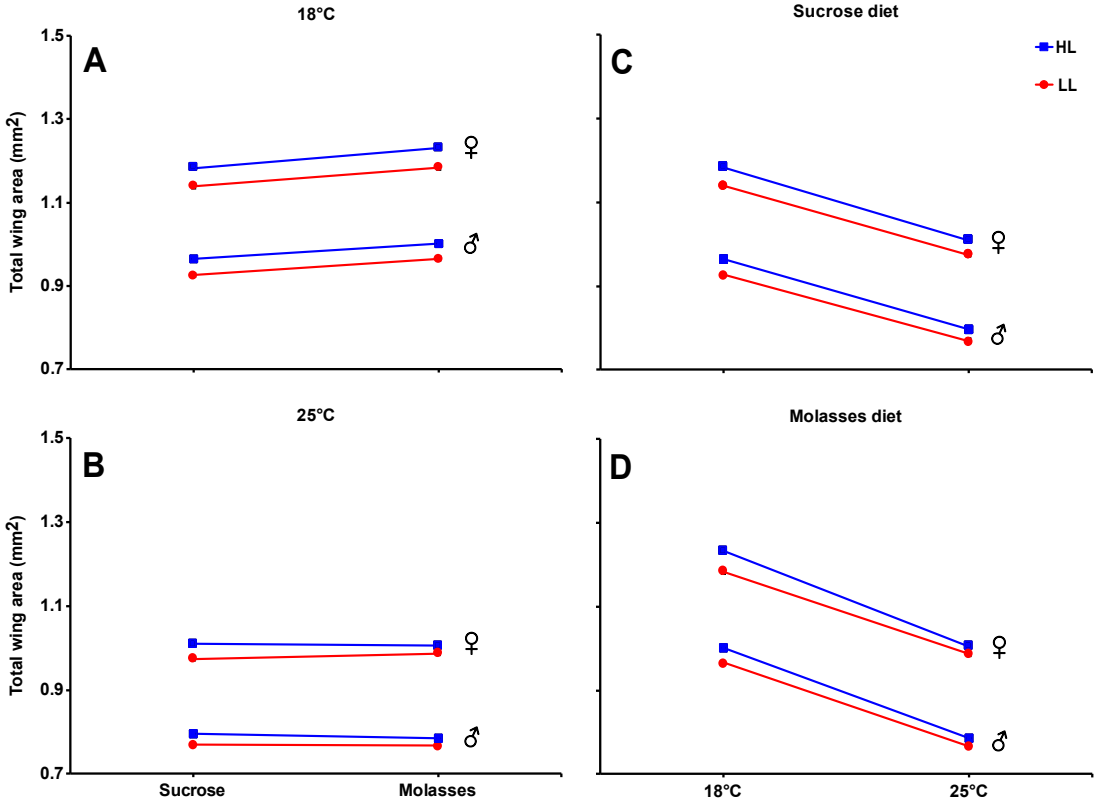

18°C
Sucrose diet
A
C
B
D
HL
LL
♀
♂
Total wing area (mm2)
1.5
1.3
1.1
0.9
0.7
Sucrose
Molasses
18°C
25°C
25°C
Molasses diet

Figure S6

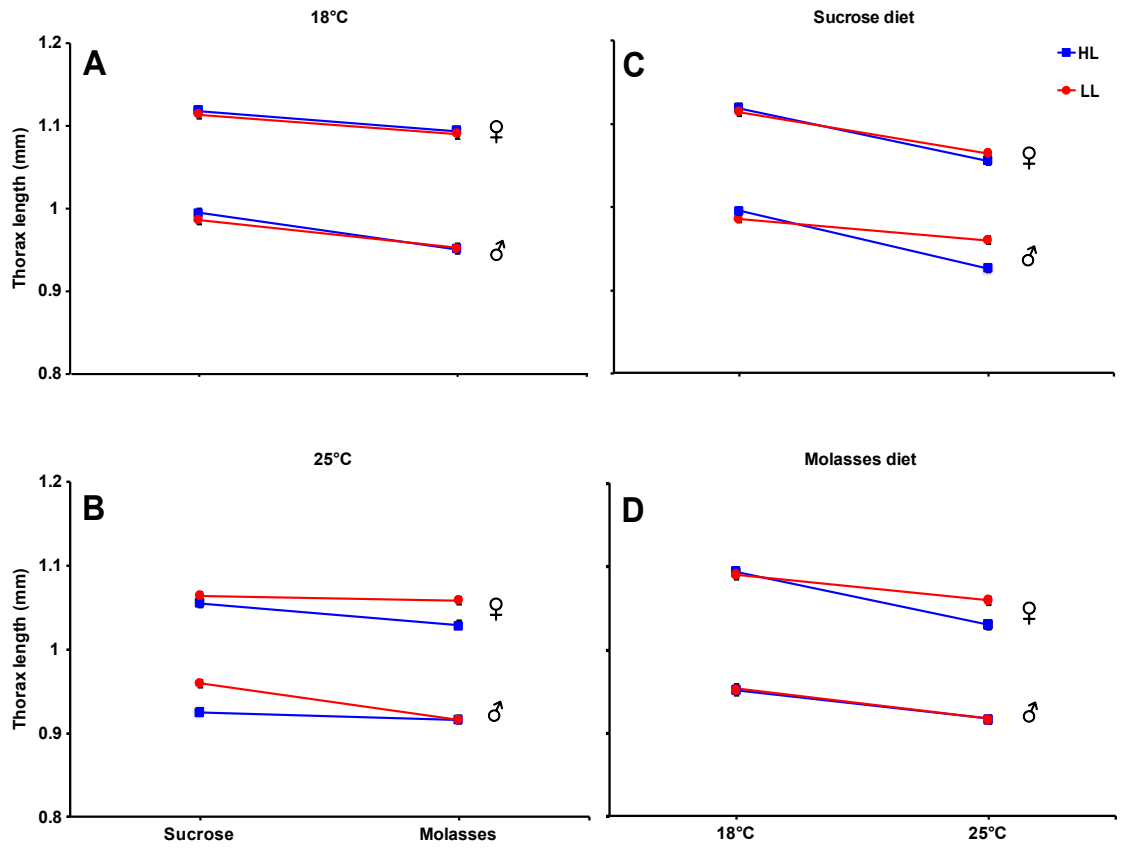

18°C
Sucrose diet
25°C
Molasses diet
A
B
C
D
HL
LL
♀
♂
Thorax length (mm)
1.2
1.1
1
0.9
0.8
Sucrose
Molasses
18°C
25°C

Figure S7

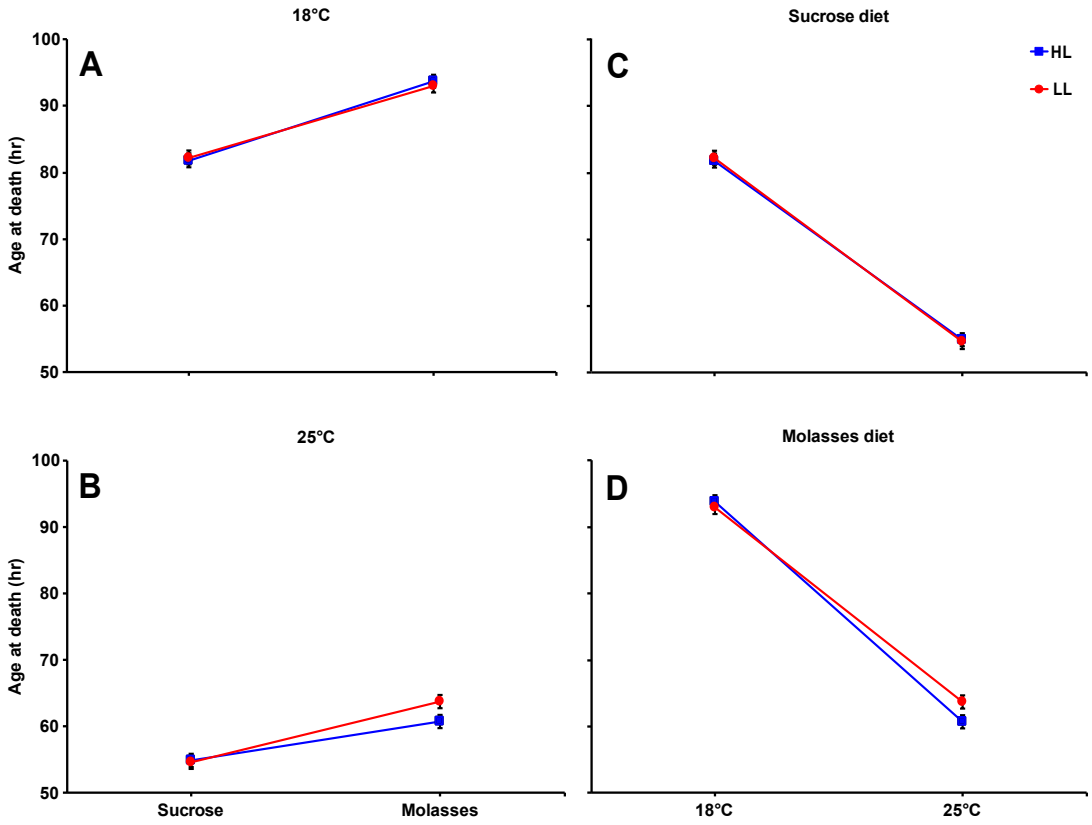

18°C
Sucrose diet
A
C
HL
LL
Age at death (hr)
100
90
80
70
60
50
25°C
Molasses diet
B
D
Sucrose
Molasses
18°C
25°C

Figure S8

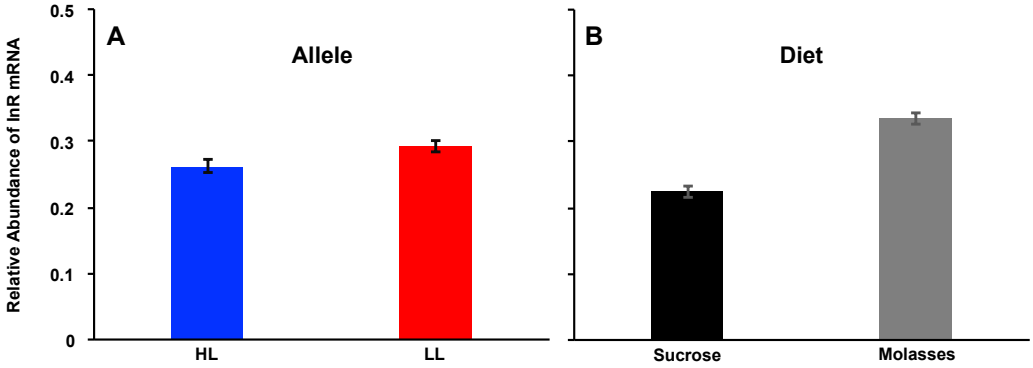

Relative Abundance of InR mRNA
0.5
0.4
0.3
0.2
0.1
0
A
Allele
HL
LL
B
Diet
Sucrose
Molasses

Table S1. Details of design of reconstituted outbred population cages. HL: high-latitude *foxo* allele; LL: low-latitude *foxo* allele. See Materials and Methods section for details.

Allele	Position	Set	Cage number	DGRP lines
LL	3R:9892517 + 9894559 (GG)	A	1	26, 57, 73, 75, 91, 101, 105, 161, 176, 280, 313, 318, 367, 371, 375, 377, 378, 379
LL	3R:9892517 + 9894559 (GG)	A	2	26, 57, 73, 75, 91, 101, 105, 161, 176, 280, 313, 318, 367, 371, 375, 377, 378, 379
LL	3R:9892517 + 9894559 (GG)	B	3	208, 373, 406, 426, 440, 491, 492, 508, 513, 535, 639, 646, 757, 761, 796, 805, 812, 852
LL	3R:9892517 + 9894559 (GG)	B	4	208, 373, 406, 426, 440, 491, 492, 508, 513, 535, 639, 646, 757, 761, 796, 805, 812, 852
HL	3R:9892517 + 9894559 (AT)	C	5	40, 41, 42, 69, 83, 109, 142, 153, 158, 177, 195, 229, 233, 365, 370, 380, 391, 405
HL	3R:9892517 + 9894559 (AT)	C	6	40, 41, 42, 69, 83, 109, 142, 153, 158, 177, 195, 229, 233, 365, 370, 380, 391, 405
HL	3R:9892517 + 9894559 (AT)	D	7	45, 332, 338, 443, 517, 531, 595, 703, 705, 707, 774, 790, 804, 820, 837, 855, 879, 890
HL	3R:9892517 + 9894559 (AT)	D	8	45, 332, 338, 443, 517, 531, 595, 703, 705, 707, 774, 790, 804, 820, 837, 855, 879, 890

Table S2. Nutritional value and composition of sucrose and molasses diets. Table S2a: nutritional values of fly food ingredients per 100 g; Table S2b: recipe for sucrose and molasses diets; Table S2c: comparison of nutritional values of sucrose and molasses diets. See Materials and Methods section for details. The sucrose diet is the standard medium used in our laboratory in Lausanne; the recipe for the molasses diet follows that recipe of the Bloomington *Drosophila* Stock Center (BDSC) but uses different products for the food ingredients.

S2a. Nutritional values of ingredients in 100g of fly food				
	Yeast	**Cornmeal**	**Sucrose**	**Molasses**
Energy (kcal)	310	345	400	290
Protein (g)	45	8	0	0
Total carbohydrates (g)	15	74	100	75

S2b. Food recipes for sucrose and molasses diets		
	Sucrose	**Molasses**
Cornmeal (g/L) (*Polenta, Migros*)	50	61.3
Yeast (g/L) (*Actilife, Migros*)	50	12.4
Sugar (g/L) (*Cristal, Migros*)	50	0
Molasses (g/L) (*Zuckerrohrmelasse, EM Schweiz*)	0	109.6
Agar (g/L) (*Drosophila Agar Type II, Genesee*)	7	6
Nipagin 10% (ml/L) (*Sigma Aldrich*)	10	14.3
Propionic acid (ml/L) (*Sigma Aldrich*)	6	6

S2c. Nutritional values of sucrose and molasses diets		
	Sucrose	**Molasses**
Energy (kcal)	527.5	567.765
Protein (g/L)	26.5	10.484
Total carbohydrate (g/L)	94.5	129.422
P:C ratio	~ 1:3.6 (≈0.28)	~1:12.3 (≈ 0.08)

Table S3. Summary of ANOVA results for wing area, thorax length, and male starvation resistance. White and grey cells show the results for females and males, respectively. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. See Results section for details.

Factor in ANOVA	Total wing area	Thorax length	Starvation resistance
Allele	$F_{1,32}=105.39^{***}$	$F_{1,32}=4.3338^{*}$	$F_{1,32}=0.70$
	$F_{1,32}=103.87^{***}$	$F_{1,32}=3.7805$	
Temperature	$F_{1,912}=2852.52^{***}$	$F_{1,422}=216.4634^{***}$	$F_{1,1553}=1711.77^{***}$
	$F_{1,918}=3962.67^{***}$	$F_{1,381}=145.4612^{***}$	
Diet	$F_{1,912}=48.36^{***}$	$F_{1,422}=31.899^{***}$	$F_{1,1553}=176.44^{***}$
	$F_{1,918}=28.15^{***}$	$F_{1,381}=88.6215^{***}$	
Allele x Temperature	$F_{1,912}=7.15^{**}$	$F_{1,422}=10.6595^{**}$	$F_{1,1553}=0.58$
	$F_{1,918}=5.89^{*}$	$F_{1,381}=8.7214^{**}$	
Temperature x Diet	$F_{1,912}=35.96^{***}$	$F_{1,422}=1.6748$	$F_{1,1553}=7.51^{**}$
	$F_{1,918}=56.66^{***}$	$F_{1,381}=3.482$	
Allele x Diet	$F_{1,912}=0.73$	$F_{1,422}=2.4425$	$F_{1,1553}=0.58^{***}$
	$F_{1,918}=1.08$	$F_{1,381}=2.4619$	
Allele x Temperature x Diet	$F_{1,912}=1.79$	$F_{1,422}=1.8863$	$F_{1,1553}=2.48$
	$F_{1,918}=0.22$	$F_{1,381}=11.1914^{***}$	
Set (Allele)	$F_{2,32}=53.59^{***}$	$F_{2,32}=8.0495^{***}$	$F_{2,32}=1.01$
	$F_{2,32}=30.53^{***}$	$F_{2,32}=7.5618^{***}$	
Cage (Set, Allele)	$F_{4,32}=64.45^{***}$	$F_{4,32}=3.4063^{**}$	$F_{4,32}=12.78^{***}$
	$F_{4,32}=29.58^{***}$	$F_{4,32}=0.7344$	

Table S4. Summary of ANOVA results for relative abundance of insulin-like receptor (InR) transcript levels. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Factor in ANOVA	Relative Abundance of InR
Allele	$F_{1,80}=4.5431^{*}$
Temperature	$F_{1,80}=0.9003$
Diet	$F_{1,80}=75.9869^{***}$
Allele x Temperature	$F_{1,80}=0.0492$
Temperature x Diet	$F_{1,80}=0.0501$
Allele x Diet	$F_{1,80}=0.4097$
Allele x Temperature x Diet	$F_{1,80}=0.0753$
Set (Allele)	$F_{2,80}=6.5294^{**}$
Cage (Set, Allele)	$F_{4,80}=5.7327^{***}$

Chapter 3

[Preliminary manuscript draft]

Allelic polymorphism at *foxo* contributes to adaptive patterns of life history differentiation in natural populations of *Drosophila melanogaster*

Contributions by E. Durmaz: experimental design, assays, statistical analysis, interpretation of results, editing and revising the manuscript.

Allelic polymorphism at *foxo* contributes to adaptive patterns of life history differentiation in natural populations of *Drosophila melanogaster*

NICOLAS J. BETANCOURT[1], SUBHASH RAJPUROHIT[1], ESRA DURMAZ[2], MARTIN KAPUN[2], THOMAS FLATT[2,*], AND PAUL SCHMIDT[1,*]

[1]*Department of Biology, University of Pennsylvania, Philadelphia, USA*

[2]*Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland*

* Co-correspondence:

Paul Schmidt, Fax: +1 215 898 8780, E-mail: schmidtp@sas.upenn.edu

Thomas Flatt, Fax: +41 21 692 4165, E-mail: thomas.flatt@unil.ch

Abstract

Understanding adaptive responses to environmental variation is a fundamental goal in evolutionary biology. The insulin/insulin-like growth factor signaling pathway (IIS) has been hypothesized to be a major determinant of life history profiles that vary adaptively across environmental gradients in *Drosophila melanogaster*. Pooled genome sequencing of populations across latitudinal gradients on multiple continents has revealed that several components in the IIS pathway vary predictably with latitude. This includes *foxo*, a gene encoding a highly conserved fork-head transcription factor that regulates IIS and with known pleiotropic effects on longevity, starvation, and size. We hypothesized that naturally occurring variation at *foxo* could be a major contributor to life history variation in natural populations. To evaluate this, we constructed recombinant outbred populations in which alternative allelic states for two SNP positions in *foxo* were fixed and the genomic background was randomized. After eight generations of recombination, flies were phenotyped for a series of fitness traits. Our results suggest that natural variation at *foxo* has pronounced effects on life history. Between *foxo* alleles, there are differences in starvation resistance and two body size traits (thorax length, wing area). However, alleles were equivalent for development time. We also hypothesized that the patterns associated with the *foxo* alleles contribute to those observed in wild populations. To test this, we assessed six populations from recent outbred collections along the latitudinal gradient in the eastern United States for the same traits. The data demonstrate that there are clear latitudinal clines for both body size and a novel cline in starvation tolerance. However, as with the *foxo* data, there was no predictable variation among populations for development time. These data suggest that variation in the IIS pathway, and at *foxo* in particular, underlies adaptive phenotypic differences in life history traits such as body size in natural populations.

Introduction

Understanding the basis for adaptive response to environmental variation is a fundamental goal in evolutionary biology. Variation in metazoan life history traits exists among species, among populations within a species, and among individuals in a given population. Because life history traits are critical components of fitness, their distribution along environmental gradients often reflect adaptive responses to varying selection pressures (De Jong & Bochdanovits 2003). *Drosophila melanogaster* is an ancestrally tropical species from sub-Saharan Africa that has recently colonized the Americas and Australia over the last few hundred years (David & Bocquet 1975; David & Capy 1988; De Jong & Bochdanovits 2003; Hoffmann & Weeks 2007; Adrion *et al.* 2015). Thus, the radiation of these flies into temperate regions is thought to have resulted in several climatic adaptations, particularly for life history traits (David & Capy 1988; Hoffmann *et al.* 2003; Schmidt *et al.* 2005a; b; Paaby & Schmidt 2009). As such, predictable patterns for these traits including body size, development time, starvation tolerance, and other traits over latitudinal gradients in this model species (Coyne & Beecham 1987; Weeks *et al.* 2002; De Jong & Bochdanovits 2003; Schmidt *et al.* 2005a; b; Paaby *et al.* 2010; 2014; Kapun *et al.* 2016b). However, there has been a by and large failure to explain many of these observed patterns in light of the natural allelic variation present in wild populations (Paaby & Schmidt 2008; Schmidt *et al.* 2008).

It is well established that adult flies from temperate populations are larger than those from tropical populations in almost every measurable parameter (Noach *et al.* 1996; Gilchrist & Partridge 1999; Calboli *et al.* 2003; Kennington & Hoffmann 2010; Kapun *et al.* 2016b). Indeed, positive clines for body size, from the equator to the poles, exist in almost every continent it has been researched (De Jong & Bochdanovits 2003; Schmidt *et al.* 2005a). For example, there is a well-characterized body size cline in Australia from Queensland to Tasmania (James *et al.* 1995; 1997; Kennington *et al.* 2007; Kennington & Hoffmann 2010;

Kapun *et al.* 2016b) and a long established body size cline in North America (Coyne & Beecham 1987; Kapun *et al.* 2016b). Larger sizes in temperate populations have been found to be most related to increases in cell size and number. Indeed, in the Australian body size cline mentioned above, flies from the temperate populations were found to have increased cell numbers and greater mean cell sizes relative to those from tropical populations (James *et al.* 1995; 1997).

Aside from body size, development time also tends to differ between temperate and tropical populations. Despite their greater sizes, temperate fly populations often have been found to develop faster on average than tropical ones when reared at similar temperatures (James & Partridge 1995). Specifically, in the Australian cline, larvae collected from high latitudes were found to use limited food more efficiently than larvae collected from tropical populations, or low latitudes (James & Partridge 1995; Robinson & Partridge 2001). Consequently, this may have allowed temperate larvae to achieve larger overall adult body sizes upon development relative to tropical larvae. It was also hypothesized that the increases in larval growth efficiency could explain their more rapid development in addition to their larger sizes (Robinson & Partridge 2001) . The primary mechanism that selects for the faster development and larger size in temperate populations seems directly related to temperature. Indeed, this trend has been substantiated by the parallel evolutionary patterns of these traits seen across cold-adapted lab populations and warm-adapted lab populations (James & Partridge 1995; Blanckenhorn 2000).

Unlike body size and other life history traits, the cline for the stress trait, starvation tolerance, has not been characterized consistently across continents and less is known about the selection pressures that influence this trait and the genes regulating it. However, a study on the Indian subcontinent demonstrated a negative starvation cline, where flies closer to the equator had increased starvation resistance, but also found a positive cline for desiccation

tolerance going in the opposite direction (Karan *et al.* 1998). Despite these findings, in Australia and South America, no clear or consistent cline for starvation resistance was found in North America (Robinson *et al.* 2000; Hoffmann *et al.* 2001). Considering a different approach, selection line experiments have revealed that when selecting for starvation resistance, body sizes tend to increase while development time also increases (Chippindale *et al.* 1996; Harshman *et al.* 1999). This contradicts the growth-efficiency relationship observed in temperate populations in regard to larger body size being associated with quicker development time relative to tropical populations. Thus, there seems to exist complex pleiotropic interactions and inherent trade-offs between these three life history traits and the effects of the genes controlling them.

The insulin/insulin-like growth factor signaling pathway (IIS) has been hypothesized as a major determinant of life history profiles that vary adaptively across environmental gradients. Indeed, this pathway is highly conserved across organisms and several homologs exist between *Drosophila,* mice, *C. elegans,* and humans (Kenyon *et al.* 1993; Clancy 2001; Tatar *et al.* 2001; Garofalo 2002; Oldham & Hafen 2003; Tatar 2003). An exhaustive dissection of the *D. melanogaster* has confirmed the extensive role of this pathway in regulating growth and cell proliferation (Saucedo & Edgar 2002; Oldham & Hafen 2003; Nielsen *et al.* 2008). Furthermore, the insulin-signaling pathway is known to affect nutrient storage and metabolism among other traits (Garofalo 2002). Single mutations in this pathway have been demonstrated to confer substantial pleiotropic effects on life history profiles (Giannakou & Partridge 2007). Studies of known members in the IIS pathway have identified a number of genes that can extend lifespan by reducing insulin signaling. Specifically, loss of function mutations or disruptions of the *insulin-like receptor* (*InR*) or its substrate, *chico*, promote increased nuclear localization of the fork-head transcription factor, *foxo*, which ultimately reduces insulin signaling (Clancy 2001; Tatar *et al.* 2001). Similarly, overexpression of *foxo*,

which acts as a downstream regulatory transcription factor, reduces insulin signaling and ultimately increases longevity (Hwangbo *et al.* 2004; Giannakou *et al.* 2004). Lifespan is another life history trait that is associated with many tradeoffs, and recent studies have found correlations between the level of insulin signaling and a variety of fitness traits (Paaby *et al.* 2014).

Surprisingly, the functional significance of naturally occurring allelic variation at *foxo* has not been comprehensively examined. We previously identified a number of clinal SNPs in multiple genes in the central IIS pathway, including a number of promising candidates in *foxo* (Fabian *et al.* 2012). Here, we test whether two clinal *foxo* alleles, defined by nucleotide state at two SNPs across ~2kb, are functionally distinct with respect to a series of life history-associated phenotypes that vary predictably among natural populations (body size, development rate, and starvation tolerance). We then examined variation in these traits across a series of natural populations from the eastern United States, and whether functional differentiation between *foxo* alleles might explain (to a large or small extent) the observed patterns of variation in the field. We hypothesized that *foxo* alleles regulate major aspects of variation in *Drosophila* life history in a trait-specific fashion. Indeed, our results demonstrate that the two *foxo* alleles differ with respect to starvation tolerance and body size, but are equivalent with respect to development time. These patterns mirror those observed in natural populations, for which we also demonstrate clines in body size and starvation tolerance, but no variation with latitude for development time. Furthermore, our data suggest that allelic variation at *foxo* is a minor, albeit significant, contributor to genetic variance in starvation tolerance in natural populations, but a major contributor to variance in body size.

Material and Methods

Identification of SNPs/alleles that vary predictably with latitude

Fabian *et al*. (2012) identified a series of SNPs in *foxo* that exhibited high F_{ST} in pooled sequencing of natural populations derived from Florida (low latitude), Pennsylvania (mid latitude), and Maine, U.S.A. (high latitude). From this analysis, we identified a candidate *foxo* allele based on the nucleotide state at two SNPs spanning approximately ~2kb. We subsequently used the more extensive pooled population sequencing from the *Drosophila* Real Time Evolution Consortium (Dros-RTEC) to examine associations with allele frequency and latitude in natural populations collected in 2012 across a variety of locations in the U.S. (NCBI SRA BioProject PRJNA308584#; (Bergland *et al.* 2014; Kapun *et al.* 2016a). We investigated the allele frequency changes for candidate *foxo* SNPs and showed that both SNPs are also strongly clinal in this dataset. Thus, we investigated the functional effects of these SNPs as a 2-SNP allelic polymorphism by reconstituting outbred populations.

Constructing outbred population cages with foxo alleles

Based on the combination of the results of Fabian *et al*. (2012) and the DrosRTEC data set, we identified individual lines in the *Drosophila* Genetic Reference Panel (DGRP) (Mackay *et al.* 2012), that were homozygous for either the *foxo* allele that was at high frequency in high latitude populations (hereafter, the high latitude allele), and lines that were fixed for the *foxo* allele that was at high frequency in low latitude samples (hereafter, the low latitude allele). Two biological replicate population cages were established from 20 independent lines per cage per allele; these biological replicates are denoted by sets A and B, each containing independent sets of 20 inbred lines. These biological replicates were then experimentally replicated 2X by splitting each biological replicate into two experimental replicates in the first generation of founding. All replicate cages (2 *foxo* alleles x 2 biological replicates (sets A and

B, each comprised of 20 independent inbred lines) x 2 experimental replicates = 8 total population cages) were founded by 10 individuals of each sex per inbred line from density-controlled cultures; subsequently, each population cage was cultured under constant density on standard cornmeal-molasses medium for 8 discrete generations under conditions of constant temperature (25˚C) and photoperiod (12L:12D) in Percival I36VL incubators. Thus, at the end of the recombination period, we generated replicate population cages in which the focal *foxo* allele was fixed for either the high latitude or low latitude allele and the genomic background was randomized across the 20 inbred lines used to found each respective cage (Paaby *et al.* 2014; Zhao *et al.* 2015). After 8 generations of density-standardized culture under standardized environmental conditions, we established replicate density controlled vial cultures (30 ± 20 eggs/vial) for subsequent phenotyping of the high latitude and low latitude *foxo* alleles.

Selecting outbred lines from recent collections along the Eastern U.S.

Thirty isofemale lines were randomly selected from each of six previously collected outbred populations along the Eastern U.S. sampled in the years 2011-2013 from Homestead, FL (HFL), Jacksonville, FL (JFL), Charlottesville, VA (CVA), Media PA (MPA), Lancaster, MA (LMA), and Bowdoin, ME (BME) (Fig. 3; described in (Bergland *et al.* 2014; Behrman *et al.* 2015; Rajpurohit *et al.* 2017a; b).

The individual lines for each population were maintained in a standard 3-week culture regime under the same conditions as the *foxo* recombinant cages. Prior to phenotyping, each isofemale line was cultured for two generations at low density (30±20 eggs/vial) under 25˚C, 12L:12D; in the third generation, freshly eclosed flies were collected in daily cohorts and used in the phenotypic assays described below.

Phenotype Assessment

In all assessments, *foxo* recombinant cages were tested simultaneously at three independent time points and the data partitioned into blocks. For assessment of starvation resistance, virus contamination of the cages precluded running three independent blocks and a single time point was included in the analysis. For the natural populations, all lines from all populations were assayed simultaneously for all phenotypes using discrete 1d cohorts for each phenotype.

Starvation Resistance

For the *foxo* cage populations, embryos were collected from each cage in two replicate bottles. After lowering the density to ~150 eggs per bottle, they were kept at 25°C, 12L:12D, 60% RH. Similarly, eggs were collected from each outbred population's respective lines, but instead collected in food vials with controlled density ~50 eggs per vial.

In both independent procedures, the early, only the 2nd and 3rd day eclosers were collected and considered for the phenotype assays; after aging and mating for 4-5 days upon eclosion. The flies were then separated by sex and divided, 10 each, into glass vials equipped with a small cotton ball saturated with 1 mL of water, and kept at the same conditions as development. The survival upon starvation (starvation tolerance) was recorded daily at time points: 9AM, 1PM, 5PM, and 9PM. The *foxo* cages were given three replicates (a,b,c) per cage population; the natural populations were tested for all of their lines once, respectively.

Development Time

For the *foxo* cage populations, several eggs were collected from each cage in a three-hour window using petri dishes reared with cornmeal culture and activated yeast. The collected eggs were then counted and distributed, 30 each, in three replicate vials (a,b,c) per cage reared with the standard cornmeal culture.

For the natural populations, this assessment was broken into 3 separate blocks, each representing a random set of lines from their respective populations. Similar to the *foxo* cage procedure, eggs were collected in a three-hour window with egg collecting receptacles reared with cornmeal culture. The eggs were distributed 30 each in vials, one per line, for each of the populations.

In both independent procedures, the flies were kept in the same conditions for the starvation tolerance assessments. Data was recorded at time points: 9AM, 1PM, 5PM, and 9PM. Adult emergence and sex was recorded when flies successfully eclosed from their pupal cases. Eclosed flies were stored in 95% ethahol and used for body size measurements.

Body Size/ Morphology

For *foxo* flies, 10 flies for each sex were measured from each cage population. For the natural populations, 5 flies for each sex were measured for every line within their respective population. The body size measurements were recorded using an Olympus DP73 Microscope-Imaging Camera with CellSens Standard measuring software, which took measurements of pictures relative to a programmed scale. The body size parameters, thorax length and full wing area, were parameterized (Fig. S1, Supporting Information). Thorax length was measured as the longest length from the dorsal tip of the thorax to the tangent surface of the thorax near the head (Fig S1, Supporting Information). Total wing area measured as a polygon surrounding a series of set points of interest, which were easy to recognize, representative of almost the entire wing area, and consistently not damaged across all samples in males and females (Fig S1, Supporting Information). The ratio of total wing area to thorax length, as indicative of wing loading, was also calculated and subsequently analyzed.

Statistical Analysis

For the natural populations, data were analyzed separately by sex. Isofemale line was considered a random variable and all data were analyzed using a restricted maximum likelihood ANOVA with population as a fixed effect. For all other traits other than starvation tolerance, experimental block (N=3) was also included as a covariate. For the *foxo* experimental population cages, a similar nested ANOVA was run independently for both sexes in which *foxo* allele, biological replicate (set), and experimental replicate (cage) were included as predictors.

Results

Allele frequency changes in Dros-RTEC dataset and genome-wide background analysis

The analysis confirmed previously observed clinal pattern for *foxo* alleles in Dros-RTEC data, which showed the allele frequency change across latitude for examined SNPs vary clinally in North American east coast. in *D. melanogaster* (Fig. 1**)**. We observed ~60% allele frequency difference between low and of high-latitude alleles along the cline; low-latitude allele is prevalent in Florida (~70%) but at low frequency in Maine (~10%). We also analyzed the genomes of the DGRP lines used to set up and confirmed that the were fixed (F_{ST} = 1) for the focal LL alleles, without any systematic differentiation in the genome-wide background (Fig. 2) by testing all polymorphic sites following the approach by Weir & Cockerham 1984.

Natural populations – phenotype analysis and output

The total statistical output and effect tests are summarized in Table 1 for the natural population life history assays. For a visual representation of the observed clines from our data, we plotted lines of best fit between population mean trait values using latitude as a predictor variable for illustrative purposes (Fig. 3). Male and female clines are separated for

each trait and the same traits assessed across the outbred *foxo* cages have been assessed here. Also on the left side of the figure is a NOAA mean-temperature-graded map of the Eastern coast of the United States. The sampled populations we collected from have been marked and their associated latitudes have labeled beside them, respectively.

Total Wing Area

In natural populations, for the trait full wing area, the entire mixed model was found to be significant across the latitudinal gradient of the Eastern U.S (Table 1, Fig.3). Specifically, the respective populations showed differences in body size ($p < 0.001$). The common size different between sexes was also observed in natural populations ($p < 0.001$). Furthermore, an extension effect of the previous two effects, population crossed with sex, also showed highly significant differences. Though each line comes from the same population, we expected a considerable degree of genetic variation within a population, as the line effects was found in our analysis. Because each line represents an isofemale line established independently from a collection site, it is not surprising that we observe significant differences between lines within a population. Also, it is important to note that high degrees of repetition and blocks were used to accurately determine representative means and standard errors from each population, thus the variability within lines of a population only serves as a more robust representation of the population as a whole. More importantly, perhaps, is the effect test of block, which shows no significant difference between each round of independent assessment in females ($p > 0.05$). This repeatability further strengthens our confidence in our data that the cline for full wing area, and body size in general, does indeed exist in Eastern North America.

We also observed robust clines for both males and females across the latitudinal gradient. This data effectively reaffirms the clinal end point study conducted by Coyne and Beecham (1987) on Florida and Maine flies, respectively. By adding populations at intermediate

latitudes to a study of this kind, we were able to more confidently claim that a body size cline exists along the latitudinal gradient of the Eastern U.S. where body size tends to increase with latitude, which parallels much of the work done on continental body size clines in *Drosophila melanogaster* (De Jong & Bochdanovits 2003).

Thorax Length

In natural populations, for the other body size parameter trait, thorax length, the entire mixed model was also found to be significant (Fig. 3; Table 1). As in the previous morphological model, we observed differences between the respective populations ($p < 0.001$), sexes (Females: $p < 0.001$; Males: $p < 0.01$), and isofemale lines. As for the previous trait, we expect a considerable degree of genetic variation within a population, as observed for both size related traits for isofemale lines.

We demonstrate positive clines for both males and females for the ratio of total wing area to thorax length across the latitudinal gradient in the Eastern U.S (Fig. 3). Indeed, both sexes demonstrate a general increase in this ratio with increasing latitude despite considerable regional variability observed for this trait. Though both sexes exhibit variability for this trait, the female data represents a slightly more robust cline with a greater trait slope relative to the males in natural populations. Like the cline of the previous morphological trait, the cline for wing area: thorax size largely agrees with much of the work done on continental body size clines in *D. melanogaster* (Coyne & Beecham 1987; De Jong & Bochdanovits 2003).

Starvation Resistance

In natural populations, for the trait starvation tolerance, we found that populations differ in their resistance across latitudinal gradient of the Eastern U.S ($p < 0.001$; Fig.3; Table 1). Additionally, we observed a difference between the sexes, and their interaction with

population. And similar to the morphological traits, we again observed differences between isofemale lines in a given population.

For starvation resistance, we demonstrate robust positive clines for both males and females across the latitudinal gradient in the Eastern United States. The data points for males seems to especially fit well the line of regression, while the female data presents a considerably more robust cline despite not fitting the line of regression over latitude as well. Here we are seeing a clearly adaptive response over the North American latitudinal gradient, with starvation resistance increasing with latitude. This is a novel starvation resistance cline observed in North America for *D. melanogaster.* Indeed, starvation clines have yet to be found on the South American and Australian continents (Robinson *et al.* 2000; Hoffmann *et al.* 2001). And perhaps more strangely, we found the starvation tolerance cline in North America to go in the opposite direction as what was observed on the Indian subcontinent, where studies have found that Indian flies near the equator tend to be more starvation resistance than those at higher latitudes (Karan *et al.* 1998).

Development Time

For development time, the entire mixed model was found to be significant across the latitudinal gradient of the Eastern U.S (Fig.3; Table 1). Specifically, we observed differences between the respective populations both in males and females ($p < 0.001$). However, we did not observe an interaction between sex and population. Also, we observed differences for isofemale lines and experimental block, which indicates that this trait has considerable variability and that uncontrollable factors, such as time of year and air pressure, may contribute more to this trait than latitudinal gradients do.

There seems to exist no clear or consistent pattern for development time along the latitudinal gradient of the United States. The only consist pattern that exists is in between the

sexes, which both have population means distributed in a similar arrangement. We were surprised to not observe a cline for this trait in North America. Past studies have linked increased temperate population size to larval growth efficiency, which should also confer speedier development to these populations relative to tropical fly populations when reared at the same temperature (James & Partridge 1995; Blanckenhorn 2000). On the other hand, a more recently proposed selection regime based on seasonal stress tolerance and diapause posits that temperate populations should develop more slowly than tropical flies (Paaby & Schmidt 2009). However, we did not observe results consistent with either pattern here in North America.

foxo recombinant cages – phenotype analysis and output

The total statistical output and effect tests are summarized in Table 2 for males and females from the *foxo* recombinant cage population life history assays (Fig. 3). The male and female data are separated for each trait.

Full Wing Area

For the trait full wing area, the entire mixed model was shown to be statistically significant in both males and females (Fig.3; Table 2). The high latitude *foxo* allele was associated with increased wing area relative to the low latitude allele in both sexes ($p < 0.001$). This suggests that the focal *foxo* alleles have functional significance that underlies differential adaptive responses in body size that are consistent with expectations from work on several clines on body size and on work on temperate and tropical flies in general (Noach *et al.* 1996; De Jong & Bochdanovits 2003).

Thorax Length

For the trait thorax length, the entire mixed model was also shown to be statistically significant in both males and females (Fig.3; Table 2). The high latitude *foxo* allele was associated with larger thorax length both in females and males ($p < 0.01$). Similar results were observed for the ratio of total wing area to thorax length, with this ratio demonstrating a more pronounced latitudinal cline (Fig. 3).

Starvation Resistance

For the stress trait starvation resistance, the entire mixed model shown to be statistically significant in both males and females as in the previous two assessed morphological traits (Fig.3; Table 2). Overall, candidate flies fixed for the low latitude *foxo* allele demonstrated reduced starvation tolerance relative to flies fixed for the high latitude, temperate allele both in females and males ($p < 0.01$). This relationship is interesting and newly attributed to this transcription factor, *foxo*. The mechanism for increased starvation resistance, however, may simply be correlated to increased body sizes and fat reserves. The low-latitude allele flies have consistently demonstrated smaller body size in both the thorax and wing area across both sexes, and now have also demonstrated consistently less starvation tolerance than high-latitude allele flies.

Development Time

Unlike the previously measured traits, the entire mixed model for development time was found to be not significant with respect to *foxo* allele both in females and males ($p > 0.05$). Development time varied significantly across blocks and replicates, but was not distinct between the high and low latitude *foxo* alleles (Fig.3; Table 2). Overall this model seems to

demonstrate that there is no functional significance associated with *foxo* allele in terms of predictable effects on development time, despite previous models' implication for functional significance on body size and starvation tolerance. This is somewhat congruent with data on Australian clines, where the relationship between body size and development time is inconsistent across latitude (James *et al.* 1995; 1997). This implies that other selection pressures on other genes and pathways are playing more important roles in differentiating development time responses than what is being offered by the differential phenotypic contributions of the two nucleotide states of the linked candidate SNP in *foxo*. Also, development time was the most variable trait studied in all models, and could perhaps be most affected by inbreeding depression effects in lab.

Discussion

Past studies on *foxo* have validated the fork-head transcription factor as a major regulator of lifespan in *Drosophila, c. elegans,* mice and humans (Garofalo 2002; Giannakou *et al.* 2007; Alic *et al.* 2014; Morris *et al.* 2015), and more recent studies have elucidated a role for *foxo* in reproductive diapause regulation in *C. pipiens* and *D. melanogaster* (Tatar & Yin 2001; Sim *et al.* 2015). Because this gene encodes a transcription factor for the fundamental insulin-signaling pathway in most multicellular organisms, it is not surprising that *foxo* exhibits extensive pleiotropic effects. Here, we have presented evidence of a novel functional polymorphism in *foxo,* which varies predictably with latitude and confers predictable effects on life history traits, namely for body size and starvation resistance. Specifically, between the two-nucleotide states of the tested candidate SNP (high vs. low latitude, Fig. 3), flies with the low-latitude SNP were shown to be significantly smaller than flies with the high-latitude SNP. This was the case for both body size parameters; however, the data was more significant and robust for the full wing area parameter. Additionally, data from the males in both

parameters was more robust and conclusive in determining the functional significance of the polymorphism in *foxo*. Thus, taken together with the male data, we believe the female data also offers robust evidence for the functional significance of this polymorphism in determining body size across latitudinal gradients, which seems to increase with latitude.

Functionally, the mechanism behind the polymorphism conferring increased body size most likely has to do with increased cell sizes or cell numbers being exhibited in flies at higher latitudes (James *et al.* 1995; 1997). However, past studies have shown that at least for wing area, cell number seems to be a more important in determining variation than cell size (Zwaan 2000). In this light of this, it is interesting to note that the full wing area differences were more significant between the *foxo* alleles across the sexes than they were for thorax length. Thus we hypothesize that the clinal polymorphism in *foxo* is likely to be functionally significant in determining variable cell numbers in *D. melanogaster* populations over latitudinal gradients.

Not only that, but here we also presented an additional functional significance of the clinal polymorphism in *foxo.* Our data demonstrate that between the low- and high-latitude variants, there were indeed significant differences in starvation resistance for both sexes. Ultimately, this data taken together suggests that *foxo* definitively plays a functional role in determining variation of starvation tolerance over latitudinal gradients, albeit perhaps only in North America. How this occurs mechanistically is unclear; however, variation in the IIS pathway has long been shown to affect nutrient storage, metabolism, and cell growth (Saucedo & Edgar 2002; Oldham & Hafen 2003). Variation in these aspects, particularly in nutrient storage and metabolism, could be responsible for the predictable change we see in starvation tolerance along the latitudinal gradient of the United States.

Despite the functional significances we found in the clinal polymorphism for *foxo* in morphology and starvation tolerance, we failed to recognize any functional significance of

this SNP in regards to development time. Male data was shown to be especially variable and inconsistent, where the alleles displayed no significant differences. Due to the high degrees of variability across both sexes in this phenotype and the lack of a consistent pattern across alternate nucleotide states, we conclude that the candidate SNP in *foxo* is not relevant in determining variation for development time over latitudinal gradients.

In qualifying our first hypothesis, we believe our data from the *foxo* recombinant SNP cages clearly demonstrate that natural variation at *foxo* at least in part contributes to the adaptive differences in life history profiles we see in natural populations, namely for body size and starvation tolerance in *D. melanogaster*. To bolster our study, we also tested outbred populations collected along the Eastern United States for the same series of traits. Our results reconfirm the long-known existence of a positive body size cline along the latitudinal gradient of North America (Coyne & Beecham 1987). However, because we also used intermediate populations in between Maine and Florida, as opposed to only using endpoint populations, the body size cline that we demonstrate is perhaps the most robust offered in North America. The two measured body size parameters displayed similar trends, however, the parameter for full wing area offered especially robust clines for both males and females relative to thorax length, which was more variable across populations. More importantly, it is very reassuring that the functional significance between the low- and high-latitude alleles matches the body size cline we observed in North America. These results taken together, powerfully conclude that variation in *foxo* is indeed in part responsible for body size variation between tropical and temperate flies across the latitudinal gradient in the United States.

Furthermore, additional assessment of the natural populations revealed a novel positive starvation cline in North America. This contradicts the negative starvation cline found years ago on the Indian subcontinent (Karan *et al.* 1998) and also comes as somewhat of a surprise considering that starvation resistance clines have not been found in either Australia or South

America (Robinson *et al.* 2000; Hoffmann *et al.* 2001). The mechanism behind this relationship is unclear; however, a recently proposed selection regime for temperate populations based on seasonally induced diapause and stress tolerance has associated increased body size with increased stress resistance and slower development for those populations (Paaby & Schmidt 2009). In light of this relationship, it is not surprising that clines for body size and starvation resistance match in the United States. Additionally, the increased stress resistance may be due causally to increased cell size or cell number, but further research is warranted to elucidate this connection.

For the final life history trait assessed, development time, natural population data, as with the *foxo* data, demonstrated no clear or consistent patterns of development time over the latitudinal gradient. Though we were relieved that the lack of a pattern in the *foxo* alleles matched the lack of a cline in the natural populations, we were surprised at the results across the board. We expected to see some sort of relationship of development time with latitude as we had seen in the previous traits. However, our expectations were unclear from the start. There are two conflicting hypotheses for temperature-based temperate fly selection regimes in which one proposes larval growth efficiency as the major factor that is being selected on (Robinson & Partridge 2001), and the other regime focuses more on selecting for increased stress tolerance and diapause activation (Paaby & Schmidt 2009). As a result, the former predicts enhanced larval growth efficiency to be associated with increased size and decreased development time while the latter predicts enhanced stress tolerance to be associated with larger body size and slower development. In our data, we saw neither of these relationships and propose that the true selection regime in North America is a composite of these two, perhaps favoring the stress tolerance regime due to existence of a robust starvation cline in the U.S. Alternately, selection on other factors such as those associated with diet and larval crowding may be more important than temperature and the latitudinal gradient in determining

variation for development time. Indeed, work on the Australian cline has not always reported consistent correlations between body size and development time (James *et al.* 1995; 1997).

In short, we have elucidated novel functional significance for a SNP in *foxo*, the fork-head transcription factor that regulates the insulin signaling pathway in *D. melanogaster.* This is just another step forward in characterizing the many members of the IIS pathway, which have been known to confer pleiotropic effects on several life history traits such as diapause regulation and regulation of lifespan (Clancy 2001; Tatar *et al.* 2001). Indeed, further work is warranted on members of this pathway and we challenge other researchers to identify more functional SNPs that vary predictably with latitude in order to further elucidate the effects the IIS pathway. It may also be interesting to see the interactive effects of several members of this pathway, to see if they interact additively or in epistasis. For example, fixing a latitudinal variant SNP in *foxo, chico, and InR,* all for "derived-temperate SNPs," may produce extreme phenotypes that would help further elucidate the variable effects of the insulin signaling pathway on organisms in wild populations.

From our findings, we conclude that variation in the IIS pathway, particularly at *foxo*, at least in part contributes to the differential functional responses in *D. melanogaster*. In particular, our analysis indicates functional significance in *foxo* in determining differential body size and starvation tolerance at opposite ends of the cline.

Acknowledgements

We thank the members of the Schmidt and Flatt labs for discussion and/or assistance in the lab. Our research was funded by the Swiss National Science Foundation (SNSF grants PP00P3_133641; PP00P3_165836; 310030E-164207 to TF), the Austrian Science Foundation (FWF P21498-B11 to TF), the National Institutes of Health (NIH 5R01GM100366 to PS), the

National Science Foundation (NSF DEB 0921307 to PSS), and the Department of Biology at the University of Pennsylvania.

References

Adrion JR, Hahn MW, Cooper BS (2015) Revisiting classic clines in *Drosophila melanogaster* in the age of genomics. *Trends in Genetics*, **31**, 434–444.

Alic N, Giannakou ME, Papatheodorou I *et al.* (2014) Interplay of d*FOXO* and two ETS-family transcription factors determines lifespan in *Drosophila melanogaster*. (SK Kim, Ed,). *PLoS Genetics*, **10**, e1004619.

Behrman EL, Watson SS, O'Brien KR, Heschel MS, Schmidt PS (2015) Seasonal variation in life history traits in two *Drosophila* species. *Journal of Evolutionary Biology*, **28**, 1691–1704.

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*. (D Bolnick, Ed,). *PLoS Genetics*, **10**, e1004775.

Blanckenhorn WU (2000) The evolution of body size: what keeps organisms small? *The Quarterly Review of Biology*, **75**, 385–407.

Calboli F, Kennington WJ, Partridge L (2003) QTL mapping reveals a striking coincidence in the positions of genomic regions associated with adaptive variation in body size in parallel clines of *Drosophila melanogaster* on different continents. *Evolution*, **57**, 2653–2658.

Chippindale AK, Chu TJF, Rose MR (1996) Complex Trade-Offs and the Evolution of Starvation Resistance in *Drosophila melanogaster*. *Evolution*, **50**, 753.

Clancy DJ (2001) Extension of Life-Span by Loss of CHICO, a *Drosophila* Insulin Receptor Substrate Protein. *Science*, **292**, 104–106.

Coyne JA, Beecham E (1987) Heritability of two morphological characters within and among natural populations of *Drosophila melanogaster*. *Genetics*, **117**, 727–737.

David JR, Bocquet C (1975) Evolution in a cosmopolitan species: genetic latitudinal clines in *Drosophila melanogaster* wild populations. *Experientia*, **31**, 164–166.

David JR, Capy P (1988) Genetic variation of *Drosophila melanogaster* natural populations. *Trends in Genetics*, **4**, 106–111.

De Jong G, Bochdanovits Z (2003) Latitudinal clines in *Drosophila melanogaster*: body size, allozyme frequencies, inversion frequencies, and the insulin-signalling pathway. *Journal of Genetics*, **82**, 207–223.

Fabian DK, Kapun M, Nolte V *et al.* (2012) Genome-wide patterns of latitudinal differentiation among populations of *Drosophila melanogaster* from North America. *Molecular Ecology*, **21**, 4748–4769.

Garofalo RS (2002) Genetic analysis of insulin signaling in *Drosophila*. *Trends in Endocrinology & Metabolism*, **13**, 156–162.

Giannakou ME, Partridge L (2007) Role of insulin-like signalling in *Drosophila* lifespan. *Trends in Biochemical Sciences*, **32**, 180–188.

Giannakou ME, Goss M, Jacobson J *et al.* (2007) Dynamics of the action of *dFOXO* on adult mortality in *Drosophila*. *Aging Cell*, **6**, 429–438.

Giannakou ME, Goss M, Jünger MA *et al.* (2004) Long-lived *Drosophila* with overexpressed d*FOXO* in adult fat body. *Science*, **305**, 361–361.

Gilchrist AS, Partridge L (1999) A Comparison of the Genetic Basis of Wing Size Divergence in Three Parallel Body Size Clines of *Drosophila melanogaster*. *Genetics*, **153**, 1775–1787.

Harshman LG, Hoffmann AA, Clark AG (1999) Selection for starvation resistance in *Drosophila melanogaster*: physiological correlates, enzyme activities and multiple stress responses. *Journal of Evolutionary Biology*, **12**, 370–379.

Hoffmann AA, Weeks AR (2007) Climatic selection on genes and traits after a 100 year-old invasion: a critical look at the temperate-tropical clines in *Drosophila melanogaster* from eastern Australia. *Genetica*, **129**, 133–147.

Hoffmann AA, Hallas R, Sinclair C, Mitrovski P (2001) Levels of variation in stress resistance in *Drosophila* among strains, local populations, and geographic regions: Patterns for desiccation, starvation, cold resistance, and associated traits. *Evolution*, **55**, 1621–1630.

Hoffmann AA, Sørensen JG, Loeschcke V (2003) Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. *Journal of Thermal Biology*, **28**, 175–216.

Hwangbo DS, Gersham B, Tu M-P, Palmer M, Tatar M (2004) *Drosophila dFOXO* controls lifespan and regulates insulin signalling in brain and fat body. *Nature*, **429**, 562–566.

James AC, Partridge L (1995) Thermal Evolution of Rate of Larval Development in *Drosophila-Melanogaster* in Laboratory and Field Populations. *Journal of Evolutionary Biology*, **8**, 315–330.

James AC, Azevedo RB, Partridge L (1995) Cellular basis and developmental timing in a size cline of *Drosophila melanogaster*. *Genetics*, **140**, 659–666.

James AC, Azevedo RB, Partridge L (1997) Genetic and environmental responses to temperature of *Drosophila melanogaster* from a latitudinal cline. *Genetics*, **146**, 881–890.

Kapun M, Fabian DK, Goudet J, Flatt T (2016a) Genomic evidence for adaptive inversion clines in *Drosophila melanogaster*. *Molecular Biology and Evolution*, **33**, 1317–1336.

Kapun M, Schmidt C, Durmaz E, Schmidt PS, Flatt T (2016b) Parallel effects of the inversion *In(3R)Payne* on body size across the North American and Australian clines in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, **29**, 1059–1072.

Karan D, Dahiya N, Munjal AK *et al.* (1998) Desiccation and starvation tolerance of adult *Drosophila*: Opposite latitudinal clines in natural populations of three different species. *Evolution*, **52**, 825.

Kennington WJ, Hoffmann AA (2010) The genetic architecture of wing size divergence at varying spatial scales along a body size cline in *Drosophila melanogaster*. *Evolution*, **64**, 1935–1943.

Kennington WJ, Hoffmann AA, Partridge L (2007) Mapping regions within cosmopolitan inversion *In(3R)Payne* associated with natural variation in body size in *Drosophila melanogaster*. *Genetics*, **177**, 549–556.

Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R (1993) A *C. elegans* mutant that lives twice as long as wild type. *Nature*, **366**, 461–464.

Mackay TFC, Richards S, Stone EA *et al.* (2012) The *Drosophila melanogaster* Genetic Reference Panel. *Nature*, **482**, 173–178.

Morris BJ, Willcox DC, Donlon TA, Willcox BJ (2015) *FOXO*3: A major gene for human longevity--A mini-review. *Gerontology*, **61**, 515–525.

Nielsen MD, Luo X, Biteau B, Syverson K, Jasper H (2008) 14-3-3ε antagonizes *Foxo* to control growth, apoptosis and longevity in *Drosophila*. *Aging Cell*, **7**, 688–699.

Noach EJK, de Jong G, Scharloo W (1996) Phenotypic plasticity in morphological traits in two populations of *Drosophila melanogaster*. *Journal of Evolutionary Biology*, **9**, 831–844.

Oldham S, Hafen E (2003) Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control. *Trends in Cell Biology*, **13**, 79–85.

Paaby AB, Schmidt PS (2008) Functional significance of allelic variation at methuselah, an

aging gene in *Drosophila* (MW Hahn, Ed,). *PLoS ONE*, **3**, e1987.
Paaby AB, Schmidt PS (2009) Dissecting the genetics of longevity in *Drosophila melanogaster*. *Fly*, **3**, 29–38.
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**, 3395–3409.
Paaby AB, Blacket MJ, Hoffmann AA, Schmidt PS (2010) Identification of a candidate adaptive polymorphism for *Drosophila* life history by parallel independent clines on two continents. *Molecular Ecology*, **19**, 760–774.
Rajpurohit S, Hanus R, Vrkoslav V *et al.* (2017a) Adaptive dynamics of cuticular hydrocarbons in *Drosophila*. *Journal of Evolutionary Biology*, **30**, 66–80.
Rajpurohit S, Zhao X, Schmidt PS (2017b) A resource on latitudinal and altitudinal clines of ecologically relevant phenotypes of the Indian *Drosophila*. *Scientific data*, **4**, 170066.
Robinson SJW, Partridge L (2001) Temperature and clinal variation in larval growth efficiency in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, **14**, 14–21.
Robinson SJW, Zwaan B, Partridge L (2000) Starvation resistance and adult body composition in a latitudinal cline of *Drosophila melanogaster*. *Evolution*, **54**, 1819.
Saucedo LJ, Edgar BA (2002) Why size matters: altering cell size. *Current Opinion in Genetics & Development*, **12**, 565–571.
Schmidt PS, Matzkin L, Ippolito M, Eanes WF (2005a) Geographic variation in diapause incidence, life-history traits, and climatic adaptation in *Drosophila melanogaster*. *Evolution*, **59**, 1721–1732.
Schmidt PS, Paaby AB, Heschel MS (2005b) Genetic variance for diapause expression and associated life histories in *Drosophila melanogaster*. *Evolution*, **59**, 2616–2625.
Schmidt PS, Zhu C-T, Das J *et al.* (2008) An amino acid polymorphism in the couch potato gene forms the basis for climatic adaptation in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, **105**, 16207–16211.
Sim C, Kang DS, Kim S, Bai X, Denlinger DL (2015) Identification of *FOXO* targets that generate diverse features of the diapause phenotype in the mosquito *Culex pipiens*. *Proceedings of the National Academy of Sciences*, 201502751.
Tatar M (2003) The endocrine regulation of aging by insulin-like signals. *Science*, **299**, 1346–1351.
Tatar M, Yin CM (2001) Slow aging during insect reproductive diapause: why butterflies, grasshoppers and flies are like worms. *Experimental Gerontology*, **36**, 723–738.
Tatar M, Kopelman A, Epstein D *et al.* (2001) A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function. *Science*, **292**, 107–110.
Weeks AR, McKechnie SW, Hoffmann AA (2002) Dissecting adaptive clinal variation: markers, inversions and size/stress associations in *Drosophila melanogaster* from a central field population. *Ecology Letters*, **5**, 756–763.
Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. *Evolution*, **38**, 1358.
Zhao X, Bergland AO, Behrman EL *et al.* (2015) Global transcriptional profiling of diapause and climatic adaptation in *Drosophila melanogaster*. *Molecular Biology and Evolution*, **33**, msv263–720.
Zwaan BJ (2000) Cellular basis of wing size variation in *Drosophila melanogaster*: a comparison of latitudinal clines on two continents. *Heredity*, **84**, 338.

Author Contributions
PS and TF conceived the study and designed research; SR and NB established populations; SR, NB, MK and ED performed research and analyzed data; NB and PS wrote the paper; ED edited and revised the paper.

Data Accessibility
Data deposited at Dryad: doi link to be added upon publication.

Table 1. ANOVA results for the assayed phenotypic traits among natural populations. ANOVA results for development time, thorax length, wing area, wing area:thorax length ratio, and starvation tolerance. White and grey cells show the results for females and males, respectively. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. See Results for details.

Factor	Development Time	Thorax Length	Wing Area	Wing Area:Thorax Length Ratio	Starvation Tolerance
Population	$F_{5,133}$=6.75***	$F_{5,135}$=4.71***	$F_{5,137}$=14.15***	$F_{5,136}$=13.91***	$F_{5,117}$=11.04***
	$F_{5,132}$=6.42***	$F_{5,140}$=3.28**	$F_{5,136}$=11.54***	$F_{5,138}$=11.45***	$F_{5,114}$=5.63***
Block	$F_{2,650}$=69.69***	$F_{2,364}$=0.05	$F_{2,387}$=2.72	$F_{2,365}$=3.687*	*NA*
	$F_{2,540}$=75.52***	$F_{2,366}$=1.96	$F_{2,395}$=4.71**	$F_{2,372}$=3.27*	*NA*

Table 2. ANOVA results for the effects of high and low latitude *foxo* alleles on phenotype. ANOVA results for development time, thorax length, wing area, wing area:thorax length ratio, and starvation tolerance. White and grey cells show the results for females and males, respectively. Sums of squares are shown in parenthesis. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. See Results for details.

Factor	Development Time	Thorax Length	Wing Area	Wing Area:Thorax Length Ratio	Starvation Tolerance
Allele	*F*=0.67 (57.12)	*F*=8.17** (8185.11)	*F*=57.74*** (1.95E+11)	*F*=39.83*** (97159.11)	*F*=8.38** (1128.84)
	F=3.23 (111.80)	*F*=9.97** (5689.75)	*F*=41.35*** (8.69E+10)	*F*=34.19*** (52392.04)	*F*=8.14** (1039.23)
Set (Allele)	*F*=2.44 (416.40)	*F*=5.78** (11580.03)	*F*=16.50*** (1.12E+11)	*F*=7.99*** (38974.63)	*F*=9.41*** (2535.86)
	F=4.45* (307.58)	*F*=4.95** (5644.90)	*F*=10.23*** (4.30E+10)	*F*=6.36** (19500.92)	*F*=7.79*** (1988.76)
Cage(Set, Allele)	*F*=7.04*** (2400.37)	*F*=3.72** (14914.63)	*F*=18.15*** (2.46E+11)	*F*=15.60*** (152264.86)	*F*=4.63** (2492.85)
	F=2.99* (413.11)	*F*=4.00** (9119.65)	*F*=12.00*** (1.01E+11)	*F*=13.95*** (85498.41)	*F*=2.36 (1202.37)

Figure legends

Fig. 1. Allele frequency changes for *foxo* SNPs in the Dros-RTEC dataset. Figure shows allele frequency differences conditioned to increase from south to north, with the frequency in Florida being set to zero. The *foxo* candidate SNPs are highlighted by two vertical black lines in the gene plot (A) or in bold red in the clinal plot (B). Note that the coordinates refer to the *Drosophila melanogaster* reference version 6.

Fig. 2. F_{ST} Manhattan plots for the biological replicates A and B, constructed from independent sets of inbred lines from the DGRP panel. The *foxo* candidate SNPs are highlighted in red, demonstrating that there are no confounding genomic background signals in either set.

Fig. 3. **Phenotypic analysis of natural populations collected across the latitudinal gradient in the eastern U.S**. (A-D) and the homozygous high- and low-latitude *foxo* genotypes (E-H). In all panels, females are depicted by filled symbols and males by open symbols. Starvation tolerance increases with increasing latitude (A); similarly, the high latitude *foxo* allele is associated with increased starvation resistance (E). Development time does not vary predictably with latitude (B), and is also equivalent between *foxo* alleles (F). Wing area (C) and the ratio of wing area to thorax length (D) exhibit a positive latitudinal cline in the sampled populations; these patterns of size variation in the natural populations are mirrored in both magnitude and direction by the observed differences in size parameters between the low and high latitude *foxo* alleles (G, H).

Figure 1

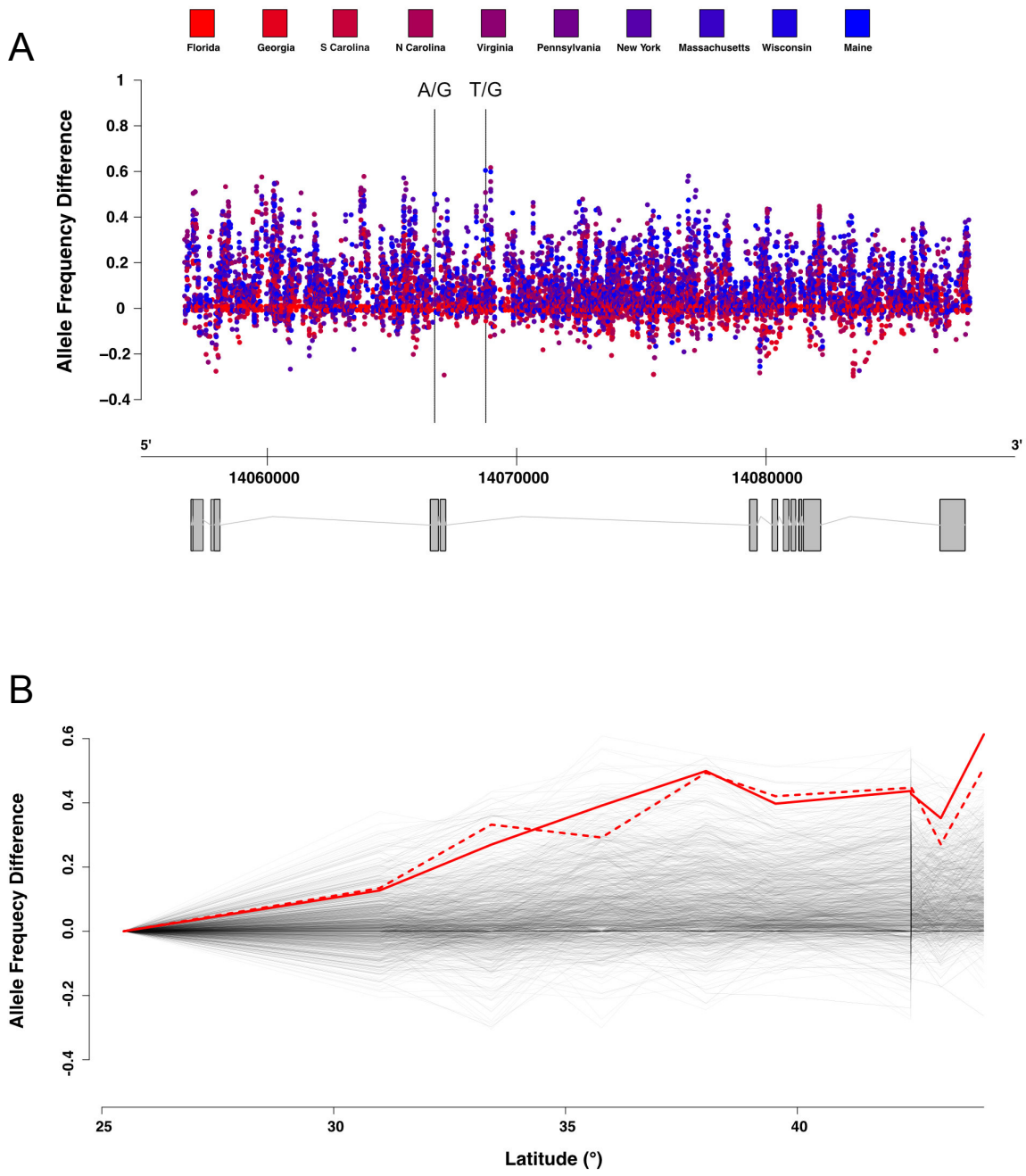

A
Florida
Georgia
S Carolina
N Carolina
Virginia
Pennsylvania
New York
Massachusetts
Wisconsin
Maine
A/G
T/G
Allele Frequency Difference
1
0.8
0.6
0.4
0.2
0
−0.2
−0.4
5'
3'
14060000
14070000
14080000
B
Allele Frequecy Difference
0.6
0.4
0.2
0.0
-0.2
-0.4
25
30
35
40
Latitude (°)

Figure 2

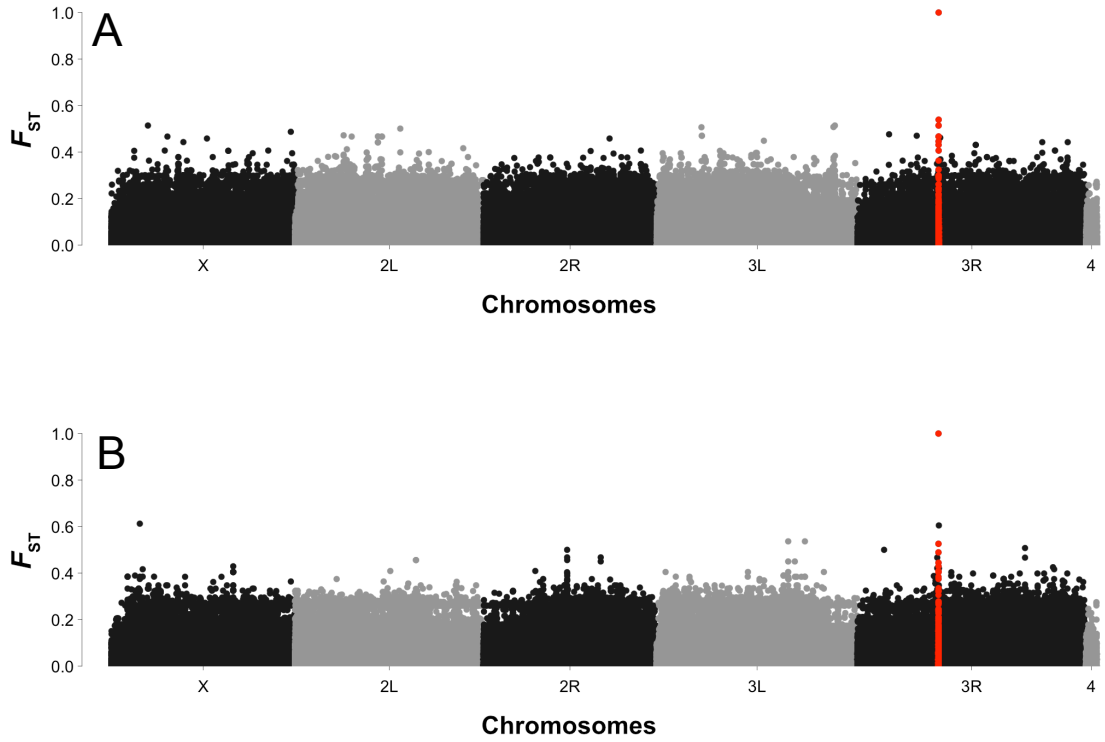
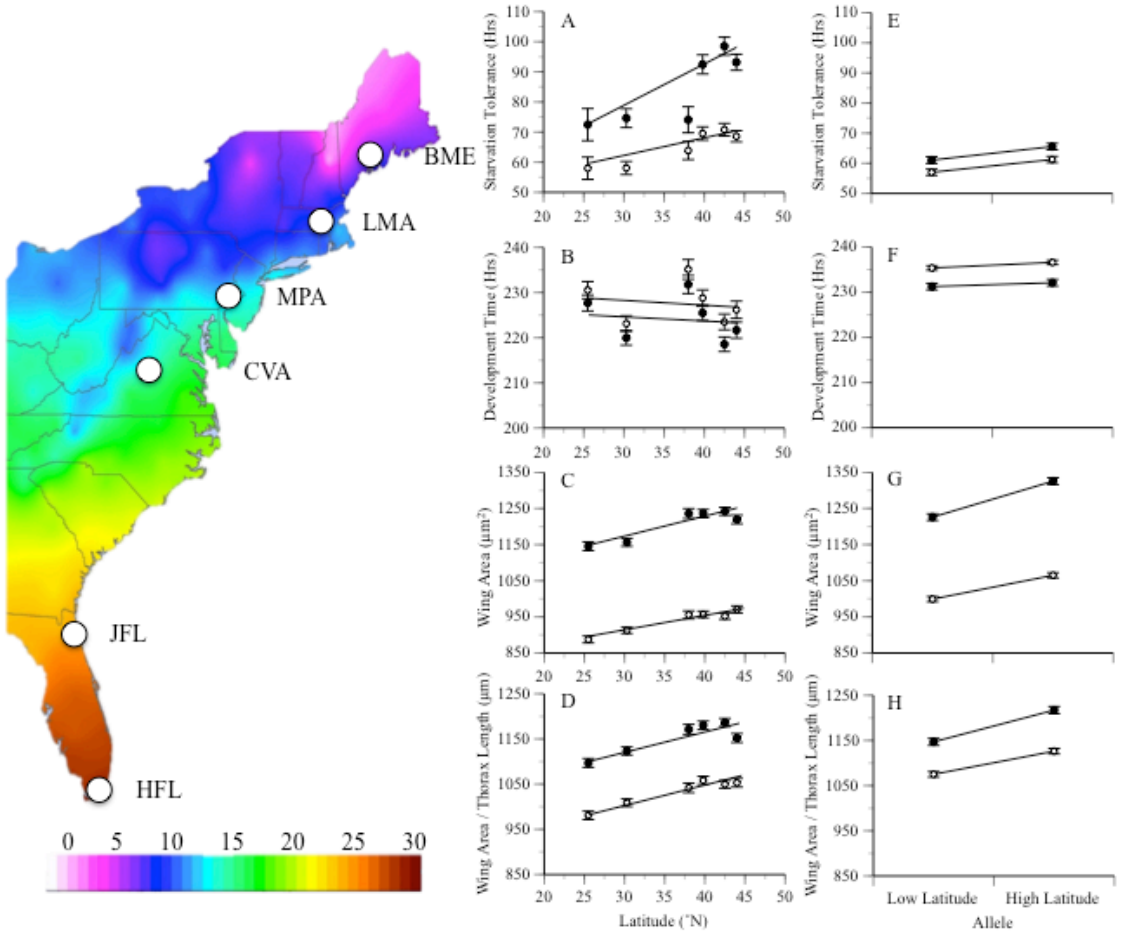


Figure 3

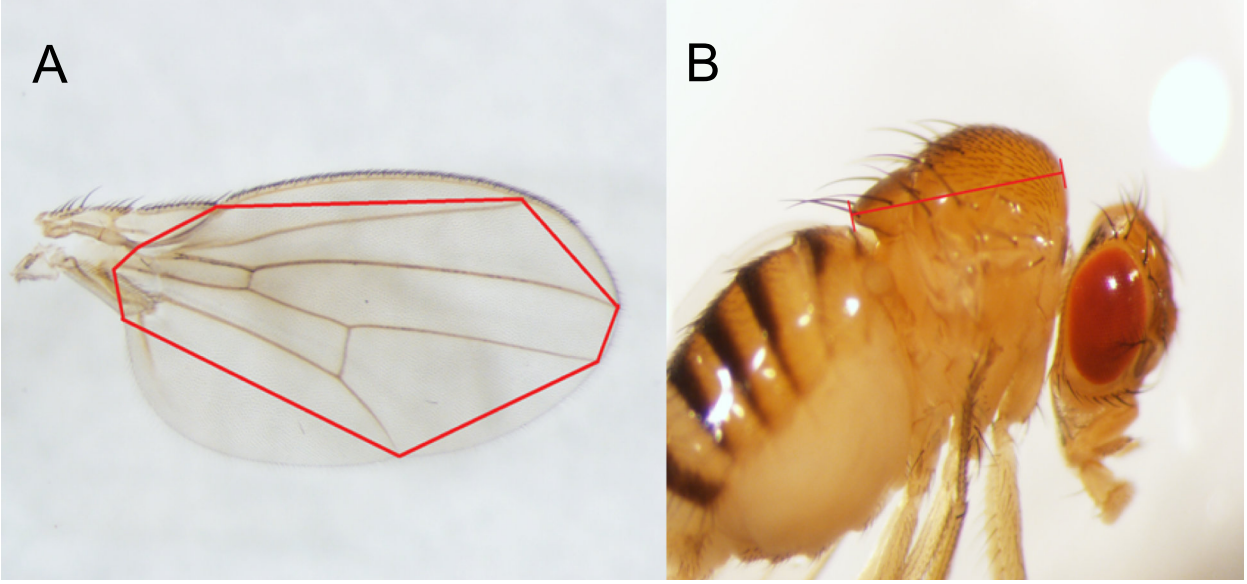

BME
LMA
MPA
CVA
JFL
HFL
0 5 10 15 20 25 30
A
B
C
D
E
F
G
H
Starvation Tolerance (Hrs)
Development Time (Hrs)
Wing Area (μm²)
Wing Area / Thorax Length (μm)
Latitude (°N)
Low Latitude
High Latitude
Allele

Supporting Information for Betancourt *et al*., Allelic polymorphism at *foxo* contributes to adaptive patterns of life history differentiation in natural populations of *Drosophila melanogaster*

Supporting Information Figure Legends

Figure S1. Body size parameters. The area of polygon in between the eight points of interest represent the parameter we used for measuring wing area (A). The length of the red line between the tip of the thorax and the tangent point of the thorax near the head represent the parameter we used for measuring thorax length (B).

Figure S1



Chapter 4

[Brief progress report]

Determining the causative effects of a clinal nucleotide polymorphism with CRISPR/Cas9 in *D. melanogaster*

Contributions by E. Durmaz: experimental design, bioinformatic analysis, assays, interpretation of results.

Chapter 4

Determining the causative effects of a clinal nucleotide polymorphism with CRISPR/Cas9 in *D. melanogaster*

ESRA DURMAZ[1], CLARE BENSON[2], KATHRIN GARSCHALL[1], ALISTAIR MCGREGOR[3] & THOMAS FLATT[1]

[1] *Department of Ecology and Evolution, University of Lausanne, CH-1015 Lausanne, Switzerland*

[2] *School of Biological Sciences, University of Manchester, M13 9PL, Manchester, United Kingdom*

[3] *Evolution of Animal Development and Morphology Department of Biological and Medical Sciences, Oxford Brookes University, OX3 0BP, Oxford, United Kingdom*

Summary

Homologous replacement of naturally occurring alleles in a controlled, isogenic background is "gold standard" for mapping as well as establishing the causative effects of natural genetic variants. In this mini-chapter, I am giving a brief progress report on our ongoing attempt to use the CRISPR/Cas9 genome editing technique to manipulate and experimentally study a clinally (latitudinally) varying genotype consisting of two single nucleotide polymorphisms (SNPs) in the insulin signaling transcription factor *foxo* in *D. melanogaster*.

Introduction

In a previous analysis, our team has identified genome-wide patterns of clinal differentiation among populations from the North American east coast and identified hundreds of clinally varying SNPs (based on SNP F_{ST} outliers) (Fabian *et al.* 2012). Many candidates were found to be located in genes belonging to several metabolic pathways, most notably in the insulin/insulin-like growth factor signaling (IIS) pathway, a pathway known from studies of mutants and transgenes in model organisms to have major regulatory effects on growth, size, reproduction and lifespan (Tatar 2003; De Jong & Bochdanovits 2003; Bochdanovits & de Jong 2004; Nässel *et al.* 2015). However, with very few exceptions practically nothing is known yet about the effects of naturally occurring alleles affecting this major nutrient sensing and energy pathway.

In collaboration with the team of Prof. Paul Schmidt (University of Pennsylvania), I have investigated the life-history effects of a clinally varying 2-SNP haplotype (*3R*:9892517; *3R*:9894559) in the gene *foxo*, a central forkhead box-O transcription factor involved in IIS. By reconstituting outbred populations, I have found that this polymorphism exerts pleiotropic life-history effects, mainly on body size, starvation resistance and fat storage. All of these traits are known to be affected in loss-of-function mutants or transgenic overexpression constructs of *foxo* (**Chapter 2**). Similarly, we have investigated the clinal life-history phenotypes in natural populations of *Drosophila melanogaster* and examined the contribution of *foxo* alleles to clinal life-history variation in body size (**Chapter 3**).

Bioinformatic analyses of the genomes of the inbred lines used in our outbred population approach suggest that the only consistently strong signal of differentiation (F_{ST}=1) between experimental populations differing in allelic state (A,T vs. GG) occurs at the 2-SNP positions of interest, so that our comparison of allelic states is unlikely confounded by other between-group differences in genomic background (**Chapter 3**). However, the "gold standard" for

establishing causal effects of natural alleles is their homologous replacement into a common genetic background, for example via CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9), a powerful genome editing method that is rapidly advancing and improving (Cong *et al.* 2013; Sander & Joung 2014; Turner 2014; Zhang *et al.* 2014b; Zhang 2015; D'Agostino & D'Aniello 2017). To establish causative effects of our candidate polymorphism in a maximally controlled genomic background we therefore decided to apply this novel state-of-the-art method, in collaboration with Alistair McGregor (Oxford Brookes University), and Ariane Ramaekers (Vlaams Instituut voor Biotechnologie), two experts in using this system in *Drosophila*.

The CRISPR/Cas9 system is involved in the prokaryotic acquired immune system, conferring resistance to foreign genetic elements (Horvath & Barrangou 2010; Garneau *et al.* 2010; Jinek *et al.* 2012; Gasiunas & Siksnys 2013). This system has recently been used to develop a powerful and flexible gene-editing platform for higher organisms. This new CRISPR/Cas9 method is very versatile, efficient and straightforward, especially when compared to previous genome editing techniques such as transcription activator-like effectors (TALENs) or zinc finger nucleases (ZFNs) (Gaj *et al.* 2013; Zhang 2014; Gupta & Musunuru 2014; D'Agostino & D'Aniello 2017). Another benefit of this system is that CRISPR/Cas9 can edit multiple targets at once, thus allowing for multiple-site manipulations in the genome (Cong *et al.* 2013; Wang *et al.* 2013; Chylinski *et al.* 2014; Mahfouz *et al.* 2014). More specifically, the novel CRISPR/Cas9 method allows the experimenter to engineer genomes, knockdown or activate target genes and edit transcription levels (D'Agostino & D'Aniello 2017). Changing, disrupting or editing genomic sequences in a variety of cell types and organisms such as viruses, plants, insects, worms, fish, birds, mice and humans gives the researchers a chance to investigate causative effects of genetic polymorphisms. Over the last years, there has been a growing body of literature reporting improved protocols, and the

method has successfully been used in a wide variety of organisms (Mali *et al.* 2013b; Friedland *et al.* 2013; Bassett *et al.* 2013; Mali *et al.* 2013a; Zhang *et al.* 2014a; Wei *et al.* 2014; Platt *et al.* 2014; Fan *et al.* 2014; Gratz *et al.* 2014b; Ho *et al.* 2015; Liang *et al.* 2015; Gratz *et al.* 2015; Véron *et al.* 2015; Cai *et al.* 2015; Zhou *et al.* 2016; Cinesi *et al.* 2016).

At the molecular level, genome editing with CRISPR/Cas9 requires (1) a universal endonuclease (Cas9), (2) a protospacer adjacent motif (PAM) in the genetic region of interest, and (3) a 20bp long sequence specific guide RNA (sgRNA) (**Figure 1**). The sgRNA guides Cas9 to the homologous region of editing, followed by binding of Cas9 which induces the double stranded break (DSB) 3bp upstream of the PAM region, with the resulting break being repaired by the cellular machinery. Two different repair mechanisms can prevent the potential loss of genetic information due to DSBs (Gaj *et al.* 2013). First, the more common non-homologous end joining (NHEJ) provides a mechanism whereby a break is repaired without the use of a template by random addition or removal of nucleotides, thus being prone to small deletions and random insertions (Lieber 2010; Deriano & Roth 2013). Second, in homology directed repair (HDR), damage is repaired by the use of a template that is homologous to sequences up- and downstream of the break. A desired change in the sequence can thus be introduced in the donor template which would serve as sister chromatid in HDR, and precise gene-editing can be directed to the site of the DSB (**Figure 1**) (Kadyk & Hartwell 1992; Goldfarb & Lichten 2010). In most studies to date, CRISPR/Cas9 gene-editing has been performed without providing a template for homologous repair, and has relied on NHEJ, so that many of the edits resulted in random indel (insertion deletion) polymorphisms in the region of interest (Gratz *et al.* 2013). This being said, a growing number of studies have attempted and managed to successfully induce a HDR response, thereby causing specific alterations of the target sequences (Bassett *et al.* 2014; Zhang *et al.* 2014a). Yet, the precise manipulation of a single nucleotide position is quite rare and limited, mainly due to the low

frequency of homology directed repair in the cell (Wang *et al.* 2013; Inui *et al.* 2014; Irion *et al.* 2014; Zimmer *et al.* 2016).

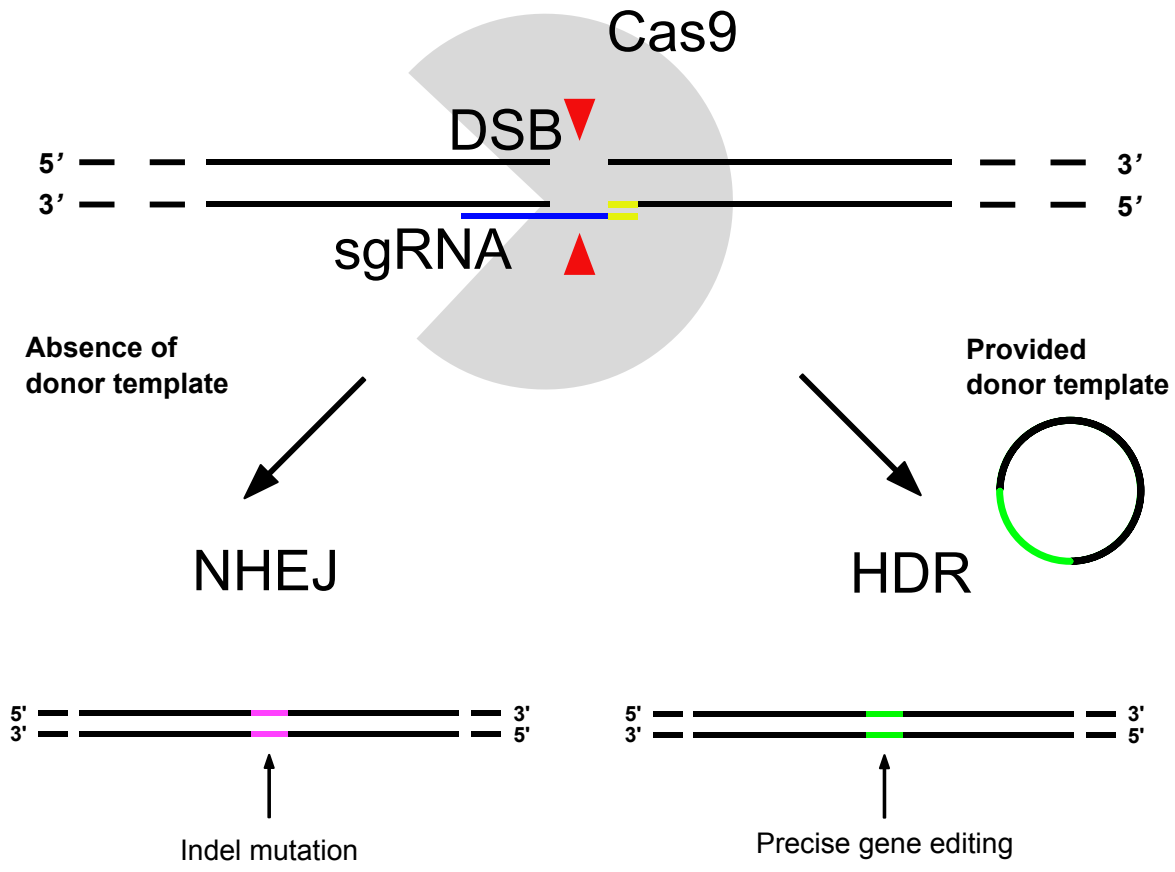


Figure 1: Schematic of the CRISPR/Cas9 genome editing. The Cas9 nuclease (in grey) is guided to editing site DNA by an sgRNA consisting of a 20-nt guide sequence (blue). The guide sequence pairs with the DNA target directly upstream of a requisite 5′-NGG adjacent motif (PAM; yellow). Cas9 mediates a double stranded break (DSB) ~3 bp upstream of the PAM (red triangle). In the error-prone NHEJ pathway, the ends of DSB are rejoined by DNA repair machinery, which can result in random indel mutations. In the HDR pathway, a repair template can be supplied in the form of ssODN or plasmid to allow precise editing.

For the purpose of our project mentioned in Chapters 2 and 3 of this thesis, we aimed to introduce a clinally varying *foxo* 2-SNP polymorphism into a common genetic background in

order to unambiguously determine its potentially causative phenotypic effects in a controlled background. Below I provide a brief summary of the status of this ongoing project.

Preliminary attempt to apply CRISPR/Cas9 to a 2-SNP variant in foxo

In a preliminary test of the CRISPR/Cas9 method in this context, we aimed to introduce the first *foxo* SNP locus (*3R*: 9892517) into a common genetic background and then – in a further step – alter the second site. This approach would allow us to investigate the individual as well as intralocus epistatic effects of our 2 *foxo* candidate SNPs. After the introduction of the clinal SNPs, we planned to test the effects of the SNPs on *Drosophila* life-history and insulin signaling states, similar to the assays we have performed on the outbred populations (see **Chapter 2**). These assays would allow us to assess the effects of clinal *foxo* SNPs, independent of their natural genetic context.

In collaboration with Alistair McGregor, we followed the protocol established by Andrew Bassett for *Drosophila*; as the starting material for CRISPR/Cas9 we chose an isofemale line with a previously sequenced genome (DGRP, #380) (Mackay *et al.* 2012; Bassett *et al.* 2013). First, to confirm the DGRP sequence, we resequenced the region of interest and utilized this information to design single guide (sg)RNAs and donor templates with the CRISPR design tool (http://crispr.mit.edu). Our designed construct had a very high quality score of 96 (out of 100), with no predicted exonic off-targets. Second, we designed a single-stranded oligodeoxynucleotide (ssODN) that carried the identical sequence as the region of interest except for the single nucleotide change (corresponding to the clinal *foxo* SNP) we aimed to introduce via HDR. Finally, we injected a cocktail of the sgRNA, mRNA for Cas9 and the ssODN into ~1000 syncytical embryos.

Due to the injections during early development, we expected embryos to be mosaics for the SNP edit; we therefore waited for two generations before screening for germ-line transformants. Since the clinal SNP we intended to manipulate is located in an exon, we did not want to interrupt the sequence by introducing a visible marker gene, such as for example green fluorescent protein (GFP). Instead of using visible markers, we thus decided to screen for transformants via sequencing of PCR products for the site of interest. Unfortunately, none of the screened individuals carried the desired alteration (or an indel polymorphism). As we observed rather high embryonic lethality (98%), we assume that the genetic modification was either arresting embryonic development or that the high quantity of foreign RNA might have been toxic.

Use of a modified CRISPR/Cas9 protocol

To increase the chance of successful editing, we have recently decided to use a different protocol and include several recently developed changes of the basic method. We are now following a protocol established by Scott Gratz (Gratz *et al.* 2014a; b), which has several modifications and improvements compared to the previous protocol. These modifications include: (i) using flies that have ubiquitous Cas9 expression (*act-cas9*; Bloomington *Drosophila* Stock Center [BDSC] #54590, *act-cas9-Lig4*; BDSC #58492), (ii) using plasmids for sgRNA and donor template, (iii) using a visible marker (DsRed) for screening and (iv) inhibition of NHEJ by using *Ligase4* (*Lig4*) mutants (BDSC #58492).

Using *act-cas9* or *act-cas9-Lig4* as a genetic background has several advantages. First, both mutants were engineered on the same genetic background as *Drosophila* reference genome (Port *et al.* 2014; Zhang *et al.* 2014c). Therefore, we could directly use the sequences for *foxo* from *Drosophila* reference genome. Second, in addition to ubiquitous expression of endonuclease, *act-cas9-Lig4* mutants have ubiquitous *Lig4* knockdown, which has been found

to significantly increase the frequency of error-free HDR as *Lig4* is an essential component of the final complex needed to complete NHEJ (Vartak & Raghavan 2015; Liang *et al.* 2017). Also, the plasmid used in this protocol contains a DsRed region flanked by *loxP* sites, to allow for more efficient screening of successful mutants which relies less heavily on molecular screening, as the DsRed marker is indicative of the successful insertion of the donor DNA template. Upon the identification of the DsRed phenotype, genetic crosses with *hs-cre* flies (BDSC #1501) successfully removes the DsRed marker (Siegal & Hartl 1996; Gratz *et al.* 2014a).

We are currently in the process of using this modified CRISPR/Cas9 protocol to manipulate and experimentally validate the *foxo* 2-SNP variant. To introduce the *foxo* SNPs into a common genetic background, we first sequenced the 1000bp upstream and downstream of both SNPs and designed multiple sgRNAs. We also used these sequences to design donor templates of ~2000bp per SNP for homology directed repair. At the moment, we are in the process of cloning sgRNAs and donor templates into plasmids. Then, we will inject a cocktail of plasmids that carry either sgRNA or donor template into *act-cas9* and *act-cas9-Lig4* embryos. Once injected, we will screen for the presence of DsRed marker and after that, we will set up genetic crosses with *hs-cre* flies to remove the marker using the Cre/*loxP* system. I am anticipating a workload of 4-5 weeks until I have positive transformants, followed by a few weeks of PCR-based screening for double-checking. Thus, not counting the actual phenotypic assays to be performed on this material, I expect that I would know after about 2 months of part-time work whether our modified protocol has worked successfully or not. This modified CRISPR/Cas9 protocol will hopefully allow us to determine additive and/or epistatic effects across the 2 SNPs of interest in a controlled genetic background.

References

Bassett AR, Tibbit C, Ponting CP, Liu JL (2014) Mutagenesis and homologous recombination in *Drosophila* cell lines using CRISPR/Cas9. *Biology Open*, **3**, 42–49.

Bassett AR, Tibbit C, Ponting CP, Liu J-L (2013) Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. *Cell Reports*, **4**, 220–228.

Bochdanovits Z, de Jong G (2004) Antagonistic pleiotropy for life-history traits at the gene expression level. *Proceedings of the Royal Society B: Biological Sciences*, **271**, S75–S78.

Cai Y, Chen L, Liu X *et al.* (2015) CRISPR/Cas9-mediated genome editing in soybean hairy roots (X Xu, Ed,). *PLoS ONE*, **10**, e0136064.

Chylinski K, Makarova KS, Charpentier E, Koonin EV (2014) Classification and evolution of type II CRISPR-Cas systems. *Nucleic Acids Research*, **42**, 6091–6105.

Cinesi C, Aeschbach LEN, Bin Yang, Dion V (2016) Contracting CAG/CTG repeats using the CRISPR-Cas9 nickase. *Nature Communications*, **7**, 1–10.

Cong L, Ran FA, Cox D *et al.* (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science*, **339**, 819–823.

De Jong G, Bochdanovits Z (2003) Latitudinal clines in *Drosophila melanogaster*: body size, allozyme frequencies, inversion frequencies, and the insulin-signalling pathway. *Journal of Genetics*, **82**, 207–223.

Deriano L, Roth DB (2013) Modernizing the nonhomologous end-joining repertoire: Alternative and classical NHEJ share the stage. *Annual Review of Genetics*, **47**, 433–455.

D'Agostino Y, D'Aniello S (2017) Molecular basis, applications and challenges of CRISPR/Cas9: a continuously evolving tool for genome editing. *Briefings in Functional Genomics*.

Fabian DK, Kapun M, Nolte V *et al.* (2012) Genome-wide patterns of latitudinal differentiation among populations of *Drosophila melanogaster* from North America. *Molecular Ecology*, **21**, 4748–4769.

Fan Z, Li W, Lee SR *et al.* (2014) Efficient gene targeting in golden syrian hamsters by the CRISPR/Cas9 system (YL Tang, Ed,). *PLoS ONE*, **9**, e109755.

Friedland AE, Tzur YB, Esvelt KM *et al.* (2013) Heritable genome editing in *C. elegans* via a CRISPR-Cas9 system. *Nature Methods*, **10**, 741–743.

Gaj T, Gersbach CA, Barbas CF III (2013) ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends in Biotechnology*, **31**, 397–405.

Garneau JE, Dupuis M-È, Villion M *et al.* (2010) The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature*, **468**, 67–71.

Gasiunas G, Siksnys V (2013) RNA-dependent DNA endonuclease Cas9 of the CRISPR system: Holy Grail of genome editing? *Trends in Microbiology*, **21**, 562–567.

Goldfarb T, Lichten M (2010) Frequent and efficient use of the sister chromatid for DNA double-strand break repair during budding yeast meiosis (RS Hawley, Ed,). *PLoS Biology*, **8**, e1000520–12.

Gratz SJ, Cummings AM, Nguyen JN *et al.* (2013) Genome engineering of *Drosophila* with the CRISPR RNA-guided Cas9 nuclease. *Genetics*, **194**, 1029–1035.

Gratz SJ, Rubinstein CD, Harrison MM, Wildonger J, O'Connor-Giles KM (2015) CRISPR-Cas9 genome editing in *Drosophila*. *Current protocols in molecular biology*, **111**, 31.2.1–20.

Gratz SJ, Ukken FP, Rubinstein CD *et al.* (2014a) Highly specific and efficient CRISPR/Cas9-catalyzed homology-directed repair in *Drosophila*. *Genetics*, **196**, 961–971.

Gratz SJ, Wildonger J, Harrison MM, O'Connor-Giles KM (2014b) CRISPR/Cas9-mediated genome engineering and the promise of designer flies on demand. *Fly*, **7**, 249–255.

Gupta RM, Musunuru K (2014) Expanding the genetic editing tool kit: ZFNs, TALENs, and CRISPR-Cas9. *Journal of Clinical Investigation*, **124**, 4154–4161.
Ho TT, Zhou N, Huang J *et al.* (2015) Targeting non-coding RNAs with the CRISPR/Cas9 system in human cell lines. *Nucleic Acids Research*, **43**, e17–e17.
Horvath P, Barrangou R (2010) CRISPR/Cas, the immune system of bacteria and archaea. *Science*, **327**, 167–170.
Inui M, Miyado M, Igarashi M *et al.* (2014) Rapid generation of mouse models with defined point mutations by the CRISPR/Cas9 system. *Scientific Reports*, **4**.
Irion U, Krauss J, Nuesslein-Volhard C (2014) Precise and efficient genome editing in zebrafish using the CRISPR/Cas9 system. *Development*, **141**, 4827–4830.
Jinek M, Chylinski K, Fonfara I *et al.* (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, **337**, 816–821.
Kadyk LC, Hartwell LH (1992) Sister chromatids are preferred over homologs as substrates for recombinational repair in *Saccharomyces cerevisiae*. *Genetics*, **132**, 387–402.
Liang P, Xu Y, Zhang X *et al.* (2015) CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. *Protein & cell*, **6**, 363–372.
Liang X, Potter J, Kumar S, Ravinder N, Chesnut JD (2017) Enhanced CRISPR/Cas9-mediated precise genome editing by improved design and delivery of gRNA, Cas9 nuclease, and donor DNA. *Journal of Biotechnology*, **241**, 136–146.
Lieber MR (2010) The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annual review of biochemistry*, **79**, 181–211.
Mackay TFC, Richards S, Stone EA *et al.* (2012) The *Drosophila melanogaster* Genetic Reference Panel. *Nature*, **482**, 173–178.
Mahfouz MM, Piatek A, Stewart CN Jr (2014) Genome engineering via TALENs and CRISPR/Cas9 systems: challenges and perspectives. *Plant Biotechnology Journal*, **12**, 1006–1014.
Mali P, Esvelt KM, Church GM (2013a) Cas9 as a versatile tool for engineering biology. *Nature Methods*, **10**, 957–963.
Mali P, Yang L, Esvelt KM *et al.* (2013b) RNA-guided human genome engineering via Cas9. *Science*, **339**, 823–826.
Nässel DR, Liu Y, Luo J (2015) Insulin/IGF signaling and its regulation in *Drosophila*. *General and Comperative Endocrinology*, **221**, 255–266.
Platt RJ, Chen S, Zhou Y *et al.* (2014) CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell*, **159**, 440–455.
Port F, Chen HM, Lee T, Bullock SL (2014) Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in *Drosophila*. *Proceedings of the National Academy of Sciences*, **111**, E2967–E2976.
Sander JD, Joung JK (2014) CRISPR-Cas systems for editing, regulating and targeting genomes. *Nature Biotechnology*, **32**, 347–355.
Siegal ML, Hartl DL (1996) Transgene coplacement and high efficiency site-specific recombination with the Cre/loxP system in *Drosophila*. *Genetics*, **144**, 715–726.
Tatar M (2003) The endocrine regulation of aging by insulin-like signals. *Science*, **299**, 1346–1351.
Turner TL (2014) Fine-mapping natural alleles: quantitative complementation to the rescue. *Molecular Ecology*, **23**, 2377–2382.
Vartak SV, Raghavan SC (2015) Inhibition of nonhomologous end joining to increase the specificity of CRISPR/Cas9 genome editing. *The FEBS journal*, **282**, 4289–4294.
Véron N, Qu Z, Kipen PAS, Hirst CE, Marcelle C (2015) CRISPR mediated somatic cell genome engineering in the chicken. *Developmental Biology*, DBIO15168.
Wang H, Yang H, Shivalila CS *et al.* (2013) One-step generation of mice carrying mutations

in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell*, **153**, 910–918.
Wei W, Xin H, Roy B *et al.* (2014) Heritable genome editing with CRISPR/Cas9 in the Silkworm, *Bombyx mori* (Z Gong, Ed,). *PLoS ONE*, **9**, e101210.
Zhang C, Xiao B, Jiang Y *et al.* (2014a) Efficient editing of malaria parasite genome using the CRISPR/Cas9 system. *mBio*, **5**, e01414–14.
Zhang F (2015) CRISPR/Cas9: Prospects and challenges. *Human Gene Therapy*, **26**, 409–410.
Zhang F, Wen Y, Guo X (2014b) CRISPR/Cas9 for genome editing: progress, implications and challenges. *Human molecular genetics*, **23**, R40–R46.
Zhang X, Koolhaas WH, Schnorrer F (2014c) A versatile two-step CRISPR- and RMCE-based strategy for efficient genome engineering in *Drosophila*. *G3; Genes|Genomes|Genetics*, **4**, 2409–2418.
Zhang Y (2014) Genome editing with ZFN, TALEN and CRISPR/Cas systems: The applications and future prospects. *Advancements in Genetic Engineering*, **03**.
Zhou X, Wang L, Du Y *et al.* (2016) Efficient generation of gene-modified pigs harboring precise orthologous human mutation via CRISPR/Cas9-induced homology-directed repair in zygotes. *Human mutation*, **37**, 110–118.
Zimmer CT, Garrood WT, Puinean AM *et al.* (2016) A CRISPR/Cas9 mediated point mutation in the alpha 6 subunit of the nicotinic acetylcholine receptor confers resistance to spinosad in *Drosophila melanogaster*. *Insect Biochemistry and Molecular Biology*, **73**, 62–69.

Chapter 5

[Manuscript published in Journal of Evolutionary Biology]

Parallel effects of the inversion *In(3R)Payne* on body size across the North American and Australian clines in *Drosophila melanogaster*

Contributions by E. Durmaz: experimental design, assays, statistical analysis, interpretation of results.

JOURNAL OF Evolutionary Biology

doi: 10.1111/jeb.12847

Parallel effects of the inversion *In(3R)Payne* on body size across the North American and Australian clines in *Drosophila melanogaster*

M. KAPUN*[1], C. SCHMIDT*[1], E. DURMAZ*, P. S. SCHMIDT† & T. FLATT*

*Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland
†Department of Biology, University of Pennsylvania, Philadelphia, PA, USA

Keywords:

adaptation;
body size;
clines;
D. melanogaster;
inversions;
life history;
spatially varying selection.

Abstract

Chromosomal inversions are thought to play a major role in climatic adaptation. In *D. melanogaster*, the cosmopolitan inversion *In(3R)Payne* exhibits latitudinal clines on multiple continents. As many fitness traits show similar clines, it is tempting to hypothesize that *In(3R)P* underlies observed clinal patterns for some of these traits. In support of this idea, previous work in Australian populations has demonstrated that *In(3R)P* affects body size but not development time or cold resistance. However, similar data from other clines of this inversion are largely lacking; finding parallel effects of *In(3R)P* across multiple clines would considerably strengthen the case for clinal selection. Here, we have analysed the phenotypic effects of *In(3R)P* in populations originating from the endpoints of the latitudinal cline along the North American east coast. We measured development time, egg-to-adult survival, several size-related traits (femur and tibia length, wing area and shape), chill coma recovery, oxidative stress resistance and triglyceride content in homokaryon lines carrying *In(3R)P* or the standard arrangement. Our central finding is that the effects of *In(3R)P* along the North American cline match those observed in Australia: standard arrangement lines were larger than inverted lines, but the inversion did not influence development time or cold resistance. Similarly, *In(3R)P* did not affect egg-to-adult survival, oxidative stress resistance and lipid content. *In(3R)P* thus seems to specifically affect size traits in populations from both continents. This parallelism strongly suggests an adaptive pattern, whereby the inversion has captured alleles associated with growth regulation and clinal selection acts on size across both continents.

Introduction

One of the central goals of evolutionary biology is to understand how organisms adapt to environmental heterogeneity (Hoffmann & Sgrò, 2011; Savolainen *et al.*, 2013). A promising approach towards this end is to investigate systematic, gradual phenotypic and genotypic changes along environmental (e.g. climatic) gradients, so-called clines that are thought to be driven by spatially varying selection (Mayr, 1963; Endler, 1977; de Jong & Bochdanovits, 2003; Charlesworth & Charlesworth, 2010).

A classical model system for studying clinality is *Drosophila melanogaster* (de Jong & Bochdanovits, 2003; Hoffmann & Weeks, 2007; Adrion *et al.*, 2015), an ancestrally tropical vinegar (fruit) fly that has migrated out of sub-Saharan Africa about 10 000–15 000 years ago and subsequently colonized the rest of the world as a human commensal (David & Capy, 1988; Keller, 2007). As a result of its colonization history, this species had to adapt to a wide range of climatic and ecological conditions, including temperate and seasonal habitats. This is evidenced by patterns of clinal differentiation of

Correspondence: Thomas Flatt, Department of Ecology and Evolution, University of Lausanne, CH-1015 Lausanne, Switzerland.
Tel.: +41 21 692 4203; fax: +41 21 692 4165;
e-mail: thomas.flatt@unil.ch
[1]Equal contribution.

numerous life history, morphological and physiological traits across latitude: clinally varying traits include development time (James & Partridge, 1995), body size (Coyne & Beecham, 1987; Imasheva *et al.*, 1994; James *et al.*, 1995, 1997; Zwaan *et al.*, 2000; Gockel *et al.*, 2001; Gibert *et al.*, 2004; Klepsatel *et al.*, 2014; Fabian *et al.*, 2015), wing loading (Stalker, 1980; Azevedo *et al.*, 1998), pigmentation (Telonis-Scott *et al.*, 2011), ovariole number (Capy *et al.*, 1993; Gibert *et al.*, 2004; Klepsatel *et al.*, 2014), diapause propensity (Schmidt *et al.*, 2005; Schmidt & Paaby, 2008), cold and heat resistance (Hoffmann & Shirriffs, 2002) and desiccation resistance (Hoffmann & Parsons, 2009).

Consistent with spatially varying selection, many of these traits exhibit parallel clinal patterns across latitude on multiple continents, even though demography (e.g. admixture) can also contribute to patterns of clinality (Bergland *et al.*, 2016; Kao *et al.*, 2015; Flatt, 2016). For example, qualitatively identical latitudinal clines have been reported across several continents for body size (Coyne & Beecham, 1987; James *et al.*, 1995; van't Land *et al.*, 1999; Klepsatel *et al.*, 2014; Fabian *et al.*, 2015), pigmentation (David *et al.*, 1985; Munjal *et al.*, 1997; Telonis-Scott *et al.*, 2011) and chill coma recovery time (Gibert *et al.*, 2001; Hoffmann *et al.*, 2002; Ayrinhac *et al.*, 2004).

Despite much work on phenotypic clines in *Drosophila*, and although several single genetic markers are known to covary latitudinally with trait clines (de Jong & Bochdanovits, 2003; Hoffmann & Weeks, 2007; Adrion *et al.*, 2015; and references therein), little is known about the genetics underlying clinal trait variation (for some exceptions see Schmidt *et al.*, 2008; Paaby *et al.*, 2014) and the mechanisms by which clines are formed and maintained. Recent progress comes from genomewide studies of the Australian and North American clines that have identified hundreds of clinally varying single-nucleotide polymorphisms (SNPs) (Kolaczkowski *et al.*, 2011; Fabian *et al.*, 2012; Bergland *et al.*, 2014, 2015; Reinhardt *et al.*, 2014; Kapun *et al.*, 2016). While some proportion of these clinal variants is expected to causally contribute to clinal trait variation, other variants might be subject to hitchhiking (genetic draft) or admixture (Fabian *et al.*, 2012; Bergland *et al.*, 2016; Kapun *et al.*, 2016). Thus, identifying the true genic targets of clinal selection remains a considerable challenge (Adrion *et al.*, 2015; Flatt, 2016).

Information on potentially functionally relevant genomic sites or regions might be gleaned from the genomewide distribution of clinal SNPs. Remarkably, even though clinally varying SNPs occur throughout the genome, *the majority* of clinal variants is located on the right arm of the third chromosome (*3R*), especially within the region spanned by a large (~8 Mb), cosmopolitan chromosomal inversion, *In(3R)Payne* (also called *In(3R)P*) (Kolaczkowski *et al.*, 2011; Fabian *et al.*, 2012; Kapun *et al.*, 2016).

The *In(3R)P* inversion is of particular interest for four reasons. First, in several geographical areas (e.g. North American east coast, Australian east coast, India, Japan), this inversion exhibits steep, parallel latitudinal clines: the inverted karyotype reaches intermediate frequencies at low latitudes but is rare or absent at high latitudes (Mettler *et al.*, 1977; Inoue & Watanabe, 1979; Stalker, 1980; Knibb *et al.*, 1981; Knibb, 1982; Das & Singh, 1991; Matzkin *et al.*, 2005; Fabian *et al.*, 2012; Kapun *et al.*, 2014, 2016; Rane *et al.*, 2015). For example, along the North American cline, this arrangement reaches a frequency of ~50% in southern Florida but is absent in Maine (Mettler *et al.*, 1977; Knibb, 1982; Fabian *et al.*, 2012; Kapun *et al.*, 2014, 2016); thus, flies from high-latitude populations are fixed or nearly fixed for the standard arrangement. Second, in Australia and North America, the latitudinal slopes of the *In(3R)P* clines have remained stable across > 40 years of observation, consistent with the clines being maintained by spatially varying selection (Anderson *et al.*, 2005; Umina *et al.*, 2005; Kapun *et al.*, 2014, 2016); in Australia, the intercept of the clinal slope has recently shifted – possibly as a consequence of climate change (Anderson *et al.*, 2005; Umina *et al.*, 2005). Third, recent evidence suggests that the North American cline of *In(3R)P* is maintained non-neutrally and independent of population structure or admixture (Kapun *et al.*, 2016). Fourth, several inversions in *Drosophila* have previously been found to be associated with development time, egg-to-adult survival, size-related traits, fecundity and fertility, stress resistance (to cold, heat, starvation) and lifespan (Sperlich & Pfriem, 1986; Hoffmann *et al.*, 2004; Hoffmann & Weeks, 2007; Hoffmann & Rieseberg, 2008; and references therein). Thus, although many alleles within *In(3R)P* might be in linkage disequilibrium (LD) and thus subject to hitchhiking, the observation that the majority of clinal SNPs resides in the genomic region spanned by this inversion suggests that clinal trait variation might at least partly be driven by *In(3R)P* (de Jong & Bochdanovits, 2003; Fabian *et al.*, 2012; Kapun *et al.*, 2016).

Indeed, several association mapping studies have linked *In(3R)P* to clinal size variation among Australian populations (Weeks *et al.*, 2002; Rako *et al.*, 2006; Kennington *et al.*, 2007). Similarly, using quantitative trait locus (QTL) mapping, Calboli *et al.* (2003) found that the largest QTL peak for body size for the endpoints of the Australian and South American clines overlaps the region of *In(3R)P*. However, little is known about associations between *In(3R)P* and clinal phenotypes (including size) for other continents; finding parallel phenotypic effects of *In(3R)P* across multiple clines would considerably strengthen the case for spatially varying (clinal) selection. Moreover, effects of this inversion polymorphism on clinal fitness-related traits other than size remain largely unknown (cf. Rako *et al.*, 2006).

Here, we investigate – for the first time – the phenotypic effects of *In(3R)P* in populations that approximate the endpoints of the North American east coastal cline (southern Florida vs. Maine). We measured several fitness-related traits thought to be clinal (development time, egg-to-adult survival, proxies of body size [femur length, tibia length, wing area and wing shape], chill coma recovery time, oxidative stress resistance and triglyceride content [a correlate of starvation resistance]) in isochromosomal homokaryon lines carrying *In(3R)P* or the standard chromosomal arrangement.

Our results for the effects of *In(3R)P* on several measures of body size mirror those previously observed in populations from the Australian cline (Weeks *et al.*, 2002; Rako *et al.*, 2006; Kennington *et al.*, 2007) – this strongly suggests the existence of parallel adaptive effects of *In(3R)P* on clinal size variation across both continents that are driven by spatially varying selection.

Materials and methods

Fly stocks and maintenance

We used isofemale lines collected from populations that approximate the endpoints of the clinal gradient running along the North American east coast: a set of lines from subtropical southern Florida (Homestead and Jacksonville) and one from a temperate population in Maine (Bowdoin) (see Table 1; also see Schmidt *et al.*, 2005; Schmidt & Paaby, 2008; Fabian *et al.*, 2012 for further details on these populations). As we failed to detect phenotypic differences between the two Florida populations (not shown), we combined lines from both populations for statistical analysis. Isofemale lines were kept for long-term maintenance under constant conditions at 18 °C and 60% relative air humidity, at a photoperiod of 12 h:12 h light: dark.

All isofemale lines were screened for the presence of six cosmopolitan inversions (*In(2L)t, In(2R)NS, In(3L)P, In(3R)K, In(3R)Mo and In(3R)P*; see Lemeunier & Aulard, 1992) by extracting DNA from pools of 5–10 individuals from each line with a salt–chloroform extraction protocol and using PCR markers described in Matzkin *et al.* (2005) and Corbett-Detig *et al.* (2012). Consistent with previous data (Mettler *et al.*, 1977; Knibb, 1982; Kapun *et al.*, 2016), *In(3L)P* and *In(3R)P* segregated at intermediate frequencies in the subtropical samples from Florida but were absent in Maine. *In(3R)Mo*, in contrast, showed the opposite trend: it segregated at 11% frequency in Maine but was absent in Florida. None of the other inversions showed clinality (Table 1; also see below).

Generation of isochromosomal lines

To isolate wild-type chromosomes either carrying the inverted *In(3R)P* arrangement or the standard arrangement from isofemale lines (see above), we used a compound (second and third chromosome) balancer (*SMB6*; *TM6B*; Bloomington *Drosophila* Stock Center [BDSC], stock #5687) in an *ebony* (e^1) mutant background (Fig. S1). For a given isofemale line, we crossed a wild-type male from that line to a female carrying the balancer. F1 pupae heterozygous for the balancer were selected visually based on the dominant *tubby* (Tb^1) mutant phenotype. Upon eclosion, F1 adults were backcrossed to the balancer line to amplify the isolated wild-type chromosome. After four days of egg laying, F2 adults were screened for the presence or absence of *In(3R)P* using PCR markers described in Matzkin *et al.* (2005). Isochromosomal homokaryon lines were generated by selecting against balancer phenotypes in F3 crosses.

We isolated 41 *3R* chromosomes carrying *In(3R)P* ('Florida inverted', FI) and 30 carrying the standard arrangement ('Florida standard', FS) from the two Florida populations and 20 chromosomes carrying the standard arrangement from Maine ('Maine standard', MS). In total, we were able to generate 14 FI (34.1% of all FI isolates), 13 FS (43.3% of FS isolates) and 6 MS (30% of MS isolates) isochromosomal homokaryon lines for phenotyping (see below). For the remaining isolates, we failed to obtain homokaryons, possibly due to recessive deleterious or lethal variants in the wild-type chromosomes; we maintained these lines as heterozygotes over a balancer chromosome but excluded them from the phenotypic assays reported here. We verified *3R* karyotype using PCR on 3–5 single individuals per isolated chromosome, as described above.

Table 1 Summary of samples used in this study and estimates of inversion frequencies.

							Inversion frequencies					
Location	State	*n*	Latitude	Longitude	Date	Collector	*In(2L)t*	*In(2R)NS*	*In(3L)P*	*In(3R)K*	*In(3R)Mo*	*In(3R)P*
Homestead	Florida	51	25.5°N	−71.06 °E	5/2011	P. Schmidt	0.38	0.06	0.42	0.09	0.00	0.63
Jacksonville	Florida	32	30.3°N	−81.6°E	8/2011	R. Cogni	0.63	0.05	0.20	0.42	0.09	0.31
Bowdoin	Maine	35	42.3°N	−80.5°E	10/2012	P. Schmidt	0.43	0.03	0.00	0.03	0.11	0.00

n = number of isofemale lines screened to isolate *3R* homokaryons.

During the isolation process we did not control for inversions on chromosomal arms other than *3R*: apart from *In(2L)t*, which segregated in ~30% of isolated lines, other inversions were either absent or present at only very low frequencies. Given that *In(2L)t* segregated at approximately equal proportions among the three sets of isochromosomal lines, we did not control for its effects in our analyses.

Phenotypic assays

General methods

Isochromosomal lines were used to measure several pre-adult life-history traits (development time and egg-to-adult survival), stress-related and physiological traits (chill coma recovery time, oxidative stress resistance and triglyceride content) and proxies of body size (femur length, tibia length, wing area and wing shape). Isochromosomal lines were assigned randomized identifiers; assays were performed blind with respect to identifiers to eliminate potential bias. Vials or bottles were maintained and experiments performed at 25 °C and 60% relative humidity, under a photoperiod of 12 h:12 h light: dark.

To avoid nongenetic parental and environmental effects, assays were performed on flies from the F2 generation. Prior to the assays, we let 100 flies from each line oviposit for 2 days on standard (cornmeal–agar–yeast) medium. Eclosing F1 individuals were distributed into three replicate bottles (~200 flies per bottle) and aged for 5 days; flies were then transferred to new bottles and allowed to lay eggs for 3 h. For each line, we collected 200 eggs and placed them into bottles containing 25 mL of standard medium. The positions of experimental bottles were randomized once per day to avoid potential effects caused by environmental heterogeneity inside the incubator. Eclosing F2 adults were collected every 6 h during the day and every 12 h overnight and aged for 3 days before being used for phenotypic assays.

Pre-adult life history (development time and egg-to-adult survival)

To assess egg-to-adult development time and egg-to-adult survival (proportion viability), we recorded eclosion times for each individual and estimated developmental time in hours relative to the time point of egg laying.

Chill coma recovery

Adults were aged for two days after eclosion prior to the chill coma recovery assay. Twenty-four hours before the start of the assay, we anesthetized flies with CO_2 and created new subsets of up to 20 flies per sex and line in new vials with standard medium. To induce chill coma, flies were transferred to empty vials without anaesthesia and vials placed on ice at 0 °C for 3 h. Flies were subsequently transferred to petri dishes at room temperature and visually monitored until they woke up. For each individual, the time elapsed between removals from ice and waking was recorded; a fly was deemed 'awake' as soon as it was able to stand on all its legs. Flies from this assay were stored for triglyceride measurements at −20 °C.

Oxidative stress resistance

Adults were aged for two days after eclosion and split in two replicate subsets of 10 flies per sex and line 24 h before the start of the assay. To induce oxidative stress, flies were transferred to media-free vials containing filter paper saturated with 5 mL of 30 mM methyl viologen (paraquat) (Sigma-Aldrich, Steinheim, Germany) in 5% sucrose solution (Paaby & Schmidt, 2008). To prevent evaporation, each vial was sealed with parafilm. We monitored mortality every two hours until ~ 90% of all flies had died. We continued monitoring flies in 8-h intervals until all flies were dead. Corpses were preserved for morphometric measurements in ethanol.

Triglyceride content

As starvation resistance is often correlated with lipid content (Hoffmann & Harshman, 1999; Schmidt *et al.*, 2005; Goenaga *et al.*, 2013), we measured whole-body triglyceride (triacylglyceride [TAG]) content as a proxy. For each sample, we generated homogenates using 2 pooled flies and estimated serum TAG levels in micrograms per fly from blanks and standards run with each plate, using an enzymatic assay kit (Serum Triglyceride Determination Kit; Sigma-Aldrich) (also see McGowan *et al.*, 1983; Tennessen *et al.*, 2014).

Size-related traits and morphometric analysis

For morphometric measurements, we removed the first right leg and right wing of each fly. Both body parts were mounted on slides with CC/Mount™ tissue mounting medium (Sigma-Aldrich) and sealed with cover slips. Images of legs and wings were taken with a digital camera (Leica DFC 290, Leica Microsystems GmbH, Wetzlar, Germany) attached to a stereo dissecting microscope (Leica MZ125). Femur and tibia length were measured as the distance between two sets of landmarks with IMAGEJ (http://imagej.nih.gov/ij/; v.1.47d), following the approach described in Debat *et al.* (2011).

To minimize measurement error, we repeated all measurements three times and used the average lengths for statistical analysis. For wing measurements, we used IMAGEJ (v.1.47d) to define two orientation landmarks at the distal side of the humeral break at the posterior end of the costal cell (C) and the notch at the sinus between the alula (Al) and the axillary cell (Ax) of the wing (Fig. S2). These landmarks were used to infer semi-landmarks and to fit B-splines along the

outline of the wing and along wing veins with WINGS4 and CPR software (van der Linde & Houle, 2009; http://bio.fsu.edu/dhoule/wings.html). Males and females were analysed separately, and landmark data for every image were processed manually. We applied multivariate outlier detection based on principal components analysis (PCA) of landmark coordinates using CPR and excluded extreme outliers caused by broken wings or images of insufficient quality. As a proxy for wing size, we used total wing area, based on spline functions along the wing outline. Wing shape variation was analysed using LORY software (http://bio.fsu.edu/dhoule/lory.html), following the methods described by Márquez *et al.* (2012). We obtained point estimates of shape deformation by locally evaluating Jacobian matrices of interpolation functions at pseudo-landmarks using LORY. Log ($-log_2$) - transformed determinants of Jacobian matrices contain information about local space contractions or expansions relative to a reference configuration and can be used as discrete summary variables that describe shape variation.

Deformations of individual configurations were analysed relative to Procrustes-transformed landmark coordinates, averaged across all individuals for each sex. We fitted elastic body splines (EBS) as interpolation functions at 122 (females) and 124 (males) evenly distributed pseudolandmarks and calculated log-transformed Jacobian determinants for each individual. To visualize shape differences, we averaged Jacobian determinants across all individuals for each pseudolandmark, group (FI, FS and MS) and sex. To interpolate shape values between landmarks, we performed 'kriging' (Gaussian process regression) using the R package KRIGING and plotted wings by showing interpolated Jacobian determinants for each group and sex using custom software (available upon request from M.K). Finally, to examine the variation in allometry between body parts among the three karyotypic groups (FI, FS and MS), we calculated the ratios of (1) femur length to tibia length, (2) femur length vs. wing area and (3) tibia length vs. wing area.

Statistical analysis

Statistical analyses were performed using JMP (SAS, Raleigh, NC, USA; v.11.1.1) and R (https://www.r-project.org/; v.3.2.1) software. Given that the *In(3R)P* is absent in Maine, we could not analyse the data with a fully factorial (orthogonal) model, testing the effects of karyotype (standard vs. inverted), geography (Florida vs. Maine) and the karyotype by geography interaction. We thus created a compound grouping factor g with three levels ('Florida inverted', FI; 'Florida standard', FS; 'Maine standard', MS).

We first performed multivariate analysis of variance (MANOVA) to test the effects of karyotype and geography on multivariate phenotype (i.e. a linear combination of all measured traits, except wing shape [due to its high dimensionality] and size ratios), using the following model: $Y_i = g + s + g \times s$, where Y_i denotes the matrix of measured individual traits averaged by line and sex for the i^{th} line, g is the nominal fixed grouping factor (with levels FI, FS, MS), s denotes the fixed effect of sex, and $g \times s$ denotes the interaction term. We also used MANOVA to analyse the multivariate wing shape based on multiple Jacobian determinants, separately for each sex, using the following model: $Y_i = g + l_{(g)}$, where $l_{(g)}$ represents the effect of line nested within the grouping factor g.

Next, we analysed each trait (including size ratios; see above) separately using a nested mixed-effects analysis of variance (ANOVA) model of the following form: $y_i = g + s + g \times s + l_{(g)}$, where y_i is the measured phenotype for the ith individual, g denotes the grouping factor, s denotes sex, and $l_{(g)}$ is the random effect of line nested in g, estimated using restricted maximum likelihood (REML). The random line effect was included to account for variation among lines, but we were not primarily interested in the variance component estimates of this effect; we therefore do not report these estimates.

To analyse the egg-to-adult survival (proportion viability), we used the following ANOVA model: *arcsine square root* $(y_i) = g + s + g \times s$, where y_i is the proportion of egg-to-adult survival of the i^{th} line and g and s denote the grouping factor and sex, respectively; note that, in this analysis, 'line' was the lowest level of replication.

To tease apart the effects of karyotype and geography, we performed post hoc tests using Tukey's honest significant difference (HSD) tests implemented in JMP, whenever the effect of the grouping factor g was significant; Tukey's HSD method corrects for multiple testing (i.e. the family-wise error rate). (For MANOVAS, we used planned contrasts instead as post hoc tests were not available in JMP.) We were specifically interested in using these tests to determine the effects of *In(3R)P* karyotype; the effects of geography were only of secondary interest. Significant differences between FI and FS and between FI and MS, with the comparison FS vs. MS being nonsignificant, imply a clear-cut effect of karyotype, and that the standard homokaryons from Florida and Maine have qualitatively identical effects. A pattern where FI vs. FS, FI vs. MS, and FS vs. MS are all significantly different implies that inverted vs. standard karyotypes differ in their effect, but that the two standard arrangement genotypes from Florida and Maine differ as well. In this situation, the effects of karyotype and geography cannot be completely separated; nonetheless, the significant difference between FI and FS indicates an effect of *In(3R)P* karyotype. Under either scenario, it thus seems safe to conclude that *In(3R)P* karyotype affects the phenotype of interest.

To compare our results for the differential effects of *In(3R)P* karyotype on wing area in North America to those from Australia (Queensland; Rako *et al.*, 2006), we calculated Cohen's standardized effect size *d* (Cohen, 1988) (1) from lines means and standard deviations for the FI and FS lines from Florida (this study) and (2) from approximate values of line means and standard deviations of inverted and standard lines obtained from Fig. 1 in Rako *et al.* (2006), using the online tool WebPlotDigitizer (Rohatgi, 2015).

In contrast to size data, the assumptions of normality and homoscedasticity underlying ANOVA were not always fulfilled for other traits. As data for development time, egg-to-adult survival, chill coma recovery and oxidative stress resistance represent failure time or time-to-event data that can violate ANOVA assumptions,

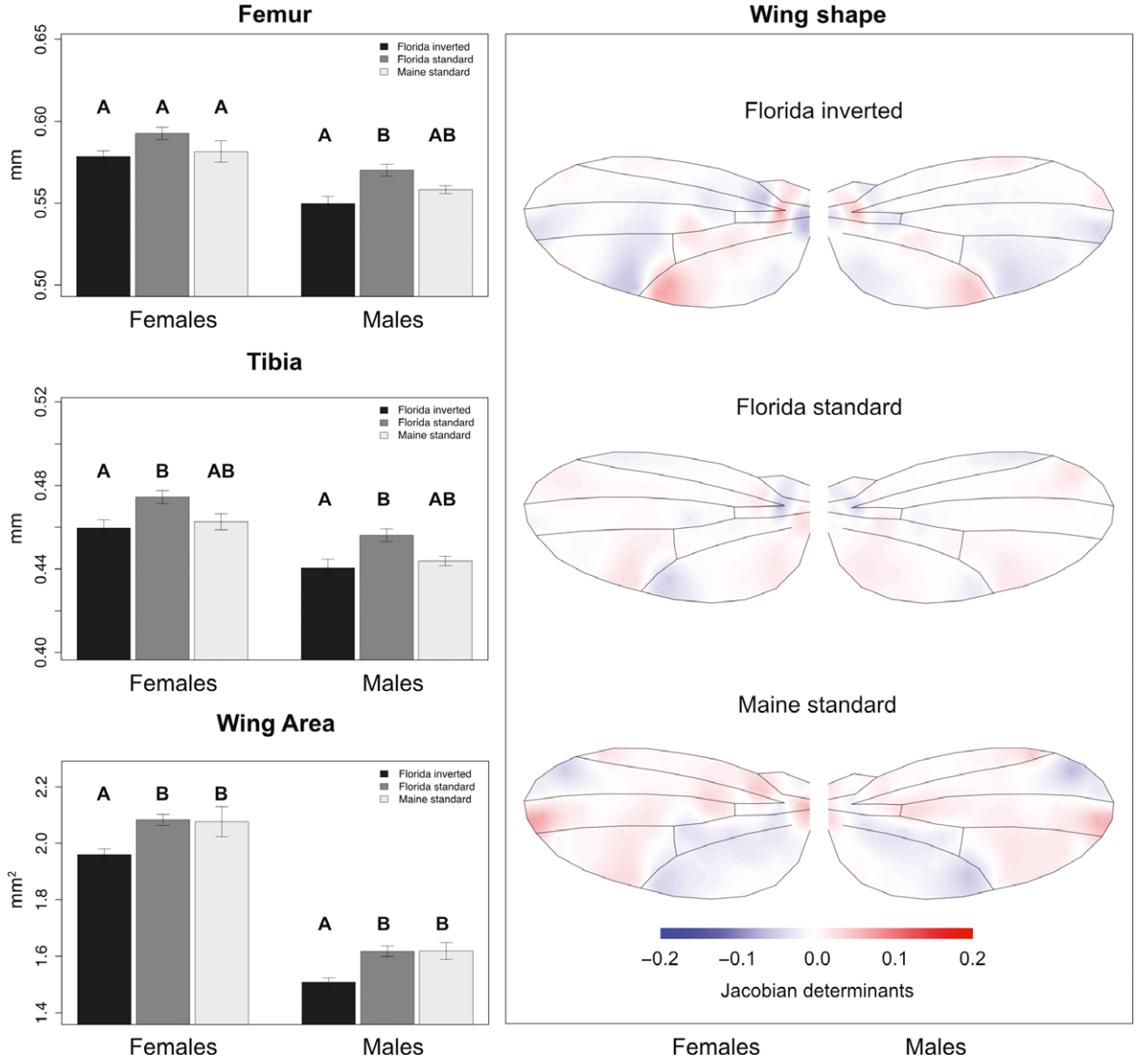


Fig. 1 The effects of *In(3R)P* on size-related traits. The left panel shows trait values averaged across line means for the three different groups differing in *In(3R)P* karyotype ('Florida inverted', FI; 'Florida standard', FS; 'Maine standard', MS). Error bars show standard errors. Letters above bars show the outcomes of Tukey's HSD post hoc tests, carried out for each sex separately: groups that not containing the same letter are significantly different ($P < 0.05$). The right panel shows average wing outlines and Jacobian determinants for each of the three groups (FI, FS and MS). Jacobian determinants, interpolated with kriging, represent local expansion (positive values; red) or contractions (negative values; blue) relative to the grand mean.

we additionally analysed these traits using mixed-effects Cox (proportional hazards) regression implemented in the R package COXME (Therneau, 2012), following the same model structure as defined above. These analyses yielded outcomes that were qualitatively identical to those based on ANOVA (not shown).

Results

Effects on multivariate phenotype

To account for potential phenotypic correlations among traits, we performed MANOVA analysis of the multivariate phenotype, that is a linear combination of all measured traits (except wing shape; see below). Examination of contrasts for the grouping factor *g* (FI vs. FS, FI vs. MS, FS vs. MS) indicated that inverted *In(3R)P* and standard arrangement differ in their effects on multivariate phenotype (Table S1; also see below and Table S3). The karyotypic effect of *In(3R)P* was most clearly revealed by the significant difference between the FI and FS groups. Inspection of contrasts also suggested that geographical origin (Florida vs. Maine) might affect multivariate phenotype (Table S1). In particular, the significant difference between FS and MS might be consistent with an effect of geography; however, a nonmutually exclusive alternative is that standard arrangements from Florida and Maine differ genotypically in their effects upon phenotype.

Effects on pre-adult life history and stress resistance

Pre-adult life-history traits (developmenst time and egg-to-adult survival) were neither affected by *In(3R)P* karyotype nor by geography (Table 2). Similarly, karyotype and geography had no measurable effect on any of the stress resistance or physiological traits (chill coma recovery time, oxidative stress resistance and triglyceride content) (Table 2).

Effects on size, shape and allometry

In contrast to life history and stress resistance, inverted and standard chromosomal arrangements differed in their effects on size-related traits. Inverted and standard lines from Florida differed significantly for both femur and tibia length, suggesting an effect of *In(3R)P* on body size (Table 2). The tibiae of inverted homokaryons were significantly shorter than those of noninverted lines for both sexes; the same effect was seen for femur length but only in males (Fig. 1, Table 2). Although for both traits standard arrangement lines from Maine did not differ from the two Florida karyotypes (Fig. 1, Table 2), we failed to identify a clear effect of geography when comparing lines from Florida and Maine without accounting for karyotype (not shown). These observations indicate that *In(3R)P* karyotype affects size, even though geographical differences independent of karyotype might also make a contribution.

The notion that *In(3R)P* inverted vs. standard arrangements have differential effects on size was clearly confirmed by an analysis of variation in wing size: for both sexes, Florida inverted lines had significantly smaller wings than Florida standard and Maine standard lines, whereas standard arrangement lines from Florida and Maine did not differ from each other (Fig. 1, Table 2). Despite different measurement methods and sample sizes, we found that the effect sizes for wing size differences between inverted and standard karyotypes from low-latitude populations in North America (Florida; our data) and Australia (Queensland; Rako *et al.*, 2006) were large (i.e. Cohen's $d > 1.4$) and qualitatively very similar (Florida: $d = 1.74$; Queensland, Australia: $d = 1.64$) across both continents (Table S2).

Table 2 Mixed-effects ANOVA tables for phenotypic analyses.

	Factors		
Trait	Group (*g*)	Sex (*s*)	$g \times s$
Development time (h)	$F_{2,31} = 1.07$	$F_{1.3554} = 402.52$***	$F_{2,3554} = 0.06$
Egg-to-adult survival (%)	$F_{2,62} = 2.88$	$F_{1,62} = 3.12$	$F_{2,62} = 0.577$
Wing area (mm^2)	$F_{2,29} = 10.24$**	$F_{1,1075} = 3551.66$***	$F_{2,1075} = 0.89$
Femur length (mm)	$F_{1,29} = 6.3$**	$F_{1,1053} = 525.04$***	$F_{2,1053} = 5.1$**
Tibia length (mm)	$F_{1,29} = 6.39$**	$F_{1,1053} = 318.66$***	$F_{2,1053} = 0.23$
Femur-to-tibia ratio	$F_{1,28} = 0.9$	$F_{1,1059} = 0.9$	$F_{2,1059} = 0.9$
Femur-to-wing area ratio	$F_{1,29} = 7.72$**	$F_{1,1056} = 2268$***	$F_{2,1056} = 2.58$
Tibia-to-wing area ratio	$F_{1,29} = 5.77$**	$F_{1,1055} = 2119$***	$F_{2,1055} = 3.4$*
Chill coma recovery (time to recovery, h)	$F_{1,28} = 1.29$	$F_{1,1041} = 20.3$***	$F_{2,1040} = 9.09$**
Oxidative stress resistance (age at death, h)	$F_{1,29} = 0.56$	$F_{1,1183} = 0.65$	$F_{2,1183} = 0.03$
Triglyceride content (μg)	$F_{1,29} = 0.61$	$F_{1,488} = 264.76$***	$F_{2,488} = 2.68$

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Significant among-group effects for the grouping factor *g* were analysed using Tukey's HSD post hoc tests; results of these tests are shown in Fig. 1. See Materials and methods and Results sections for further details.

MANOVA applied to a linear combination of femur length, tibia length and wing area, thus accounting for potential intercorrelations among size-related traits, also revealed significant among-group contrasts consistent with effects of karyotype and geography on size (Table S3).

We next analysed among-group variation in wing shape. Contrasts from MANOVA performed on Jacobian determinants of pseudolandmarks showed significant effects of karyotype and geography on wing shape for both sexes (Table S4). Florida inverted and Maine standard lines differed most strongly in their effects on wing shape, with Florida standard lines being intermediate. In both sexes, areas that showed largest variation for wing shape were located at the proximal part of the wing around the humeral break, around the terminal end of the distal (L5) wing vein, and at the distal end of the 1st posterior (1P) wing cell (Fig. 1, Fig. S2).

We also examined whether the three groups differ in allometry by analysing among-group variation in the size ratios of leg parts (femur length vs. tibia length) and different body parts (femur length vs. wing area, tibia length vs. wing area). While we failed to detect effects for the ratio of femur: tibia length, both group and sex affected the ratios of leg parts to wing area, with the ratios being larger for males than females (Table 2, Fig. S3). This suggests that in males wing size is smaller relative to leg size. For both measures of leg: wing size, Florida inverted lines exhibited larger ratios than Maine standard lines, irrespective of sex. The effect of *In(3R)P* karyotype was most clear-cut for the femur length: wing area ratio in males: Florida inverted lines had a greater ratio than both Florida and Maine standard lines, whereas standard lines from Florida and Maine did not differ from each other (Table 2, Fig. S3).

Together, our results indicate that *In(3R)P* affects multiple aspects of body size, shape and allometry but does not seem to have detectable effects upon pre-adult life history, stress resistance (e.g. chill coma recovery, oxidative stress resistance) and fat content.

Discussion

Chromosomal inversion polymorphisms are commonly found in *D. melanogaster* populations (Lemeunier & Aulard, 1992) but evidence for selection acting on them is surprisingly scarce (Kapun *et al.*, 2016). In support of a role for selection, *In(3R)Payne*, a cosmopolitan inversion that is clinally distributed along latitudinal gradients in Australia and North America, has been associated with body size clines in Australian populations (Weeks *et al.*, 2002; Rako *et al.*, 2006; Kennington *et al.*, 2007). However, comparable phenotypic data from other continents are not available, and whether the observations from the Australian cline represent a local phenomenon or a general pattern remains unclear. Moreover, effects of this inversion on traits other than size remain largely unknown (cf. Rako *et al.*, 2006). Here, we have investigated the phenotypic effects of *In(3R)P* in populations originating from the endpoints of the latitudinal cline running along the North American east coast.

In(3R)P has parallel effects on size across the North American and Australian clines

Our study provides the first evidence for an association between *In(3R)P* and the body size cline (cf. Coyne & Beecham, 1987) in North America. For the endpoints of the Australian cline, Rako *et al.* (2006) reported that flies carrying *In(3R)P* had smaller wings than standard arrangement flies. Similarly, for several proxies of body size, we found that inverted flies from the North American cline are smaller than flies carrying the standard chromosomal arrangement. Our findings thus mirror previous observations from the Australian cline (Weeks *et al.*, 2002; Rako *et al.*, 2006; Kennington *et al.*, 2007) and suggest that *In(3R)P* has parallel – very likely adaptive – effects on body size along both clinal gradients (cf. Kapun *et al.*, 2016).

Another size trait known to exhibit clinal variation on multiple continents – and thus likely to be subject to spatially varying selection – is wing 'loading' (the intercept of the relationship between body and wing size) (Azevedo *et al.*, 1998; Gilchrist *et al.*, 2000). Stalker (1980), for example, reasoned that larger wings relative to body size (i.e. low wing loading) might result in increased lift and would thus compensate for lower beat frequencies at lower temperatures experienced at higher latitudes. Perhaps consistent with this prediction, we observed lowest wing loading for standard arrangement lines from Maine, intermediate loading in standard arrangement lines from Florida and highest loading in inverted lines from Florida. It is noteworthy in this context that QTL mapping has identified a major peak for male flight duration within the region spanned by *In(3R)P* (Luckinbill *et al.*, 2005; see discussion in Rako *et al.*, 2006).

We also found karyotypic and geographical variation in wing shape. Inverted lines from Florida and standard arrangement lines from Maine differed most strongly in wing shape, whereas standard lines from Florida showed an intermediate pattern. Consistent with observations by Gilchrist *et al.* (2000), who investigated wing shape variation along size clines from three continents (albeit without examining *In(3R)P*), we observed large shape deformations in the anterior distal region between the medial and cubital vein. Moreover, we identified large shape differences at the discal cell and the 3rd posterior cell along the distal vein (L5), indicating shape expansion in Florida inverted lines but shape contraction in Maine standard lines. In contrast, shape differentiation was minimal along the leading edge of

the wing. This is in good agreement with kinetic analyses of wing aerodynamics: the anterior–posterior wing region might potentially be functionally constrained as it maintains the rotation axis close to the leading edge (Dickinson *et al.*, 1999; Gilchrist *et al.*, 2000). However, the evolutionary mechanisms that maintain variation in wing shape remain poorly understood; while wing size is subjected to directional selection, wing shape seems to be the result of optimizing (stabilizing) selection (potentially due to selection for 'canalization' [Flatt, 2005;]) rather than directional selection (Gilchrist & Partridge, 2001). Additional data will be required to unravel the potentially adaptive effects of *In(3R)P* on variation in wing shape.

In(3R)P and the genetic basis of size and shape

Further support for potentially causal links between *In(3R)P* and size-related traits comes from studies of the genetic basis of size and shape variation in *Drosophila* (see de Jong & Bochdanovits, 2003; Mirth & Shingleton, 2012; and references therein). Gockel *et al.* (2002) and Calboli *et al.* (2003), for example, used QTL analysis to map genetic variation associated with thorax length and wing size and found that the third chromosome accounts for a major proportion of size variation between the endpoints of the Australian and South American clines. Weeks *et al.* (2002) identified three indel (insertion deletion) and microsatellite polymorphisms within the region spanned by *In(3R)P* that are strongly associated with body size variation among Australian populations. Similarly, Kennington *et al.* (2007) found that microsatellite alleles associated with decreased wing size are in strong LD with *In(3R)P*. Moreover, the gene *Dca* (*Drosophila cold acclimation*; also known as *smp-30*), which is located close to the proximal breakpoint of *In(3R)P* and likely associated with this inversion through hitchhiking, accounts for approximately 5–10% of natural wing size variation in Australian populations (McKechnie *et al.*, 2010), and a clinal promoter polymorphism in this gene has been shown to decrease wing size (McKechnie *et al.*, 2010; Lee *et al.*, 2011).

In agreement with these findings, the region spanned by *In(3R)P* harbours several genes known to be important for growth regulation and the determination of body size (de Jong & Bochdanovits, 2003; Fabian *et al.*, 2012; Kapun *et al.*, 2016; see flybase.org for details of gene function and original source references). For example, *In(3R)P* contains multiple loci involved in insulin/insulin-like growth factor signalling (IIS), a pathway that plays a major role in regulating growth, size and shape, including *InR* (*insulin-like receptor*), *Tsc1* (*tuberous sclerosis complex 1*) and *Pi3K* (*Pi3K92E*, *phosphoinositide 3-kinase at 92E*; also known as *Dp110*) (Brogiolo *et al.*, 2001; de Jong & Bochdanovits, 2003; Oldham & Hafen, 2003; Edgar, 2006; Shingleton *et al.*, 2007; Mirth & Shingleton, 2012; Nässel *et al.*, 2015; also see below). Importantly, *InR* harbours many alleles that are strongly clinal along the North American east coast (Fabian *et al.*, 2012; Paaby *et al.*, 2014); indeed, a naturally occurring, clinal indel polymorphism in *InR* (albeit apparently not in LD with *In(3R)P*) affects body size in North American populations (Paaby *et al.*, 2014).

Whole-genome analyses of clinal variation associated with *In(3R)P* have also uncovered candidates with known effects on growth, including clinally varying alleles in *InR, Tsc1, Hmgcr* (*hydroxymethlyglutaryl coenzyme A reductase*, known to interact with IIS), *Orct2* (*organic cation transporter 2* or *calderón*, involved in IIS as well) and *Stat92E* (*signal-transducer and activator of transcription protein at 92E*, a transcription factor involved in JAK/STAT signalling) (Fabian *et al.*, 2012; Kapun *et al.*, 2016). Several of these genes, including *InR, Orct2* and *Stat92E*, also vary clinally along the Australian cline (Kolaczkowski *et al.*, 2011).

Two other interesting candidates are *hh* (*hedgehog*) and *Dad* (*Daughters against DPP*), both of which harbour clinal alleles associated with *In(3R)P* in North America (Fabian *et al.*, 2012; Kapun *et al.*, 2016). The *hh* locus encodes a signalling protein, which forms gradients in the developing wing and controls the placement and spacing of the longitudinal wing veins L3 and L4 (Blair, 2007; Matamoro-Vidal *et al.*, 2015). Perhaps consistent with the involvement of this gene, we identified strong variation in the spacing of these veins among karyotypes (see Fig. 1). *Dad* encodes a negative regulator of *Dpp* (*Decapentaplegic*), a morphogen that modulates the placement of the L2 and L5 wing veins (Tsuneizumi *et al.*, 1997; Matamoro-Vidal *et al.*, 2015); notably, we observed strong shape variation among karyotypes within the 3rd posterior cell along the L5 vein.

Thus, multiple lines of evidence suggest that *In(3R)P* harbours clinal variants in several major genes known to affect growth, size and shape. Although the causative effects of *In(3R)P*-linked alleles at these loci on size and shape remain unknown, these variants represent promising candidates for functional testing (cf. Kapun *et al.*, 2016).

In(3R)P has no measurable effects on pre-adult life history or stress resistance

Little is known about whether *In(3R)P* affects traits other than size. For example, with regard to Australian populations, a study by Anderson *et al.* (2003) reported an association between cold resistance and *In(3R)P*, and McColl *et al.* (1996) found an association between the response to thermal selection and the *hsr-omega* and *hsp68* genes, both located in the region spanned by *In(3R)P* (Anderson *et al.*, 2003). However, Rako *et al.* (2006), using a more direct genetic association approach based on *In(3R)P* homokaryon lines, failed to find an effect of *In(3R)P* on cold resistance. These find-

ings are in good agreement with ours: we also did not detect any measurable effects of *In(3R)P* on cold resistance. Although several genes known to be involved in cold resistance are located within the region of *In(3R)P* (Anderson *et al.*, 2003), it is unknown whether alleles at these loci are in LD with this inversion (cf. Weeks *et al.*, 2002; Rako *et al.*, 2006).

Rako *et al.* (2006) also found no effects of *In(3R)P* on development time for the Australian cline, an observation that is again consistent with ours. Given the usually tight physiological and genetic correlations between development time and body size (e.g. in artificial selection or experimental evolution experiments; see de Jong & Bochdanovits, 2003; and references therein), it is perhaps surprising that *In(3R)P* does not affect development time. However, clinal patterns for this trait often seem to be weak (James & Partridge, 1995) or absent (Fabian *et al.*, 2015); in line with this, development time and body size do not seem to be associated among populations along the Australian cline (James *et al.*, 1995). This raises the interesting but unresolved question of how, in terms of physiological mechanisms, *In(3R)P* affects size.

We also measured several traits that were not assayed by Rako *et al.* (2006), including egg-to-adult survival, oxidative stress resistance and triglyceride content; however, again, we could not find any measurable effects of *In(3R)P* on these traits. For the South American cline, Robinson *et al.* (2000) also failed to find a cline for fat content (and starvation resistance), albeit without examining *In(3R)P*. Together with the previous findings from Australia, our results therefore suggest that *In(3R)P* might have quite specific effects on size-related – but not necessarily other fitness-related – traits; yet, two important caveats remain. First, this inversion might have subtle effects on the non-significant traits we have measured but our statistical power for finding these effects was perhaps insufficient. Secondly, there are other major fitness-related traits known to be clinal (e.g. ovariole number, fecundity, lifespan, reproductive diapause) that we have not measured as a function of *In(3R)P* karyotype.

The adaptive significance of *In(3R)P*

The *In(3R)P* polymorphism exhibits steep, persistent latitudinal frequency clines between subtropical/tropical and temperate, seasonal environments on multiple continents (e.g. North America, Australia, Indian subcontinent, Japan), but – intriguingly – does not seem to be clinal within the tropics proper (e.g. sub-Saharan Africa, South-East Asia) (Aulard *et al.*, 2002; Glinka *et al.*, 2005). This strongly suggests that the inverted arrangement is selectively favoured in warm, low-latitude habitats, whereas the standard arrangement is favoured in temperate, seasonal and high-latitude habitats.

Recent findings indeed support the notion that latitudinal clines of *In(3R)P* are maintained by spatially varying selection: in North America, the latitudinal cline of *In(3R)P* has remained stable for > 40 years, deviates from neutral expectation and is maintained independent of isolation by distance and admixture (Kapun *et al.*, 2016). Moreover, the majority (> 90%) of the most strongly clinally varying single-nucleotide polymorphisms (SNPs) contained in *In(3R)P* are shared between the North American and Australian clines, consistent with parallel effects of spatially varying selection across both continents (Kapun *et al.*, 2016).

Interestingly, in areas where *In(3R)P* is known to be clinal (e.g. North America, Australia, India, Japan), body size also exhibits latitudinal clines (see Introduction). Together with the observation that *In(3R)P* is associated with body size in both Australia and North America, this suggests that *In(3R)P* clines might be driven by selection on body size. While the selective forces shaping body size clines still remain largely unknown (Partridge & Coyne, 1997), thermal experimental evolution experiments in *Drosophila* have shown that adaptation to warm vs. cool conditions favours small vs. large size (Partridge *et al.*, 1994). Thus, temperature might represent the most parsimonious selective agent underlying latitudinal size clines. As hypothesized by James & Partridge (1995), a possible reason for the existence of a temperature–latitude–size correlation in *Drosophila* could be that larval food resources might be more ephemeral in the tropical climates due to increased competition and that this would cause selection to favour rapid development and thus smaller adult size. In temperate habitats, in contrast, resources might be more stable and selection might thus favour longer development time and larger adult size (James & Partridge, 1995). Even though we did not find an effect of *In(3R)P* on development time, the fact that *In(3R)P* causes smaller size (through as of yet unknown developmental effects) and that its frequency is much more prevalent in warmer areas might be consistent with such a scenario.

The idea that inversions such as *In(3R)P* might be shaped by climatic adaptation is underscored by several observations. First, in North America, *In(3R)P* frequency is strongly positively associated with multiple measures of temperature and precipitation, whereas temperature dispersion (range) and seasonality seem to favour higher frequencies of the standard chromosomal arrangement (Kapun *et al.*, 2016; also see Knibb, 1982). Second, along the Australian east coast, the latitudinal cline of *In(3R)P* has shifted in position (intercept) across a time span of 20 years in response to recent climate change; as no single climatic factor could fully account for this pattern, it is likely that a combination of climatic variables, not temperature alone, has driven this shift (Umina *et al.*, 2005). Third, in support of climatic

selection, we have previously found in an experimental evolution experiment that *In(3R)Mo* and *In(3R)C*, two inversions that partly overlap with *In(3R)P*, were selectively favoured in replicate populations exposed to cold vs. warm temperatures, respectively (Kapun *et al.*, 2014). However, an important caveat is that in the same experiment *In(3R)P* itself was rapidly lost, from an initial frequency of ~20%, in both cold and warm environments. Thus, together with the findings mentioned above, unknown selective factors other than – or in addition to – temperature must play a major role in maintaining this inversion. It will clearly be of great interest – as well as a major challenge – to determine the selective factors affecting *In(3R)P* in future work.

Conclusions

Here, we have demonstrated that the chromosomal inversion *In(3R)P* affects several size-related traits in North American populations of *D. melanogaster*. Remarkably, these effects go in the same direction – and are of similar magnitude (e.g. see Table S2) – as those that have been previously reported for the Australian cline (Rako *et al.*, 2006). In conjunction with the Australian data, our results thus suggest a major role of *In(3R)P* in shaping clinal size variation across *both* continents, thereby considerably strengthening the case for spatially varying selection acting on body size via genetic variants contained within this inversion. However, the effects we have identified here remain correlational; future efforts will be required to dissect the functional links between size and the causative genetic variants harboured by this inversion.

Acknowledgments

We thank two anonymous reviewers and Kathrin Garschall for helpful comments; David Houle, Brian Hollis and Fiona Hollis for advice on morphometrics; Tad Kawecki, Jérôme Goudet and Tanja Schwander for statistical advice; and Daria Martynow for help in the laboratory. Our research was supported by the Swiss National Science Foundation (SNSF) (grant PP00P3_133641 to T.F.), the National Institutes of Health (NIH) (grant 5R01GM100366 to P.S.S.), the National Science Foundation (NSF) (grant DEB 0921307 to P.S.S.) and the Department of Ecology and Evolution at the University of Lausanne.

References

Adrion, J.R., Hahn, M.W. & Cooper, B.S. 2015. Revisiting classical clines in Drosophila melanogaster in the age of genomics. *Trends Genet.* **31**: 434–444.

Anderson, A.R., Collinge, J.E., Hoffmann, A.A., Kellett, M. & McKechnie, S.W. 2003. Thermal tolerance trade-offs associated with the right arm of chromosome 3 and marked by the *hsr-omega* gene in *Drosophila melanogaster*. *Heredity* **90**: 195–202.

Anderson, A.R., Hoffmann, A.A., McKechnie, S.W., Umina, P.A. & Weeks, A.R. 2005. The latitudinal cline in the *In(3R) Payne* inversion polymorphism has shifted in the last 20 years in Australian *Drosophila melanogaster* populations. *Mol. Ecol.* **14**: 851–858.

Aulard, S., David, J.R. & Leumeunier, F. 2002. Chromosomal inversion polymorphism in Afrotropical populations of *Drosophila melanogaster*. *Genet. Res.* **79**: 49–63.

Ayrinhac, A., Debat, V., Gibert, P., Kister, A.G., Legout, H., Moreteau, B. *et al.* 2004. Cold adaptation in geographical populations of *Drosophila melanogaster*: phenotypic plasticity is more important than genetic variability. *Funct. Ecol.* **18**: 700–706.

Azevedo, R.B.R., James, A.C., McCabe, J. & Partridge, L. 1998. Latitudinal variation of wing: thorax size ratio and wing-aspect ratio in *Drosophila melanogaster*. *Evolution* **52**: 1353–1362.

Bergland, A.O., Behrman, E.L., O'Brien, K.R., Schmidt, P.S. & Petrov, D.A. 2014. Genomic evidence of rapid and stable adaptive oscillations over seasonal time scales in *Drosophila*. *PLoS Genet.* **10**: e1004775.

Bergland, A.O., 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**: 1157–1174.

Blair, S.S. 2007. Wing vein patterning in *Drosophila* and the analysis of intercellular signaling. *Annu. Rev. Cell Dev. Biol.* **23**: 293–319.

Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R. & Hafen, E. 2001. An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. *Curr. Biol.* **11**: 213–221.

Calboli, F.C.F., Kennington, W.J. & Partridge, L. 2003. Qtl mapping reveals a striking coincidence in the positions of genomic regions associated with adaptive variation in body size in parallel clines of *Drosophila melanogaster* on different continents. *Evolution* **57**: 2653–2658.

Capy, P., Pla, E. & David, J.R. 1993. Phenotypic and genetic variability of morphometrical traits in natural populations of *Drosophila melanogaster* and *D. simulans*. I. Geographic variations. *Genet. Sel. Evol.* **25**: 517–536.

Charlesworth, B. & Charlesworth, D. 2010. *Elements of Evolutionary Genetics*. Roberts and Company Publishers, Greenwood Village.

Cohen, J. 1988. *Statistical Power Analysis for the Behavioral Sciences*. Lawrence Erlbaum Associates, Hillsdale.

Corbett-Detig, R.B., Cardeno, C. & Langley, C.H. 2012. Sequence-based detection and breakpoint assembly of polymorphic inversions. *Genetics* **192**: 131–137.

Coyne, J.A. & Beecham, E. 1987. Heritability of two morphological characters within and among natural populations of *Drosophila melanogaster*. *Genetics* **117**: 727–737.

Das, A. & Singh, B.N. 1991. Genetic differentiation and inversion clines in Indian natural populations of *Drosophila melanogaster*. *Genome* **34**: 618–625.

David, J.R. & Capy, P. 1988. Genetic variation of *Drosophila melanogaster* natural populations. *Trends Genet.* **4**: 106–111.

David, J.R., Capy, P., Payant, V. & Tsakas, S. 1985. Thoracic trident pigmentation in *Drosophila melanogaster*: differentiation of geographical populations. *Genet. Sel. Evol.* **17**: 211–224.

Debat, V., Bloyer, S., Faradji, F., Gidaszewski, N., Navarro, N., Orozco-Terwengel, P. *et al.* 2011. Developmental stability: a major role for *cyclin G* in *Drosophila melanogaster*. *PLoS Genet.* **7**: e1002314.

Dickinson, M.H., Lehmann, F.O. & Sane, S.P. 1999. Wing rotation and the aerodynamic basis of insect flight. *Science* **284**: 1954–1960.

Edgar, B. 2006. How flies get their size: genetics meets physiology. *Nat. Rev. Genet.* **7**: 907–916.

Endler, J.A. 1977. *Geographic Variation, Speciation, and Clines*. Princeton University Press, Princeton.

Fabian, D.K., Kapun, M., Nolte, V., Kofler, R., Schmidt, P.S., Schlötterer, C. *et al.* 2012. Genome-wide patterns of latitudinal differentiation among populations of *Drosophila melanogaster* from North America. *Mol. Ecol.* **21**: 4748–4769.

Fabian, D.K., Lack, J.B., Mathur, V., Schlötterer, C., Schmidt, P.S., Pool, J.E. *et al.* 2015. Spatially varying selection shapes life history clines among populations of *Drosophila melanogaster* from sub-Saharan Africa. *J. Evol. Biol.* **28**: 826–840.

Flatt, T. 2005. The evolutionary genetics of canalization. *Quart. Rev. Biol.* **80**: 287–316.

Flatt, T. 2016. Genomics of clinal variation in *Drosophila*: disentangling the interactions of selection and demography. *Mol. Ecol.* **25**: 1023–1026.

Gibert, P., Moreteau, B., Pétavy, G., Karan, D. & David, J.R. 2001. Chill-coma tolerance, a major climatic adaptation among *Drosophila* species. *Evolution* **55**: 1063–1068.

Gibert, P., Capy, P., Imasheva, A., Moreteau, B., Morin, J.P., Pétavy, G. *et al.* 2004. Comparative analysis of morphological traits among *Drosophila melanogaster* and *D. simulans*: genetic variability, clines and phenotypic plasticity. *Genetica* **120**: 165–179.

Gilchrist, A.S. & Partridge, L. 2001. The contrasting genetic architecture of wing size and shape in *Drosophila melanogaster*. *Heredity* **86**: 144–152.

Gilchrist, A.S., Azevedo, R.B.R., Partridge, L. & O'Higgins, P. 2000. Adaptation and constraint in the evolution of *Drosophila melanogaster* wing shape. *Evol. Dev.* **2**: 114–124.

Glinka, S., Stephan, W. & Das, A. 2005. Homogeneity of common cosmopolitan inversion frequencies in Southeast Asian *Drosophila melanogaster*. *J. Genet.* **84**: 173–178.

Gockel, J., Kennington, W.J., Hoffmann, A.A., Goldstein, D.B. & Partridge, L. 2001. Nonclinality of molecular variation implicates selection in maintaining a morphological cline of *Drosophila melanogaster*. *Genetics* **158**: 319–323.

Gockel, J., Robinson, S.J.W., Kennington, W.J., Goldstein, D.B. & Partridge, L. 2002. Quantitative genetic analysis of natural variation in body size in *Drosophila melanogaster*. *Heredity* **89**: 145–153.

Goenaga, J., Fanarar, J.J. & Hasson, E. 2013. Latitudinal variation in starvation resistance is explained by lipid content in natural populations of *Drosophila melanogaster*. *Evol. Biol.* **40**: 601–612.

Hoffmann, A.A. & Harshman, L.G. 1999. Desiccation and starvation resistance in *Drosophila*: patterns of variation at the species, population and intrapopulation levels. *Heredity* **83**: 637–643.

Hoffmann, A.A. & Parsons, P.A. 2009. An integrated approach to environmental stress tolerance and life-history variation: desiccation tolerance in *Drosophila*. *Biol. J. Linn. Soc.* **37**: 117–136.

Hoffmann, A.A. & Rieseberg, L.H. 2008. Revisiting the impact of inversions in evolution: from population genetic markers to drivers of adaptive shifts and speciation? *Annu. Rev. Ecol. Evol. Syst.* **39**: 21–42.

Hoffmann, A.A. & Sgrò, C.M. 2011. Climate change and evolutionary adaptation. *Nature* **470**: 479–485.

Hoffmann, A.A. & Shirriffs, J. 2002. Geographic variation for wing shape in *Drosophila serrata*. *Evolution* **56**: 1068–1073.

Hoffmann, A.A. & Weeks, A.R. 2007. Climatic selection on genes and traits after a 100 year-old invasion: a critical look at the temperate-tropical clines in Drosophila melanogaster from eastern Australia. *Genetica* **129**: 133–147.

Hoffmann, A.A., Anderson, A. & Hallas, R. 2002. Opposing clines for high and low temperature resistance in *Drosophila melanogaster*. *Ecol. Lett.* **5**: 614–618.

Hoffmann, A.A., Sgrò, C.M. & Weeks, A.R. 2004. Chromosomal inversion polymorphisms and adaptation. *Trends Ecol. Evol.* **19**: 482–488.

Imasheva, A.G., Bubli, O.A. & Lazebny, O.E. 1994. Variation in wing length in Eurasian natural populations of *Drosophila melanogaster*. *Heredity* **72**: 508–514.

Inoue, Y. & Watanabe, T.K. 1979. Inversion polymorphisms in Japanese natural populations of *D. melanogaster*. *Jpn. J. Genet.* **54**: 69–82.

James, A.C. & Partridge, L. 1995. Thermal evolution of rate of larval development in *Drosophila melanogaster* in laboratory and field populations. *J. Evol. Biol.* **8**: 315–330.

James, A.C., Azevedo, R.B.R. & Partridge, L. 1995. Cellular basis and developmental timing in a size cline of *Drosophila melanogaster*. *Genetics* **140**: 659–666.

James, A.C., Azevedo, R.B.R. & Partridge, L. 1997. Genetic and environmental responses to temperature of *Drosophila melanogaster* from a latitudinal cline. *Genetics* **146**: 881–890.

de Jong, G. & Bochdanovits, Z. 2003. Latitudinal clines in *Drosophila melanogaster*: body size, allozyme frequencies, inversion frequencies, and the insulin-signalling pathway. *J. Genet.* **82**: 207–223.

Kao, J.Y., Zubair, A., Salomon, M.P., Nuzhdin, S.V. & Campo, D. 2015. Population genomic analysis uncovers African and European admixture in *Drosophila melanogaster* populations from the south-eastern United States and Caribbean Islands. *Mol. Ecol.* **24**: 1499–1509.

Kapun, M., van Schalkwyk, H., McAllister, B., Flatt, T. & Schlötterer, C. 2014. Inference of chromosomal inversion dynamics from Pool-Seq data in natural and laboratory populations of *Drosophila melanogaster*. *Mol. Ecol.* **23**: 1813–1827.

Kapun, M., Fabian, D.K., Goudet, J. & Flatt, T. 2016. Genomic evidence for adaptive inversion clines in *Drosophila melanogaster*. *Mol. Biol. Evol.* doi: 10.1093/molbev/msw016.

Keller, A. 2007. *Drosophila melanogaster's* history as a human commensal. *Curr. Biol.* **17**: R77–R81.

Kennington, W.J., Hoffmann, A.A. & Partridge, L. 2007. Mapping regions within cosmopolitan inversion *In(3R)Payne* associated with natural variation in body size in *Drosophila melanogaster*. *Genetics* **177**: 549–556.

Klepsatel, P., Gáliková, M., Huber, C.D. & Flatt, T. 2014. Similarities and differences in altitudinal vs. latitudinal variation for morphological traits in *Drosophila melanogaster*. *Evolution* **68**: 1385–1398.

Knibb, W.R. 1982. Chromosome inversion polymorphisms in *Drosophila melanogaster* II. Geographic clines and climatic associations in Australasia, North America and Asia. *Genetica* **58**: 213–221.

Knibb, W.R., Oakeshott, J.G. & Gibson, J.B. 1981. Chromosome Inversion Polymorphisms in *Drosophila melanogaster*. I. Latitudinal Clines and Associations between Inversions in Australasian Populations. *Genetics* **98**: 833–847.

Kolaczkowski, B., Kern, A.D., Holloway, A.K. & Begun, D.J. 2011. Genomic differentiation between temperate and tropical Australian populations of *Drosophila melanogaster*. *Genetics* **187**: 245–260.

van't Land, J., Van Putten, P., Zwaan, B., Kamping, A. & van Delden, W. 1999. Latitudinal variation in wild populations of *Drosophila melanogaster*: heritabilities and reaction norms. *J. Evol. Biol.*, **12**: 222–232.

Lee, S.F., Chen, Y., Varan, A.K., Wee, C.W., Rako, L., Axford, J.K. *et al.* 2011. Molecular basis of adaptive shift in body size in *Drosophila melanogaster*: functional and sequence analyses of the *Dca* gene. *Mol. Biol. Evol.* **28**: 2393–2402.

Lemeunier, F. & Aulard, S. 1992. Inversion polymorphism in *Drosophila melanogaster*. In: *Drosophila Inversion Polymorphism* (C.B. Krimbas & J.R. Powell, eds), pp. 339–405. CRC Press, Boca Raton.

van der Linde, K. & Houle, D. 2009. Inferring the nature of allometry from geometric data. *Evol. Biol.* **36**: 311–322.

Luckinbill, L.S., Reddy, S., Dudekonda, V. & Curtsinger, J.W. 2005. Analysis of two components of flight using recombinant inbred lines of *Drosophila melanogaster*. *Genetica* **124**: 235–245.

Márquez, E.J., Cabeen, R., Woods, R.P. & Houle, D. 2012. The measurement of local variation in shape. *Evol. Biol.* **39**: 419–439.

Matamoro-Vidal, A., Salazar-Ciudad, I. & Houle, D. 2015. Making quantitative morphological variation from basic developmental processes: where are we? The case of the *Drosophila* wing. *Dev. Dyn.* **244**: 1058–1073.

Matzkin, L.M., Merritt, T.J.S., Zhu, C.-T. & Eanes, W.F. 2005. The structure and population genetics of the breakpoints associated with the cosmopolitan chromosomal inversion *In(3R)Payne* in *Drosophila melanogaster*. *Genetics* **170**: 1143–1152.

Mayr, E. 1963. *Animal Species and Evolution*. The Belknap Press of Harvard University Press, Cambridge, Mass.

McColl, G., Hoffmann, A.A. & McKechnie, S.W. 1996. Response of two heat shock genes to selection for knockdown heat resistance in *Drosophila melanogaster*. *Genetics* **143**: 1615–1627.

McGowan, M.W., Artiss, J.D., Strandbergh, D.R. & Zak, B. 1983. A peroxidase-coupled method for the colorimetric determination of serum triglycerides. *Clin. Chem.* **29**: 538–542.

McKechnie, S.W., Blacket, M.J., Song, S.V., Rako, L., Carroll, X., Johnson, T.K. *et al.* 2010. A clinally varying promoter polymorphism associated with adaptive variation in wing size in *Drosophila*. *Mol. Ecol.* **19**: 775–784.

Mettler, L.E., Voelker, R.A. & Mukai, T. 1977. Inversion clines in populations of *Drosophila melanogaster*. *Genetics* **87**: 169–176.

Mirth, C.K. & Shingleton, A.W. 2012. Integrating body and organ size in drosophila: recent advances and outstanding problems. *Front. Endocrin.* **3**: 1–13.

Munjal, A.K., Karan, D., Gibert, P., Moreteau, B., Parkash, R. & David, J.R. 1997. Thoracic trident pigmentation in *Drosophila melanogaster*: latitudinal and altitudinal clines in Indian populations. *Genet. Sel. Evol.* **29**: 601–610.

Nässel, D.R., Liu, Y. & Luo, J. 2015. Insulin/IGF signaling and its regulation in *Drosophila*. *Gen. Comp. Endocrinol.* **221**: 255–266.

Oldham, S. & Hafen, E. 2003. Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control. *Trends Cell Biol.* **13**: 79–85.

Paaby, A.B. & Schmidt, P.S. 2008. Functional significance of allelic variation at *methuselah*, an aging gene in *Drosophila*. *PLoS ONE* **3**: e1987.

Paaby, A.B., Bergland, A.O., Behrman, E.L. & Schmidt, P.S. 2014. A highly pleiotropic amino acid polymorphism in the Drosophila insulin receptor contributes to life-history adaptation. *Evolution* **68**: 3395–3409.

Partridge, L. & Coyne, J.A. 1997. Bergmann's rule in ectotherms: is it adaptive? *Evolution* **51**: 632–635.

Partridge, L., Barrie, B., Fowler, K. & French, V. 1994. Evolution and development of body size and cell size in *Drosophila melanogaster* in response to temperature. *Evolution* **48**: 1269–1276.

Rako, L., Anderson, A.R., Sgrò, C.M., Stocker, A.J. & Hoffmann, A.A. 2006. The association between inversion *In(3R) Payne* and clinally varying traits in *Drosophila melanogaster*. *Genetica* **128**: 373–384.

Rane, R.V., Rako, L., Kapun, M., Lee, S.F. & Hoffmann, A.A. 2015. Genomic evidence for role of inversion *3RP* of *Drosophila melanogaster* in facilitating climate change adaptation. *Mol. Ecol.* **24**: 2423–2432.

Reinhardt, J.A., Kolaczkowski, B., Jones, C.D., Begun, D.J. & Kern, A.D. 2014. Parallel geographic variation in *Drosophila melanogaster*. *Genetics* **197**: 361–373.

Robinson, S.J.W., Zwaan, B. & Partridge, L. 2000. Starvation resistance and adult body composition in a latitudinal cline of *Drosophila melanogaster*. *Evolution* **54**: 1819–1824.

Rohatgi, A. 2015. WebPlotDigitizer. http://arohatgi.info/WebPlotDigitizer.

Savolainen, O., Lascoux, M. & Merilä, J. 2013. Ecological genomics of local adaptation. *Nat. Rev. Genet.* **14**: 807–820.

Schmidt, P.S. & Paaby, A.B. 2008. Reproductive diapause and life-history clines in north American populations of *Drosophila melanogaster*. *Evolution* **62**: 1204–1215.

Schmidt, P.S., Matzkin, L., Ippolito, M. & Eanes, W.F. 2005. Geographic variation in diapause incidence, life-history traits, and climatic adaptation in *Drosophila melanogaster*. *Evolution* **59**: 1721–1732.

Schmidt, P.S., Zhu, C.T., Das, J., Batavia, M., Yang, L. & Eanes, W.F. 2008. An amino acid polymorphism in the *couch potato* gene forms the basis for climatic adaptation in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **105**: 16207–16211.

Shingleton, A.W., Frankino, W.A., Flatt, T., Nijhout, H.F. & Emlen, D.J. 2007. Size and shape: the developmental regulation of static allometry in insects. *BioEssays* **29**: 536–548.

Sperlich, D. & Pfriem, P. 1986. Chromosomal polymorphism in natural and experimental populations. In: *The Genetics and Biology of Drosophila* (M. Ashburner, H.L. Carson & J.R. Thompson, eds), Volume **3e**, pp. 257–309. Academic Press, New York.

Stalker, H.D. 1980. Chromosome studies in wild populations of *Drosophila melanogaster*. II. Relationship of inversion frequencies to latitude, season. Wing-loading and flight activity. *Genetics* **95**: 211–223.

Telonis-Scott, M., Hoffmann, A.A. & Sgrò, C.M. 2011. The molecular genetics of clinal variation: a case study of ebony and thoracic trident pigmentation in *Drosophila melanogaster* from eastern Australia. *Mol. Ecol.* **20**: 2100–2110.

Tennessen, J.M., Barry, W.E., Cox, J. & Thummel, C.S. 2014. Methods for studying metabolism in *Drosophila*. *Methods* **68**: 105–115.

Therneau, T. 2012. Package "coxme": Mixed effects Cox models. R package version 2.2–5. Available at: https://cran.r-project.org/web/packages/coxme/coxme.pdf

Tsuneizumi, K., Nakayama, T., Kamoshida, Y., Kornberg, T.B., Christian, J.L. & Tabata, T. 1997. *Daughters against dpp* modulates *dpp* organizing activity in *Drosophila* wing development. *Nature* **389**: 627–631.

Umina, P.A., Weeks, A.R., Kearney, M.R., McKechnie, S.W. & Hoffmann, A.A. 2005. A rapid shift in a classic clinal pattern in *Drosophila* reflecting climate change. *Science* **308**: 691–693.

Weeks, A.R., McKechnie, S.W. & Hoffmann, A.A. 2002. Dissecting adaptive clinal variation: markers, inversions and size/stress associations in *Drosophila melanogaster* from a central field population. *Ecol. Lett.* **5**: 756–763.

Zwaan, B.J., Azevedo, R., James, A.C. & Land, J.V. 2000. Cellular basis of wing size variation in *Drosophila melanogaster*: a comparison of latitudinal clines on two continents. *Heredity* **84**: 338.

Supporting information

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

Figure S1 Crossing scheme for isolating isochromosomal *3R* lines.
Figure S2 Details of wing morphology.
Figure S3 Effects of *In(3R)P* on allometry.
Table S1 MANOVA of multivariate phenotype based on line averages of all measured traits (except wing shape and size ratios).
Table S2 Cohen's standardized effect sizes *d* for the differential effects of the two *In(3R)P* karyotypes (inverted vs. standard arrangement) on wing size for Queensland (Australia; data from Rako *et al.*, 2006) and Florida (our study), calculated based on line means and standard deviations.
Table S3 MANOVA of multivariate size phenotype (i.e., a linear combination of wing area, femur length and tibia length).
Table S4 MANOVA of multivariate wing shape, based on Jacobian determinants of 122 (females) and 124 (males) pseudo-landmarks.

Data deposited at Dryad: doi: 10.5061/dryad.8ns67

Received 12 January 2016; revised 11 February 2016; accepted 12 February 2016

Supporting Information

Supporting Figure Legends

Figure S1. Crossing scheme for isolating isochromosomal *3R* lines. Wild-type males were crossed to females of a double balancer stock (*SMB6; TM6B*), marked with a dominant *tubby* (Tb^1) and a recessive *ebony* (e^1) mutation. F1 pupae exhibiting *Tb* were selected and backcrossed to the balancer. In the next generation, pupae showing *Tb* but not the *ebony* phenotype were selected and allowed to interbreed. Finally, wild-type pupae were selected to clear the balancer chromosome, resulting in isochromosomal *3R* homokaryon isolates. See Materials and Methods section for further details.

Figure S2. Details of wing morphology. Designations and locations of wing cells in red (A: Anal cell, Al: Alula, Ax: Axillary cell, B1: Basal cell 1, B2: Basal cell 2, C: Costal cell, D: Distal cell, M: marginal cell, 1P: 1st posterior cell, 2P: 2nd posterior cell, 3P: 3rd posterior cell, S: Submarginal cell) and wing veins in white (a-cv: Anterior cross-vein, L1: Vein L1, L2: Radial vein, L3: Medial vein, L4: Cubital vein, L5: Distal vein, L6: Vein L6, p-cv: Posterior cross-vein), following the nomenclature of Chyb & Gompel (2013). Blue arrows indicate landmarks used for fitting spline functions with Wings4. See Materials and Methods section for further details.

Figure S3. Effects of *In(3R)P* on allometry. Size ratios of femur/tibia, femur/wing and wing/tibia, averaged across line means for the groups differing in *In(3R)P* karyotype: "Florida inverted" (FI), "Florida standard" (FS) and "Maine standard" (MS). Error bars show standard errors. Letters above bars indicate differences according to Tukey HSD post-hoc tests, carried out for each sex separately: groups not containing the same letters are significantly different ($p<0.05$). See Materials and Methods and Results section for further details.

Table S1. MANOVA of multivariate phenotype based on line averages of all measured traits (except wing shape and size ratios). Contrasts between the three groups (FI versus FS; FI versus MS; FS versus MS) revealed significant differences, indicating that both karyotype and geography have an effect on multivariate phenotype. ** $p < 0.01$; *** $p < 0.001$.

Factors	**Wilk's λ**	***F* ratio**
group (*g*)	0.39	$F_{16,84}$= 3.13**
sex (*s*)	-	$F_{8,42}$= 54.3***
$g \times s$	0.82	$F_{16,84}$= 0.58

Table S2. Cohen's standardized effect sizes *d* for the differential effects of the two *In(3R)P* karyotypes (inverted versus standard arrangement) on wing size for Queensland (Australia; data from Rako *et al*., 2006) and Florida (our study), calculated based on line means and standard deviations. See the Materials and Methods and Results sections for further details.

Australia	**Mean**	**SD**
Queensland inverted	2.72	0.03
Queensland standard	2.77	0.02
Cohen's *d*	**1.65**	

North America	**Mean**	**SD**
Florida inverted (FI)	1.71	0.06
Florida standard (FS)	1.82	0.06
Cohen's *d*	**1.74**	

Table S3. MANOVA of multivariate size phenotype (i.e., a linear combination of wing area, femur length and tibia length). Contrasts between the three groups (FI versus FS; FI versus MS; FS versus MS) revealed significant differences, indicating that both karyotype and geography have an effect on multivariate wing shape phenotype. *** $p < 0.001$.

Factors	**Wilk's λ**	***F* ratio**
group	0.509	$F_{6,96}$= 6.42***
sex		$F_{3,48}$= 142.9***
group x sex	0.96	$F_{6,96}$=0.36

Table S4. MANOVA of multivariate wing shape, based on Jacobian determinants of 122 (females) and 124 (males) pseudo-landmarks. Contrasts between the three groups (FI versus FS; FI versus MS; FS versus MS) revealed significant differences, indicating that both karyotype and geography have an effect on multivariate wing shape phenotype. *** $p <$ 0.001.

Sex	**Factors**	**Wilk's λ**	***F* ratio**
Female	group (g)	0.23	$F_{244,832}$= 3.67***
	line(group) ($l(g)$)	1.9 x 10^{-8}	$F_{3538,12059}$= 3.1***
Male	group (g)	0.23	$F_{248,760}$= 3.26***
	line(group) ($l(g)$)	3.9 x 10^{-8}	$F_{3472,10680}$= 2.67***

Figure S1

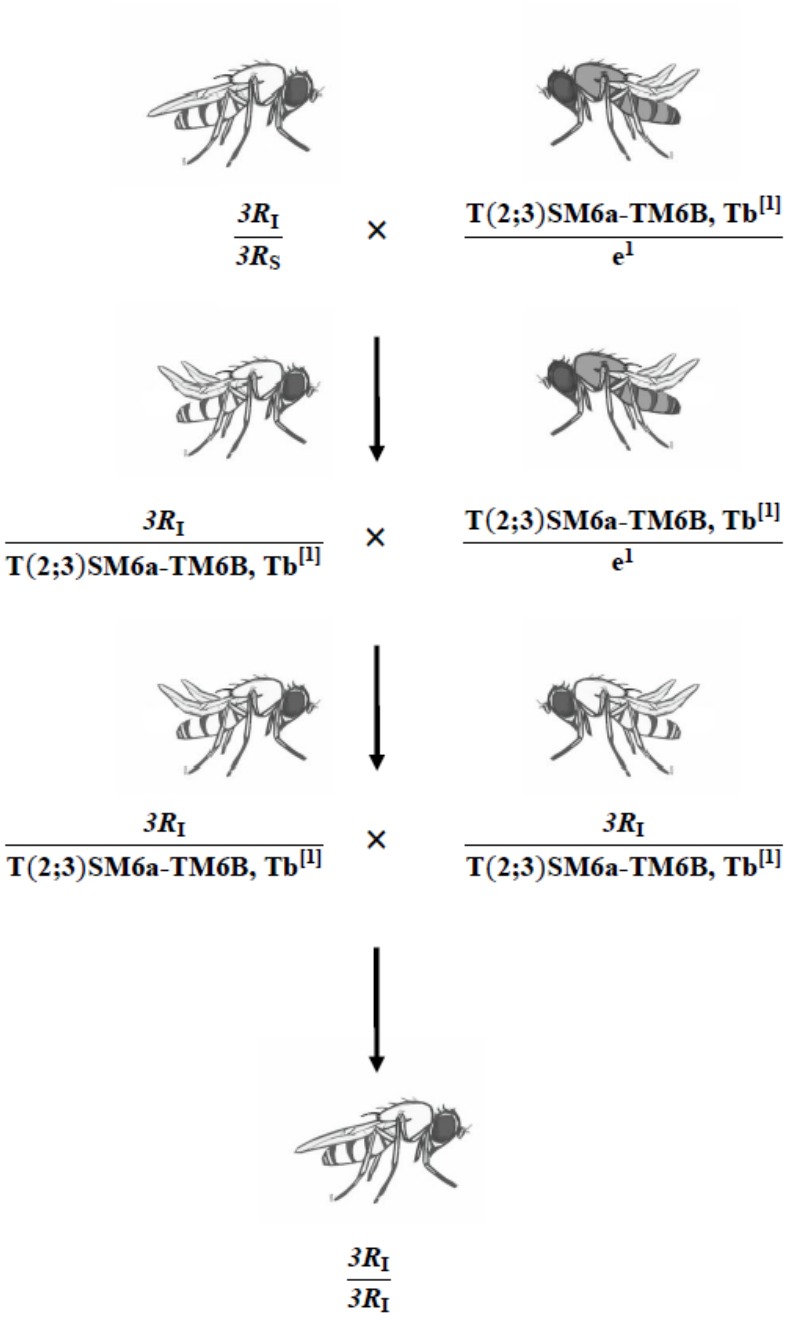

3R_I / 3R_S × T(2;3)SM6a-TM6B, Tb[1] / e1
3R_I / T(2;3)SM6a-TM6B, Tb[1] × T(2;3)SM6a-TM6B, Tb[1] / e1
3R_I / T(2;3)SM6a-TM6B, Tb[1] × 3R_I / T(2;3)SM6a-TM6B, Tb[1]
3R_I / 3R_I

Figure S2

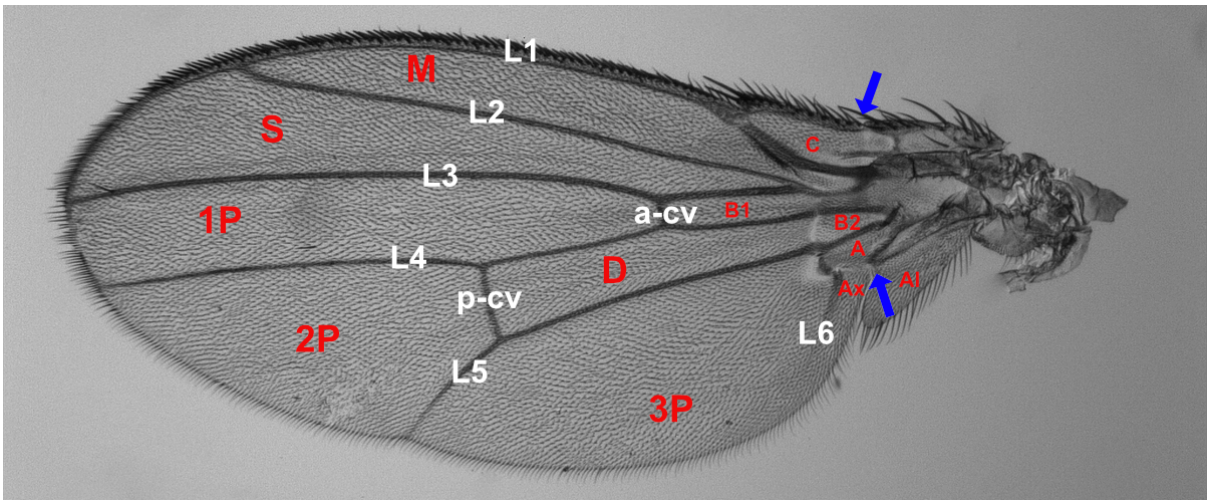
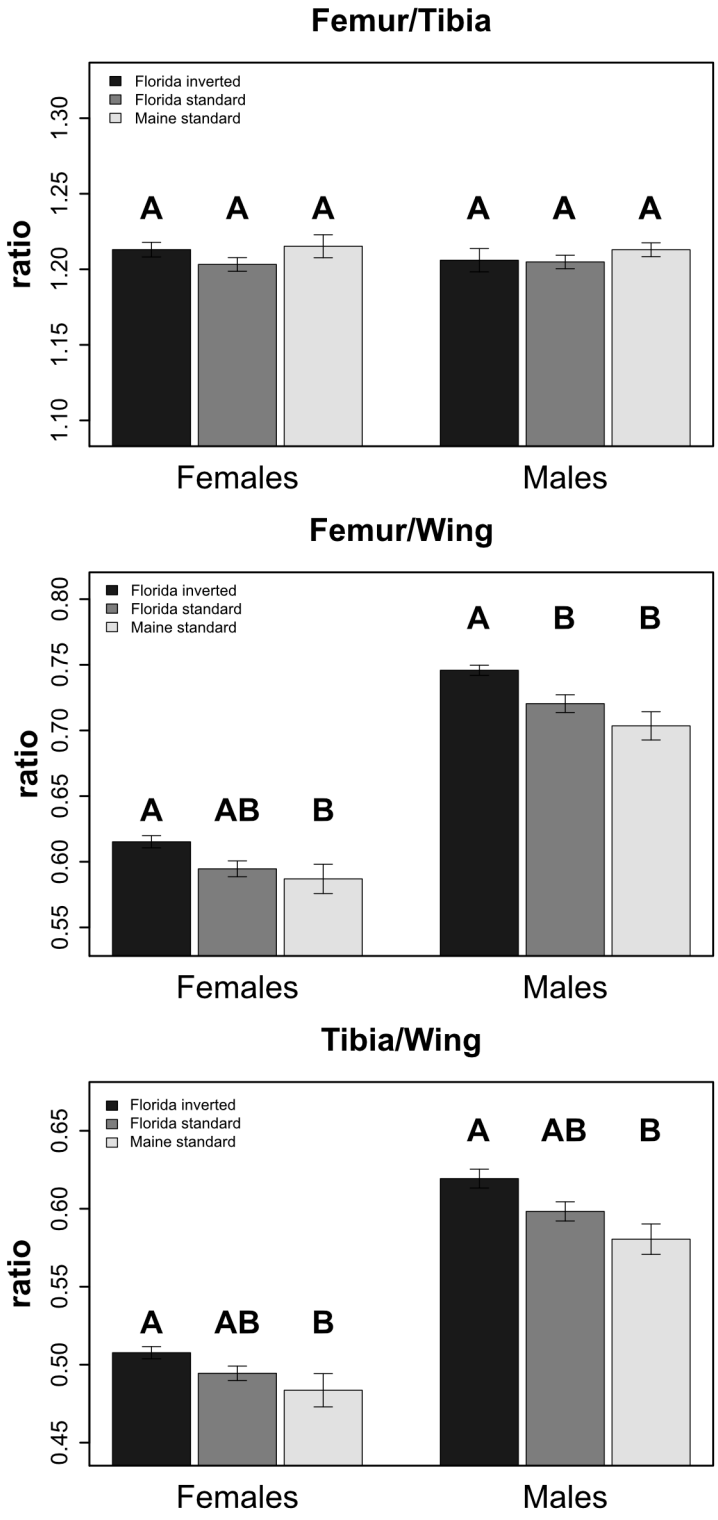


Figure S3


Femur/Tibia
Florida inverted
Florida standard
Maine standard
ratio
1.10
1.15
1.20
1.25
1.30
A A A A A A
Females
Males
Femur/Wing
Florida inverted
Florida standard
Maine standard
ratio
0.55
0.60
0.65
0.70
0.75
0.80
A AB B A B B
Females
Males
Tibia/Wing
Florida inverted
Florida standard
Maine standard
ratio
0.45
0.50
0.55
0.60
0.65
A AB B A AB B
Females
Males

References

Chyb, S. & Gompel, N. 2013. *The Atlas of Drosophila Morphology. Wild-type and Classical Mutants.* 1st edition. Academic Press, London.

Cohen, J. 1988. *Statistical power analysis for the behavioral sciences*. Lawrence Erlbaum Associates, Hillsdale.

Rako, L., Anderson, A.R., Sgrò, C.M., Stocker, A.J. & Hoffmann, A.A. 2006. The association between inversion *In(3R)Payne* and clinally varying traits in *Drosophila melanogaster*. *Genetica* **128**: 373-384.

Chapter 6

[Manuscript draft to be submitted to Journal of Evolutionary Biology]

A chromosomal inversion polymorphism underpins latitudinal clines in survival traits

Contributions by E. Durmaz: experimental design, assays, statistical analysis, interpretation of results, writing of the manuscript.

A Chromosomal Inversion Polymorphism Underpins Latitudinal Clines in Survival Traits

ESRA DURMAZ[1], CLARE BENSON[1,2], MARTIN KAPUN[1], PAUL SCHMIDT[3] & THOMAS FLATT[1,*]

[1]*Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland*

[2]*School of Biological Sciences, University of Manchester, M13 9PL, Manchester, United Kingdom*

[3]*Department of Biology, University of Pennsylvania, Philadelphia, USA*

[*]*Correspondence*: Thomas Flatt, Department of Ecology and Evolution, University of Lausanne, CH-1015 Lausanne, Switzerland. Tel.: +41 21 692 4203; Fax.: +41 21 692 4165; e-mail: thomas.flatt@unil.ch

Abstract

In many organisms, chromosomal inversions contribute to local adaptation across clinal (e.g., latitudinal) gradients, but how they affect phenotypes – especially fitness-related traits – is poorly understood. We and others have previously shown that a clinally varying inversion polymorphism in *Drosophila melanogaster*, *In(3R)Payne*, underpins body size clines along the North American and Australian east coasts but failed to find effects on other traits. Here, we examine whether across the North American cline the *In(3R)P* polymorphism contributes to clinal variation in survival traits (lifespan, survival upon starvation, and survival upon cold shock). To do so, we used homokaryon lines, either carrying the inverted or the uninverted chromosomal arrangement, isolated from populations that approximate the endpoints of the North American cline (Florida, Maine) and phenotyped flies at two growth temperatures (18°C, 25°C). Across both temperatures, and consistent with clinal expectations, high-latitude flies from Maine overall lived longer and were more stress resistant than low-latitude flies from Florida. Interestingly, the latitudinal clinality of survival traits was – at least partly – explained by the clinal distribution of *In(3R)P*: karyotypes carrying the inverted segment tended to be shorter-lived and less stress resistant than karyotypes with the uninverted arrangement. Moreover, survival traits were affected by karyotype by temperature interactions. We conclude that *In(3R)P* – beyond its effects on body size – contributes to latitudinal clines in survival traits. Yet, given that *In(3R)P* has a mostly negative impact on fitness components, it remains unclear how spatially varying selection maintains this clinal polymorphism.

Keywords: Inversions, clines, adaptation, temperature, survival, *D. melanogaster.*

Introduction

Since the pioneering work of Dobzhansky, many lines of evidence suggest that chromosomal inversion polymorphisms play a major role in climatic adaptation to altitudinal and latitudinal gradients, so-called clines (e.g., Dobzhansky, 1937; 1943; 1947a, b; Wright & Dobzhansky, 1946; Leumeunier & Aulard, 1992; Hoffmann *et al*., 2004; Kirkpatrick & Barton, 2006; Hoffmann & Rieseberg, 2008; Schaeffer, 2008; Kirkpatrick & Kern, 2012; Kapun *et al.*, 2016a; and references therein). However, while inversions have been statistically associated with many traits (e.g., Sperlich & Pfriem, 1986; Etges, 1989; De Jong & Bochdanovits, 2003; Hoffmann *et al*., 2004; Lowry & Willis, 2010), still little is known about associations between inversions and fitness-related traits, thus limiting our understanding of how these adaptive polymorphisms are maintained by selection (e.g., Hoffmann & Rieseberg, 2008).

The commonly observed latitudinal frequency clines of inversion polymorphisms in the *Drosophila melanogaster* system, often observed in a parallel fashion on multiple continents, provide an excellent opportunity to address this problem (e.g., Mettler *et al.*, 1977; Knibb *et al.*, 1981; Knibb, 1982; Leumeunier & Aulard, 1992; De Jong & Bochdanovits, 2003; Hoffmann & Weeks, 2007). For example, a large (~8 Mb), cosmopolitan inversion polymorphism on the right arm of the third chromosome, *In(3R)Payne* (also called *In(3R)P*), varies clinally along latitudinal gradients on several continents, most prominently along the Australian and North American east coasts (e.g., Mettler *et al.*, 1977; Inoue & Watanabe, 1979; Stalker, 1980; Knibb *et al.*, 1981; Knibb, 1982; Das & Singh, 1991; Anderson *et al.* 2005; Matzkin *et al.*, 2005; Fabian *et al.*, 2012; Kapun *et al.*, 2014; Rane *et al.*, 2015; Kapun *et al.*, 2016a). On all continents or subcontinents examined so far, the inverted karyotype of *In(3R)P* exhibits intermediate-to-high frequencies at low latitudes (i.e., in subtropical to tropical climates) but is rare or absent at high latitudes (i.e., in temperate, seasonal climates) (see references above). Along the North American east coast, for example, the inverted

arrangement has a frequency of ~50% in Florida but its frequency decreases along the cline to ~0% in Maine (e.g., Mettler *et al.*, 1977; Knibb, 1982; Fabian *et al*., 2012; Kapun *et al.*, 2014, 2016a). Recent population genetic evidence suggests that the North American cline in *In(3R)P* is adaptively maintained by spatially varying (clinal) selection, independent of admixture or population structure (Kapun *et al.*, 2016a).

Interestingly, several major fitness-related traits exhibit similar clinal gradients across latitude (e.g., De Jong & Bochdanovits, 2003; Hoffmann & Weeks, 2007; Adrion *et al*., 2015; and references therein), and it is thus tempting to speculate that the clinal behavior of *In(3R)P* (or that of other clinally varying inversions) might causally underlie – or contribute to – these life-history clines. For example, as compared to flies from low latitude, high-latitude flies tend to be characterized by increased body size, reduced wing loading, reduced fecundity, prolonged lifespan, increased resistance to starvation, cold and heat stress, and the plastic ability to undergo reproductive dormancy in response to cool temperature and short photoperiod (e.g., Coyne & Beecham, 1987; Azevedo *et al*., 1998; de Jong & Bochdanovits, 2003; Hoffmann *et al*., 2005; Schmidt *et al*., 2005a, b; Schmidt & Paaby, 2008; Fabian *et al*., 2015; Mathur & Schmidt, 2017). Yet, whether clinally varying inversion polymorphisms contribute to these phenotypic clines is largely unclear (e.g., De Jong & Bochdanovits, 2003; Rako *et al.*, 2006; Hoffmann *et al*., 2004; Hoffmann & Weeks, 2007; Hoffmann & Rieseberg, 2008; Kapun *et al.*, 2016a, b; and discussion therein).

Consistent with a contribution of *In(3R)P* to clinal trait differentiation, we and others have previously found that the latitudinal cline in this inversion explains – at least partly – the body size cline along the Australian (Weeks *et al.*, 2002; Rako *et al.*, 2006; Kennington *et al.*, 2007) and North American (Kapun *et al.*, 2016b) east coasts. Yet, little is known about whether *In(3R)P* also affects other traits. In Australian populations, for example, Anderson *et al.* (2003) found an association between susceptibility to cold and *In(3R)P*, but a subsequent

study by Rako *et al.* (2006) failed to find a clear effect. Similarly, for the North American cline, we also failed to detect an association between *In(3R)P* and chill coma recovery (Kapun *et al.*, 2016b). Moreover, neither study found an effect of *In(3R)P* on developmental time (Rako *et al.*, 2006; Kapun *et al.*, 2016b). Thus, whether *In(3R)P* contributes to clinal variation in fitness-related traits other than body size is not known. Although it is possible that *In(3R)P* predominantly – or exclusively – affects body size and not any other traits (cf. Kapun *et al.*, 2016b), this seems rather unlikely, for two reasons: (1) the majority of significantly clinally varying SNPs in the genome reside in the region spanned by this inversion, and (2) many of these clinal SNPs within *In(3R)P* are located in genes that are known from studies of mutants and transgenes to affect life-history traits (Kapun *et al*., 2016a; also see Fabian *et al*., 2012).

Here, we examine whether the clinal *In(3R)P* polymorphism affects three major survival traits in North American populations of *D. melanogaster*: adult lifespan, survival upon starvation, and cold resistance (measured as survival upon cold shock). All three traits are known to vary clinally in North America as a function of latitude and/or high-latitude vs. low-latitude genotypes (e.g., Schmidt *et al*., 2000; Schmidt *et al*., 2005a, b; Schmidt & Paaby, 2008; Paaby *et al*., 2014; Mathur & Schmidt, 2017). In support of previous results, we find that high-latitude flies from Maine overall live longer and are more stress resistant than low-latitude flies from Florida, thus confirming the idea that selection at high latitude favors genotypes and phenotypes with improved survival and somatic maintenance (Paaby & Schmidt, 2009; Flatt *et al*., 2013; Paaby *et al*., 2014). Interestingly, we find that the clines in these traits are, at least partly, driven by the clinal frequency gradient in *In(3R)P*: on average flies carrying the *In(3R)P* inversion from Florida live shorter and are less stress resistant than flies from Florida or Maine which possess the uninverted chromosomal segment.

Materials and methods

Fly stocks and maintenance

We isolated third-chromosome isochromosomal (homokaryon) lines, either carrying two copies of the inverted *In(3R)P* arrangement or two copies of the uninverted (standard) arrangement, from two areas approximating the endpoints of the North American cline (low latitude: Florida [Homestead and Jacksonville]; and high latitude: Maine [Bowdoin]), as previously described (see Kapun *et al*., 2016b for details). Across the cline *In(3R)P* has a frequency of ~50% in Florida but is absent in Maine, so that flies from high-latitude populations are fixed for the uninverted arrangement (e.g., Mettler *et al.*, 1977; Knibb, 1982; Fabian *et al*., 2012; Kapun *et al.*, 2014, 2016a, b). Wild-type chromosomes were isogenized using a compound balancer for the second and third chromosomes (*SM6b*; *TM6B*; Bloomington *Drosophila* Stock Center [BDSC] stock #5687) in an *ebony* (e^1) mutant background (cf. Kapun *et al*., 2016b for details). From Florida, where both the inverted and uninverted segments segregate, we isolated 9 isochromosomal lines carrying *In(3R)P* ('Florida inverted', FI) and 9 lines possessing the standard arrangement ('Florida standard', FS); from Maine, where the inverted segment is absent, we isolated 9 lines with the standard arrangement only ('Maine standard', MS). Prior to phenotyping assays, which were performed at two growth temperatures (see below), lines were kept under common garden conditions for three generations (~21°C, 10h:14h light:dark [LD], ~50% relative air humidity [RH]).

Phenotype assays

We measured three survival traits on the homokaryon lines: lifespan, survival upon starvation, and survival upon cold shock (see below). Assays were performed at two growth temperatures (18°C or 25°C), at 12:12h LD and 60% RH, on a cornmeal/sugar/yeast/agar diet. To obtain

(F1) flies for phenotypic assays, we let ~20-25 females and males mate and lay eggs into vials containing 8 mL of medium at room temperature (n = 46 vials for each of the 27 lines [= 3 karyotypes · 9 lines]; total = 1242 vials). Depending on their fecundity, females in each vial were allowed to lay eggs for up to ~24 hours; egg density was inspected regularly by eye and adjusted to ~40-50 eggs per vial. Vials were then transferred to their respective developmental temperature treatment (i.e., 18°C vs. 25°C; 23 replicate vials per temperature and isochromosomal line).

For lifespan assays, we collected cohorts of newly eclosed adult females and males within a 24-h period. Flies were sexed and counted under light CO_2 anesthesia and transferred to demography cages (see Tatar *et al*., 2001 for details of cage design) 24 hours after eclosion. Each cage was initiated with 75 females and 75 males. We set up 2 replicate cages per line and temperature (n = 2 cages · 27 lines · 2 temperatures = 108 cages; 108 cages · 150 flies = 16,200 flies in total). Every second day at 25°C and every third day at 18°C, we changed food vials and removed and recorded dead flies until all flies in the experiment had died. Flies that got stuck to the food medium were censored from analysis.

For assays of survival upon starvation, we used 5-7 day-old mated individuals. Eclosing adults were collected in 48-hour cohorts and maintained in mixed-sex groups for 4 days in their respective thermal treatments. 24 hours prior to initiating the assay, flies were sexed under light CO_2 anesthesia and transferred to fresh vials containing 10 individuals per vial and sex. On the day of the experiment, flies were transferred to food-free vials, containing 0.5% agar/water solution. For each line, temperature and sex, we used 5 replicate vials (n = 5 vials · 27 lines · 2 temperatures · 2 sexes = 540 vials, each with 10 flies; total = 5400 flies). Age at death was scored in 8-hour intervals until all flies had died.

We used an identical experimental design for measuring survival upon cold shock; again, we used 5 replicate vials per group (n = 5 vials · 27 lines · 2 temperatures · 2 sexes = 540

vials, each with 10 flies; total = 5400 flies). On the day of the experiment, 5-7 day-old mated flies were transferred to media-free vials and the vials dipped immediately into -4°C cold, salted ice water for 90 minutes. (Depending on acclimation, several hours of exposure to temperatures between -2ºC and -5ºC typically result in >50% mortality; cf. Hoffmann, 2010.) After cold shock, flies were transferred to petri dishes (60 · 15 mm) with 2 mL fly food in one corner and left at room temperature for recovery. Survival was scored after 24 hours; flies that were alive after 24 hours were censored from analysis.

Statistical analysis

The primary interest of our analysis was to determine the effects of *In(3R)P* karyotype (inverted vs. uninverted arrangement) upon survival traits; effects of clinality / geography (i.e., Florida vs. Maine) were of secondary interest. However, the biology of this system is such that the effects of karyotype vs. geography cannot be completely disentangled: since *In(3R)P* is polymorphic (with a ~50:50% frequency of inverted vs. standard arrangement) in Florida but not in Maine, where only the uninverted arrangement is present (i.e., the frequency of the inversion is ~0%), one cannot use a fully factorial, orthogonal design to analyze data for this inversion polymorphism. Nonetheless, significant differences between FI and FS and between FI and MS, with no difference between FS and MS, imply a clear main effect of *In(3R)P* karyotype. On the other hand, a situation in which all three pairwise comparisons (FI vs. FS; FI vs. MS; FS vs. MS) are different implies that inverted vs. standard arrangements differ in their effects, yet that the two uninverted arrangement types from Florida and Maine differ too, perhaps due to an effect of geography (Florida vs. Maine). In this scenario, it is not possible to clearly separate the effects of karyotype vs. geography; nevertheless, the significant difference between FI and FS indicates an effect of *In(3R)P* karyotype. In both cases, it seems thus safe to infer that *In(3R)P* karyotype affects the trait of

interest. Lastly, a scenario in which FI = FS but where both FI and FS are significantly different from MS might imply – assuming parsimony – a main effect of geography / clinal differentiation independent of *In(3R)P* karyotype.

Due to the constraint that the effects of karyotype and geography must be analyzed jointly, we created a compound grouping factor *K* ('Karyotype', partly confounded by geography) with three levels ('Florida inverted', FI; 'Florida standard', FS; and 'Maine standard', MS) and used pairwise comparisons between the levels of *K* in order to infer the effects of karyotype, geography or both (see below). The second factor that entered our analyses was temperature *T* (18°C vs. 25°C). We analyzed our survival (mortality) data in two ways. First, we used Cox (proportional hazards) regression to fit the fixed effects of *K*, *T* and the interaction $K \cdot T$. This 'global' approach gave us main effects for *K* (averaged across temperatures) and *T* (averaged across levels of *K*) and indicated whether – importantly – there are significant $K \cdot T$ (i.e., genotype by environment) interactions. Second, to dissect the source of significance of the effects of *K* and/or $K \cdot T$, we employed Kaplan-Meier survival analysis with generalized Wilcoxon (χ^2) tests to perform pairwise comparisons (i.e., FI vs. FS; FI vs. MS; FS vs. MS) for each temperature and sex separately, followed by Bonferroni correction for multiple testing. These pairwise comparisons thus serve as post-hoc tests for the Cox models. Analyses were performed in JMP v.11.2.0 (SAS, Raleigh, NC, USA).

Results

In(3R)P shortens lifespan; high-latitude flies live longer than low-latitude flies

We first analyzed the effects of 'Karyotype' and temperature and their interaction on lifespan (Fig. 1, Fig. S1). For both females and males, Cox regression revealed effects of 'Karyotype' (likelihood ratio test [LRT]; females: $\chi^2_{(2)} = 387.8$, males: $\chi^2_{(2)} = 359.0$, both $P < 0.0001$), temperature *T* (females: $\chi^2_{(1)} = 4301.9$, males: $\chi^2_{(1)} = 2893.4$, both $P < 0.0001$) and the $K \cdot T$

interaction (females: $\chi^2_{(2)} = 19.3$, males: $\chi^2_{(2)} = 31.2$, both $P < 0.0001$). This analysis, together with pairwise generalized Wilcoxon χ^2 tests [GWT], showed that Florida inverted (FI) flies lived shorter than both Florida standard (FS) and Maine standard (MS) flies (Fig. 1, Table 1, Fig. S1), implying a clear effect of *In(3R)P* karyotype on adult survival. Moreover, at 18°C – but not at 25°C – FS flies lived shorter than MS flies (Fig. 1, Table 1, Fig. S1). These results indicate that both karyotype (inverted flies live shorter than uninverted flies from both Florida and Maine) and geography (at least at 18°C, flies from Maine live longer than both inverted and uninverted flies from Florida) affect lifespan (Fig. 1, Table 1, Fig. S1). With regard to temperature, flies lived longer at 18°C than at 25°C (see significant effect of *T* in Cox regression above; and GWT, females: $\chi^2_{(1)} = 3490.4$, males: $\chi^2_{(1)} = 2262.5$, both $P < 0.0001$) (Fig. 1, Fig. S1). At 18°C females lived longer than males, but we failed to find such a sex difference at 25°C (GWT, 18°C: $\chi^2_{(1)} = 115.7$, $P < 0.0001$, 25°C: $\chi^2_{(1)} = 0.122$, $P = 0.73$) (Fig. 1, Fig. S1). In summary, the *In(3R)P* inversion negatively impacts adult survival as compared to the uninverted arrangement, and lifespan exhibits clinal differentiation, with high-latitude flies from Maine overall living longer than flies from low-latitude.

Florida inverted flies survive starvation better than uninverted flies at 18°C; high-latitude flies are more resistant than low-latitude flies

Next, we examined survival upon starvation and found significant effects of 'Karyotype' (Cox LRT; females: $\chi^2_{(2)} = 174.9$, males: $\chi^2_{(2)} = 93.2$, both $P < 0.0001$), temperature (females: $\chi^2_{(1)} = 253.3$, males: $\chi^2_{(1)} = 660.2$, both $P < 0.0001$), and – for females – of $K \cdot T$ (females: $\chi^2_{(2)} = 18.2$, $P < 0.0001$; males: $\chi^2_{(2)} = 4.1$, $P = 0.13$) (Fig. 2, Fig. S2). Interestingly, at 18°C FI flies were more starvation resistant than FS flies for both sexes, whereas at 25°C this pattern was reversed for females, without a significant difference in males (Fig. 2, Table 2, Fig. S2). Overall, across both temperatures, high-latitude MS flies were more starvation resistant than

low-latitude FI and FS flies (Fig. 2, Table 2, Fig. S2). Survival upon starvation was greater for flies reared at 18°C than at 25°C (see significant effect of *T* in Cox model above; GWT, females: $\chi^2_{(1)} = 252.4$, males: $\chi^2_{(1)} = 848.7$, both $P < 0.0001$), and females were more resistant than males at both temperatures (GWT, 18°C: $\chi^2_{(1)} = 938.6$, 25°C: $\chi^2_{(1)} = 1339.1$, both $P<0.0001$) (Fig. 2, Fig. S2). Together, these results show that in Florida the effects of *In(3R)P* karyotype on starvation survival depend upon temperature, and that high-latitude flies from Maine are more starvation resistant than low-latitude flies from Florida.

***In(3R)P* confers cold-shock mortality in females at 25°C; high-latitude females are more cold-shock resistant than low-latitude females at 25°C**

Finally, we investigated patterns of mortality upon 24 hours of exposure to cold shock at -4°C (Fig. 3). In both females and males, we failed to find effects of 'Karyotype' (Cox LRT; females: $\chi^2_{(2)} = 2.1$, $P = 0.39$; males: $\chi^2_{(2)} \approx 0$, $P = 1.0$), whereas the $K \cdot T$ was significant for females but not males (females: $\chi^2_{(2)} = 9.2$, $P = 0.01$; in males, the interaction could not be fit since at 18°C all males survived and were censored from analysis). Temperature affected cold-shock survival in both sexes (Cox LRT; females: $\chi^2_{(1)} = 350.7$, males: $\chi^2_{(1)} = 1529.8$, both $P < 0.0001$). Pairwise comparisons between the three karyotypes with GWT showed that at 25°C FI inverted females survived cold shock less well than both FS and MS uninverted females; at the same time, uninverted high-latitude females from Maine survived cold shock better than both low-latitude karyotypes (Fig. 3, Table 3). In contrast, we found no differences among karyotypes at 18°C or for males at both temperatures (Fig. 3, Table 3). For both females and males, flies survived cold shock better at 18°C than at 25°C (see significant effect of *T* in Cox regression above; GWT, females: $\chi^2_{(1)} = 652.2$, , males: $\chi^2_{(1)} = 1875.1$, both $P < 0.0001$) (Fig. 3). At 25°C females tended to survive cold shock better than males (GWT, 25°C: $\chi^2_{(1)} = 41.0$, $P<0.0001$; since at 18°C all males were censored for analysis we did not

compare the two sexes at this temperature) (Fig. 3). Thus, *In(3R)P* confers increased mortality to cold shock, and uninverted female flies from Maine tend to survive acute cold exposure better than low-latitude flies from Florida, at least at 25°.

Discussion

High-latitude flies are long-lived and stress resistant

Natural populations of *D. melanogaster* in North America, and also on other continents, display gradients of phenotypic differentiation for fitness-related traits such as development time, fecundity, stress resistance, reproductive dormancy and longevity across latitude (e.g., Coyne & Beecham, 1987; de Jong & Bochdanovits, 2003; Hoffmann *et al*., 2005; Schmidt *et al*., 2005a, b; Schmidt & Paaby, 2008; Paaby *et al.*, 2014; Fabian *et al*., 2015; Mathur & Schmidt, 2017). These patterns of clinal trait differentiation are hypothesized to be driven by differential selection pressures at high vs. low latitude (Paaby & Schmidt, 2009): genotypes that confer stress resistance and survival at the expense of reduced fecundity might be favored at high latitudes, where seasonal stressors such as cold and food shortage impose strong selection on somatic maintenance, whereas at low latitude selection might favor alternative genotypes that confer fast development and high fecundity at the expense of reduced stress resistance and survival. In support of this adaptive scenario, we observed that overall high-latitude flies from Maine lived longer and were more resistant to starvation and cold stress than low-latitude flies from Florida, in good agreement with previous observations along the North American cline (Schmidt *et al*., 2000; Schmidt *et al*., 2005a, b; Schmidt & Paaby, 2008; Paaby *et al.*, 2014; Mathur & Schmidt, 2017).

While the genetic basis of latitudinal clines for survival traits remains poorly understood, it is noteworthy that many strongly clinally varying single nucleotide polymorphisms (SNPs) are located in genes known to be important for the determination of adult lifespan and stress

resistance, for example in the insulin/insulin-like growth factor signaling (IIS) pathway (see Fabian *et al*., 2012; Kapun *et al*. 2016a). This observation opens up the opportunity to identify naturally segregating polymorphisms that affect lifespan and stress resistance (e.g., Flatt & Schmidt, 2009; Paaby & Schmidt, 2009). Two examples serve to illustrate this point. A clinally varying indel polymorphism in the *insulin-like receptor* gene has been shown to affect lifespan and stress resistance in the predicted clinal direction, with the high-latitude genotype conferring improved stress resistance and survival (Paaby *et al*., 2010, 2014). Similarly, an amino acid polymorphism in the *couch potato* gene has been found to explain clinal variation in the ability of flies to undergo reproductive dormancy (Schmidt *et al*., 2008), a plastic state associated with improved stress resistance and lifespan (e.g., Schmidt & Paaby, 2008; Flatt *et al*., 2013).

It is also worth to point out in this context that both *InR* and *cpo* are located in the region spanned by *In(3R)P*, even though the two specific variants discussed above are apparently not in linkage disequilibrium (LD) with this inversion. Since the *In(3R)P* inversion polymorphism is *the dominant* driver of genotypic latitudinal clines in North America (Fabian *et al*., 2012; Kapun *et al*., 2016a), either due direct or indirect selection (via genetic draft / hitchhiking), it is interesting to ask whether the cline in *In(3R)P* might causally contribute to the phenotypic clines seen for survival traits. Addressing this question was the main purpose of our study.

_In(3R)P_ contributes to latitudinal clinality of survival traits

Recent population genomic evidence demonstrates that *In(3R)P* is adaptively maintained by spatially varying selection along the North American cline (Kapun *et al*., 2016a), but how this inversion polymorphism affects trait differentiation is poorly understood. In previous assays, we found that *In(3R)P* affects body size, consistent with observations from Australia (Rako *et al*., 2006), but developmental time, egg-to-adult survival, chill coma recovery, oxidative

stress resistance, and lipid content were unaffected by this inversion polymorphism (Kapun *et al*., 2016b). In agreement with our findings for North America, the Australian study also failed to find effects of *In(3R)* on developmental time and chill coma recovery (Rako *et al*., 2006). Despite these negative results for traits beyond body size, here we have found that the North American cline in *In(3R)P* underpins, at least partly, previously observed latitudinal clines for three survival traits, i.e. lifespan, starvation resistance and survival upon cold shock (Schmidt *et al*., 2000; Schmidt *et al*., 2005a, b; Schmidt & Paaby, 2008; Paaby *et al.*, 2014; Mathur & Schmidt, 2017). The effects of *In(3R)P* on these traits go in the predicted clinal direction, with flies from Maine and Florida possessing the uninverted arrangement being on average longer-lived and more stress resistant than flies from Florida carrying the inverted *In(3R)P* segment. Importantly, this establishes that the *In(3R)P* polymorphism affects – and harbors genetic variance for – multiple, clinally varying components of fitness. This is in line with the observation that the genomic region spanned by *In(3R)P* contains numerous clinally varying SNPs in genes known to affect body size, lifespan, stress resistance and other fitness-related traits (Fabian *et al*., 2012; Kapun *et al*., 2016a, b).

Although work by Rako *et al*. (2006) and us (Kapun *et al.*, 2016b) did not find an effect of *In(3R)P* on cold tolerance in terms of chill coma recovery (but see Anderson *et al*., 2003), our experiments here show that the inverted arrangement confers mortality to cold shock in females. This result is in good qualitative agreement with the data of Anderson *et al.* (2003), who found that cold-shock mortality is associated with a genetic marker (*hsr-omega*) that is in LD with *In(3R)P*, and also with the earlier findings of Tucic (1979), who found a major effect of chromosome 3 on larval and adult cold tolerance. The fact that different measures of cold tolerance (chill coma recovery vs. cold-shock mortality) can give discordant results implies that the details of assay protocols used for measuring aspects of cold tolerance matter a lot (e.g., McDonald *et al*., 2004; Andersen *et al*., 2015).

Thus, by experimentally isolating and phenotyping *In(3R)P* karyotypes from populations approximating the end points of the North American cline, our results strongly suggest that this inversion polymorphism makes a major contribution to the clinality of several survival traits. An open task for future work will be to regress means of survival traits for multiple populations spanning the cline against the population frequency of *In(3R)P* as a predictor, for this would allow to estimate the amount of phenotypic variance explained by *In(3R)P*.

How is the *In(3R)P* polymorphism maintained?

Although it is clear that *In(3R)P* is maintained by selection along the cline independent of admixture or population structure (Kapun *et al.*, 2016a), the details of the underlying selective mechanisms remain unknown. Clearly, our results cannot explain how this polymorphism is maintained since the inverted segment seems to have predominantly negative effects on the measured fitness-components: inverted homokaryons were on average smaller, shorter-lived and less stress resistant than uninverted homokaryons (also see Kapun *et al*., 2016b). So, what are the fitness benefits that maintain the inverted karyotype at low latitude?

Four major considerations should be kept in mind. First, we have only phenotyped inverted vs. uninverted homokaryons but not heterokaryons: because inversions might be maintained by overdominance or associative overdominance (e.g., Dobzhansky, 1970; Kirpatrick & Barton, 2006), it will be critical to phenotype *In(3R)P* heterokaryons in future work. Second, inversion polymorphisms can be maintained by frequency-dependent selection. For example, Nassar *et al.* (1973) reported that *In(3R)P* might subject to frequency-dependent selection under conditions of larval crowding; however, it is unclear on theoretical grounds how frequency-dependent selection would be able to maintain an inversion polymorphism for a long period of time since even small amounts of recombination or gene conversion in heterokaryons will destroy LD between the genic target(s) of balancing selection and the

inversion (Kirpatrick & Barton, 2006). Nonetheless, it would be interesting to reassess the findings of Nassar *et al.* (1973) and to directly investigate, for example, larval competitive ability as a function of *In(3R)P* karyotype. Third, there are several fitness-related traits that we have not measured on the homokaryons, including fecundity: since low-latitude flies are more fecund than high-latitude flies (Schmidt & Paaby, 2008), an important open question is whether *In(3R)P* affects fecundity. Fourth, the fitness components through which the *In(3R)P* polymorphism is maintained might be subject to genotype by environment interactions. In our assays, we phenotyped homokaryons at two growth temperatures and indeed found several karyotype by temperature interactions. Perhaps most interestingly, we observed that at 18°C Florida inversion homokaryons are significantly more resistant to starvation stress than Florida uninverted homokaryons, whereas this pattern was reversed for females at 25°C. However, this effect was very small; moreover, while the latitudinal temperature gradient is a major determinant of the cline in *In(3R)P*, other latitudinally varying environmental factors (e.g., precipitation, seasonality) seem to be important too (Kapun *et al*., 2016a). Determining how selection maintains the *In(3R)P* polymorphism will thus depend on a much more detailed understanding of the various environmental factors that might affect this system.

Conclusions

Here we have asked whether a clinally varying chromosomal inversion polymorphism in *D. melanogaster*, *In(3R)P*, contributes to the clinality of three fitness-related traits: adult lifespan, survival of starvation stress, and survival upon acute cold shock. Our main finding is that the cline in *In(3R)P* underpins, at least partly, the latitudinal clines observed for these survival traits (cf. Schmidt *et al*. 2000; Schmidt *et al*., 2005a, b; Schmidt & Paaby, 2008; Mathur & Schmidt, 2017). Together with the fact that *In(3R)P* contributes to clines in body size (Rako *et al.*, 2006; Kapun *et al.*, 2016b), these results add to our understanding of how

this inversion polymorphism affects multiple fitness-related traits subject to spatially varying (clinal) selection. Yet, given that most fitness-related traits seem to be affected negatively by *In(3R)P*, the exact nature of the selective forces that maintain this inversion polymorphism remain to be elucidated.

Acknowledgements

We thank the members of the Flatt and Schmidt labs for discussion and help in the laboratory. Our research was financially supported by the Swiss National Science Foundation (SNSF PP00P3_133641; PP00P3_165836; 310030E-164207 to TF), the National Institutes of Health (NIH 5R01GM100366 to PSS), and the National Science Foundation (NSF DEB 0921307 to PS).

References

Adrion, J.R., Hahn, M.W. & Cooper, B.S. 2015. Revisiting classic clines in *Drosophila melanogaster* in the age of genomics. *Trends Genet.*, **31**: 434-444.

Andersen, J.L., Manenti, T., Sørensen, J.G., MacMillan, H.A., Loeschcke, V. & Overgaard, J. 2015. How to assess *Drosophila* cold tolerance: chill coma temperature and lower lethal temperature are the best predictors of cold distribution limits. *Func. Ecol*. **29**: 55-65.

Anderson, A.R., Collinge, J.E., Hoffmann, A.A., Kellett, M. & McKechnie, S.W. 2003. Thermal tolerance trade-offs associated with the right arm of chromosome 3 and marked by the *hsr-omega* gene in *Drosophila melanogaster*. *Heredity* **90**: 195-202.

Anderson, A.R., Hoffmann, A.A., McKechnie, S.W., Umina, P.A. & Weeks, A.R. 2005. The latitudinal cline in the *In(3R)Payne* inversion polymorphism has shifted in the last 20 years in Australian *Drosophila melanogaster* populations. *Mol. Ecol.* **14**: 851-858.

Azevedo, R., James, A.C., McCabe, J. & Partridge, L. 1998. Latitudinal variation of wing : thorax size ratio and wing-aspect ratio in *Drosophila melanogaster*. *Evolution*, **52**: 1353-1362.

Coyne, J.A. & Beecham, E. 1987. Heritability of Two Morphological Characters Within and Among Natural Populations of *Drosophila melanogaster*. *Genetics*, **117**: 727-737.

Das, A. & Singh, B.N., 1991. Genetic differentiation and inversion clines in Indian natural populations of *Drosophila melanogaster*. *Genome* **34**: 618-625.

De Jong, G. & Bochdanovits, Z. 2003. Latitudinal clines in *Drosophila melanogaster*: body size, allozyme frequencies, inversion frequencies, and the insulin-signalling pathway. *J Genet.* **82**: 207-223.

Dobzhansky, T. 1937. *Genetics and the Origin of Species*. Columbia University Press, New York.

Dobzhansky, T. 1943. Genetics of natural populations. IX. Temporal Changes in the Composition of Populations of *Drosophila pseudoobscura*. *Genetics* **28**: 162-186.

Dobzhansky, T. 1947a. Genetics of natural populations. XIV. A Response of Certain Gene Arrangements in the Third Chromosome of a Response of Certain Gene Arrangements in the Third Chromosome of *Drosophila pseudoobscura* to natural selection. *Genetics* **32**: 142-160.

Dobzhansky, T. 1947b. Adaptive changes induced by natural selection in wild populations of *Drosophila*. *Evolution* **1**: 1-16.

Dobzhansky, T. 1970. *Genetics of the Evolutionary Process*. Columbia University Press, New York.

Etges, W.J. 1989. Chromosomal Influences on Life-History Variation Along an Altitudinal Transect in *Drosophila robusta*. *Am. Nat*. **133**: 83-110.

Fabian, D.K., Kapun, M., Nolte, V., Kofler, R., Schmidt, P.S., Schlötterer, C., *et al.* 2012. Genome-wide patterns of latitudinal differentiation among populations of *Drosophila melanogaster* from North America. *Mol. Ecol.* **21**: 4748-4769.

Fabian, D.K., Lack, J.B., Mathur, V., Schlötterer, C., Schmidt, P.S., Pool, J.E., *et al.* 2015. Spatially varying selection shapes life history clines among populations of *Drosophila melanogaster* from sub-Saharan Africa. *J. Evol. Biol.* **28**: 826-840.

Flatt, T. & Schmidt, P.S. 2009. Integrating evolutionary and molecular genetics of aging. *Biochim. Biophys. Acta* **1790**: 951-962.

Flatt, T., Amdam, G.V., Kirkwood, T.B.L. & Omholt, S.W. 2013. Life-history evolution and the polyphenic regulation of somatic maintenance and survival. *Quart. Rev. Biol*. **88**: 185-218.

Hoffmann, A.A. 2010. Physiological climatic limits in *Drosophila*: patterns and implications. *J. Exp. Biol.* **213**: 870-880.

Hoffmann, A.A. & Rieseberg, L.H. 2008. Revisiting the Impact of Inversions in Evolution: From Population Genetic Markers to Drivers of Adaptive Shifts and Speciation? *Annu. Rev. Ecol. Evol. Syst.* **39**: 21-42.

Hoffmann, A.A. & Weeks, A.R. 2007. Climatic selection on genes and traits after a 100 year-old invasion: a critical look at the temperate-tropical clines in *Drosophila melanogaster* from eastern Australia. *Genetica* **129**: 133-147.

Hoffmann, A.A., Sgrò, C.M. & Weeks, A.R. 2004. Chromosomal inversion polymorphisms and adaptation. *Trends Ecol. Evol.* **19**: 482-488.

Hoffmann, A. A., Shirriffs, J. & Scott, M. 2005. Relative importance of plastic vs genetic factors in adaptive differentiation: geographical variation for stress resistance in *Drosophila melanogaster* from eastern Australia. *Func. Ecol.* **19**: 222-227

Inoue, Y. & Watanabe, T.K., 1979. Inversion polymorphisms in Japanese natural populations of *D. melanogaster*. *Jpn. J. Genet.* **54**: 69-82.

Kapun, M., van Schalkwyk, H., McAllister, B., Flatt, T. & Schlötterer, C. 2014. Inference of chromosomal inversion dynamics from Pool-Seq data in natural and laboratory populations of *Drosophila melanogaster. Mol. Ecol.* **23**: 1813-1827.

Kapun, M., Fabian, D.K., Goudet, J. & Flatt, T. 2016a. Genomic Evidence for Adaptive Inversion Clines in *Drosophila melanogaster*. *Mol. Biol. Evol.*, **33**:1317-1336.

Kapun, M., Schmidt, C., Durmaz, E., Schmidt, P.S. & Flatt, T. 2016b. Parallel effects of the inversion *In(3R)Payne* on body size across the North American and Australian clines in *Drosophila melanogaster*. *J. Evol. Biol*., **29**: 1059-1072.

Kennington, W.J., Hoffmann, A.A. & Partridge, L. 2007. Mapping Regions Within Cosmopolitan Inversion *In(3R)Payne* Associated With Natural Variation in Body Size in *Drosophila melanogaster*. *Genetics* **177**: 549-556.

Kirkpatrick, M. & Barton, N. 2006. Chromosome inversions, local adaptation and speciation. *Genetics* **173**: 419-434.

Kirkpatrick, M. & Kern, A. 2012. Where's the Money? Inversions, Genes, and the Hunt for Genomic Targets of Selection. *Genetics* **190**: 1153-1155.

Knibb, W.R. 1982. Chromosome inversion polymorphisms in *Drosophila melanogaster* II. Geographic clines and climatic associations in Australasia, North America and Asia. *Genetica* **58**: 213-221.

Knibb, W.R., Oakeshott, J.G. & Gibson, J.B. 1981. Chromosome Inversion Polymorphisms in *Drosophila melanogaster*. I. Latitudinal Clines and Associations between Inversions in Australasian Populations. *Genetics* **98**: 833-847.

Lemeunier, F. & Aulard, S. 1992. Inversion polymorphism in *Drosophila melanogaster*. In: *Drosophila Inversion Polymorphism* (Krimbas, C.B. & Powell, J.R., eds.), pp. 339-405. CRC Press, Boca Raton.

Lowry, D.B. & Willis, J.H. 2010. A Widespread Chromosomal Inversion Polymorphism Contributes to a Major Life-History Transition, Local Adaptation, and Reproductive Isolation. *PLoS Biol.* **8**: e1000500.

Mathur, V. & Schmidt, P.S. 2017. Adaptive patterns of phenotypic plasticity in laboratory and field environments in *Drosophila melanogaster*. *Evolution*, **71**: 465-474.

Matzkin, L.M., Merritt, T.J.S., Zhu, C.-T. & Eanes, W.F. 2005. The structure and population genetics of the breakpoints associated with the cosmopolitan chromosomal inversion *In(3R)Payne* in *Drosophila melanogaster. Genetics* **170**: 1143-1152.

Macdonald, S.S., Rako, L., Batterham, P. & Hoffmann, A.A. 2004. Dissecting chill coma recovery as a measure of cold resistance: evidence for a biphasic response in *Drosophila melanogaster*. *J. Insect Physiol.* **50**: 695-700

Mettler, L.E., Voelker, R.A. & Mukai, T. 1977. Inversion Clines in Populations of *Drosophila melanogaster. Genetics* **87**: 169-176.

Nassar, R., Muhs, H.J. & Cook, R.D. 1973. Frequency-Dependent Selection at the Payne Inversion in *Drosophila melanogaster*. *Evolution* **27**: 558-564.

Paaby, A.B. & Schmidt, P.S. 2009. Dissecting the genetics of longevity in *Drosophila melanogaster*. Fly (Austin) **3**: 29-38.

Paaby, A.B., Bergland, A.O., Behrman, E.L. & Schmidt, P.S. 2014. A highly pleiotropic amino acid polymorphism in the *Drosophila* insulin receptor contributes to life-history adaptation. *Evolution* **68**: 3395-3409.

Paaby, A.B., Blacket, M.J., Hoffmann, A.A. & Schmidt, P.S. 2010. Identification of a candidate adaptive polymorphism for *Drosophila* life history by parallel independent clines on two continents. *Mol. Ecol*. **19**: 760-774.

Rako, L., Anderson, A.R., Sgrò, C.M., Stocker, A.J. & Hoffmann, A.A. 2006. The association between inversion *In(3R)Payne* and clinally varying traits in *Drosophila melanogaster. Genetica* **128**: 373-384.

Rane, R.V., Rako, L., Kapun, M., Lee, S.F. & Hoffmann, A.A. 2015. Genomic evidence for role of inversion *3RP* of *Drosophila melanogaster* in facilitating climate change adaptation. *Mol. Ecol.* **24**: 2423-2432.

Schaeffer, S.W. 2008. Selection in heterogeneous environments maintains the gene arrangement polymorphism of *Drosophila pseudoobscura*. *Evolution* **62**: 3082-3099.

Schmidt, P.S. & Paaby, A.B. 2008. Reproductive Diapause and Life-History Clines in North American Populations of *Drosophila melanogaster*. *Evolution*, **62**: 1204-1215.

Schmidt, P.S., Duvernell, D.D. & Eanes, W.F. 2000. Adaptive evolution of a candidate gene for aging in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A*. **97**:10861-10865.

Schmidt, P.S., Matzkin, L., Ippolito, M. & Eanes, W.F. 2005a. Geographic Variation in Diapause Incidence, Life-History Traits, and Climatic Adaptation in *Drosophila melanogaster*. *Evolution*, **59**: 1721-1732.

Schmidt, P.S., Paaby, A.B. & Heschel, M.S. 2005b. Genetic variance for diapause expression and associated life histories in *Drosophila melanogaster*. *Evolution*, **59**: 2616-2625.

Schmidt, P.S., Zhu, C.T., Das, J., Batavia, M., Yang, L. & Eanes, W.F. 2008. An amino acid polymorphism in the *couch potato* gene forms the basis for climatic adaptation in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A*. **105**: 16207-16211.

Sperlich, D. & Pfriem, P. 1986. Chromosomal polymorphism in natural and experimental populations. In: *The Genetics and Biology of Drosophila* (Ashburner, M, Carson, H.L. & Thompson, J.R., eds.), Volume 3e, pp. 257-309. Academic Press, New York.

Stalker, H.D. 1980. Chromosome Studies in Wild Populations of *Drosophila melanogaster*. II. Relationship of Inversion Frequencies to Latitude, Season, Wing-Loading and Flight Activity. *Genetics* **95**: 211-223.

Tatar, M., Chien, S. A. & Priest, N.K. 2001. Negligible senescence during reproductive dormancy in *Drosophila melanogaster*. *Am. Nat*. **158**: 248-258.

Tucic, N. 1979. Genetic capacity for adaptation to cold resistance at different developmental stages in *Drosophila melanogaster. Evolution* **33**: 350-358.

Weeks, A.R., McKechnie, S.W. & Hoffmann, A.A. 2002. Dissecting adaptive clinal variation: markers, inversions and size/stress associations in *Drosophila melanogaster* from a central field population. *Ecol. Lett.* **5**: 756-763.

Wright, S. & Dobzhansky. 1946. Genetics of natural populations. XII. Experimental Reproduction of Some of the Changes Caused by Natural Selection in Certain Populations of *Drosophila pseudoobscura*. *Genetics* **31**: 125-156.

Supporting Information

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

Figure S1. Survival curves as a function of *In(3R)P* karyotype and temperature. Effects of *In(3R)P* and temperature (18°C vs. 25°C) on the proportion adult survival in females and males. The different curves represent Florida inverted (black), Florida standard (red), and Maine standard (blue). See Results, Fig. 1, and Table 1 for details.

Figure S2. Starvation survival curves as a function of *In(3R)P* and temperature. . Effects of *In(3R)P* and temperature (18°C vs. 25°C) on the proportion adult survival upon starvation in females and males. The different curves show Florida inverted (black), Florida standard (red), Maine standard (blue). See Results, Fig. 2 and Table 2 for details.

Data Accessibility

Data deposited at Dryad. doi link to be added upon publication.

Table 1. Analysis of lifespan. The columns show the directionality of lifespan effects for each pairwise comparison between the three karyotypes (FI = Florida inverted, FS = Florida standard, MS = Maine standard), grouped by sex and temperature. χ^2 test statistics and *P*-values are from generalized Wilcoxon tests. Significant effects are in bold; significance after Bonferroni correction is indicated by † *(α'* = 0.05/3 = 0.016). *n* represents the number of failed individuals; the total cohort size is shown in parenthesis. See Results and Figs. 1 and S1 for further details.

Sex	Temperature	Direction	χ^2	*P*	*n*
Female	18°C	FI < FS	35.86	**<0.0001**†	2609 (2639)
		FI < MS	137.31	**<0.0001**†	2591 (2644)
		FS < MS	28.52	**<0.0001**†	2520 (2571)
	25°C	FI < FS	98.75	**<0.0001**†	2500 (2549)
		FI < MS	71.53	**<0.0001**†	2478 (2539)
		FS = MS	0.65	0.42	2508 (2566)
Male	18°C	FI < FS	85.72	**<0.0001**†	2437 (2455)
		FI < MS	247.67	**<0.0001**†	2514 (2547)
		FS < MS	37.26	**<0.0001**†	2453 (2484)
	25°C	FI < FS	100.39	**<0.0001**†	2565 (2600)
		FI < MS	109.59	**<0.0001**†	2537 (2580)
		FS = MS	0.32	0.57	2512 (2558)

Table 2. Analysis of survival upon starvation. The columns show the directionality of survival upon starvation for each pairwise comparison between the three karyotypes (FI = Florida inverted, FS = Florida standard, MS = Maine standard), grouped by sex and temperature. χ^2 test statistics and *P*-values are from generalized Wilcoxon tests. Significant effects are in bold; significance after Bonferroni correction is indicated by † *(α'* = 0.05/3 = 0.016). *n* represents the total cohort size, i.e. the number of failed individuals (no flies were censored in this assay). See Results and Figs. 2 and S2 for further details.

Sex	Temperature	Direction	χ^2	*P*	*n*
Female	18°C	FI > FS	5.35	**0.02**	881
		FI < MS	72.94	**<0.0001**†	878
		FS < MS	90.88	**<0.0001**†	899
	25°C	FI < FS	10.14	**0.0014**†	899
		FI < MS	88.68	**<0.0001**†	900
		FS < MS	25.99	**<0.0001**†	899
Male	18°C	FI > FS	10.44	**0.0012**†	900
		FI < MS	34.72	**<0.0001**†	897
		FS < MS	78.31	**<0.0001**†	897
	25°C	FI = FS	0.15	0.70	898
		FI < MS	30.44	**<0.0001**†	896
		FS < MS	31.60	**<0.0001**†	894

Table 3. Analysis of survival upon cold shock. The columns show the directionality of survival upon cold shock for each pairwise comparison between the three karyotypes (FI = Florida inverted, FS = Florida standard, MS = Maine standard), grouped by sex and temperature. χ^2 test statistics and *P*-values are from generalized Wilcoxon tests. Significant effects are in bold; significance after Bonferroni correction is indicated by † *(α'* = 0.05/3 = 0.016). *n* represents the number of failed individuals; the total cohort size is shown in parenthesis. Note that at 18°C all males survived 24 hours of cold shock and were thus all censored. See Results and Fig. 3 for further details.

Sex	Temperature	Direction	χ^2	*P*	*n*
Female	18°C	FI = FS	0.64	0.43	197 (887)
		FI = MS	0.88	0.35	199 (888)
		FS = MS	0.02	0.89	210 (893)
	25°C	FI < FS	47.39	**<0.0001**†	694 (897)
		FI < MS	77.48	**<0.0001**†	664 (896)
		FS < MS	4.18	**0.04**	580 (899)
Male	18°C	-	-	-	0 (896)
		-	-	-	0 (897)
		-	-	-	0 (895)
	25°C	FI = FS	2.01	0.16	737 (887)
		FI = MS	2.35	0.14	734 (885)
		FS = MS	0.01	0.90	719 (886)

Figure Legends

Fig. 1. *In(3R)P* shortens lifespan and lifespan varies clinally. Effects of the *In(3R)P* inversion polymorphism on adult lifespan (days) in females and males. The bar plots show means and standard errors. Black bars: Florida inverted (FI); dark grey bars: Florida standard (FS); light grey bars: Maine standard (MS). Results for pairwise comparisons among karyotypes with generalized Wilcoxon (χ^2) tests are shown in letters: groups that do not contain the same letter are significantly different from each other (P<0.05). The *In(3R)P* inversion shortens lifespan as compared to uninverted karyotypes; lifespan is clinally differentiated, with high-latitude flies from Maine living longer than flies from low-latitude. See Results and Table 1 for details; for survival curves see Fig. S1.

Fig. 2. *In(3R)P* affects starvation in a temperature-dependent manner. Effects of *In(3R)P* on age at death (hours) upon starvation in females and males. Shown are means and standard errors. Black bars: Florida inverted (FI), dark grey bars: Florida standard (FS), light grey bars: Maine standard (MS). Results for pairwise comparisons among karyotypes with generalized Wilcoxon (χ^2) tests are shown in letters: groups that do not contain the same letter are significantly different from each other (P<0.05). At 18°C Florida inverted flies survive starvation better than uninverted flies, whereas this pattern is reversed for females at 25°C. Morevoer, high-latitude flies from Maine are overall more resistant than low-latitude flies from Florida. See Results and Table 2 for details; for survival curves see Fig. S2.

Fig. 3. At 25ºC *In(3R)P* confers mortality upon cold shock. Effects of *In(3R)P* on the proportion of female and male flies surviving cold shock. Shown are means and standard errors. Black bars: Florida inverted (FI); dark grey bars: Florida standard (FS); light grey bars: Maine standard (MS). Results for pairwise comparisons among karyotypes with generalized Wilcoxon (χ^2) tests are shown in letters: groups that do not contain the same letter are significantly different from each other (P<0.05). The *In(3R)P* inversion increases sensitivity to cold shock at 25°C. Generally, at 25°C, high-latitude females from Maine are more cold-shock resistant than low-latitude females from Florida. See Results and Table 3 for details.

Figure 1

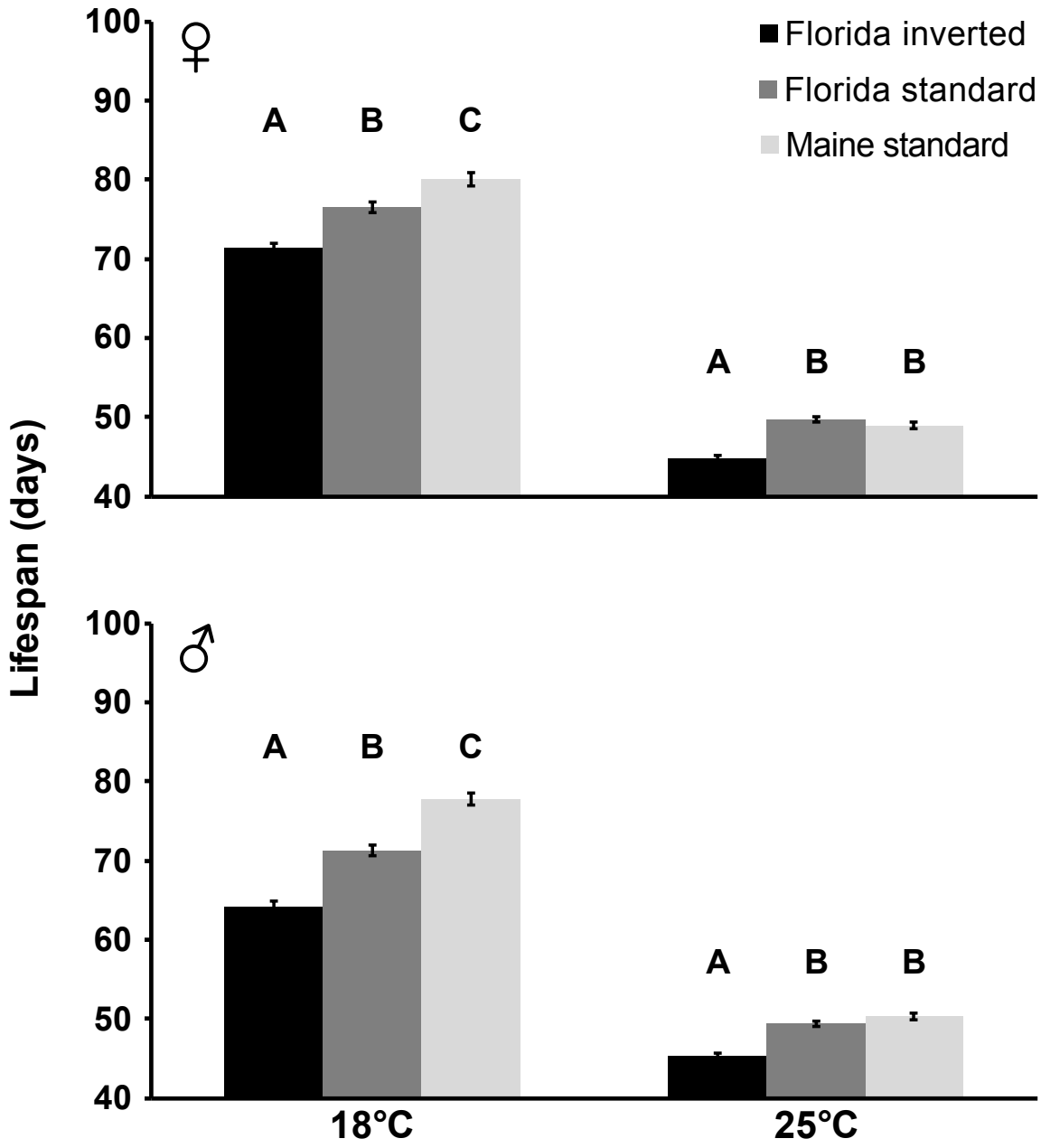

Florida inverted
Florida standard
Maine standard
♀
♂
Lifespan (days)
100
90
80
70
60
50
40
A
B
C
A
B
B
18°C
25°C

Figure 2

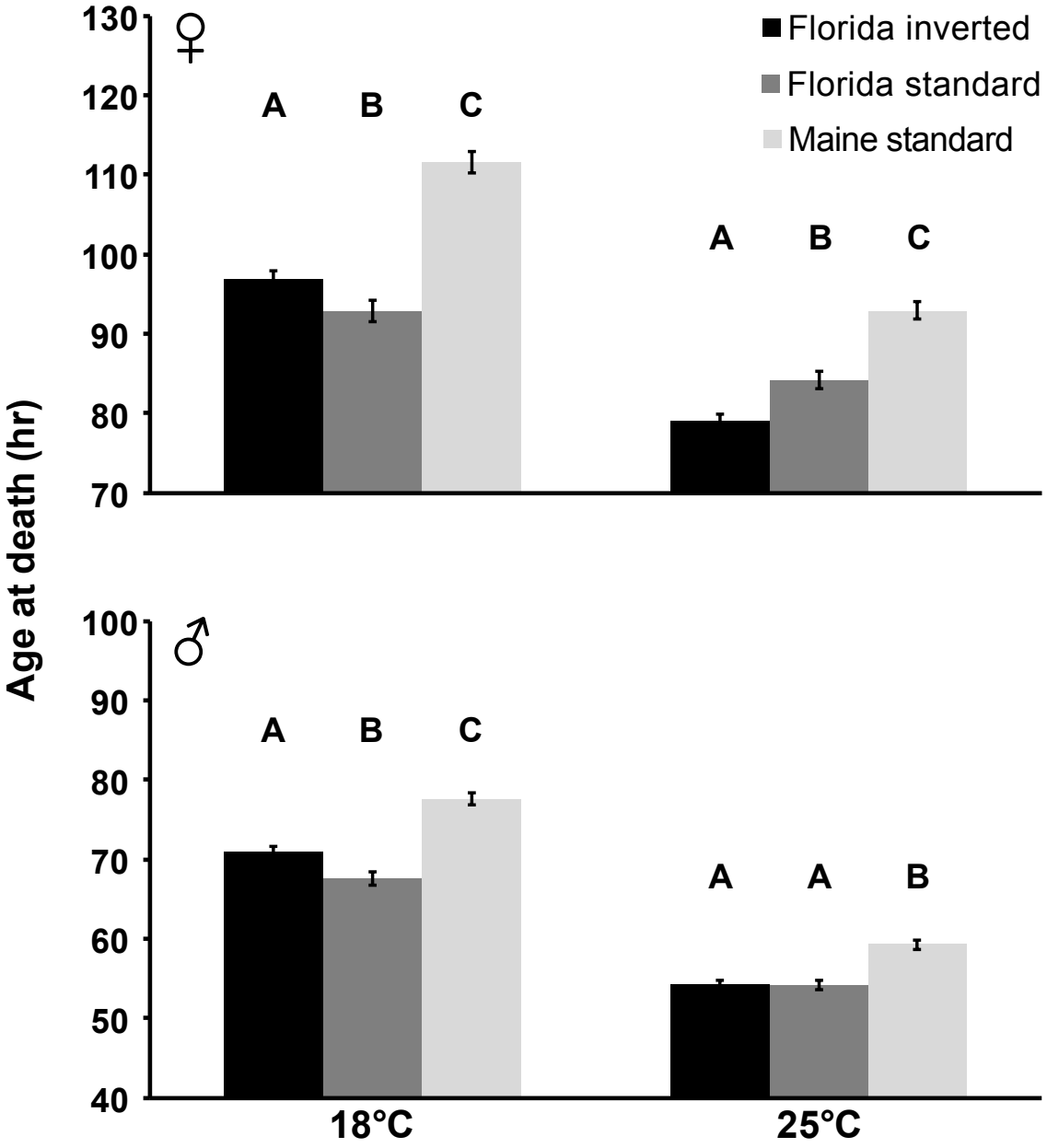
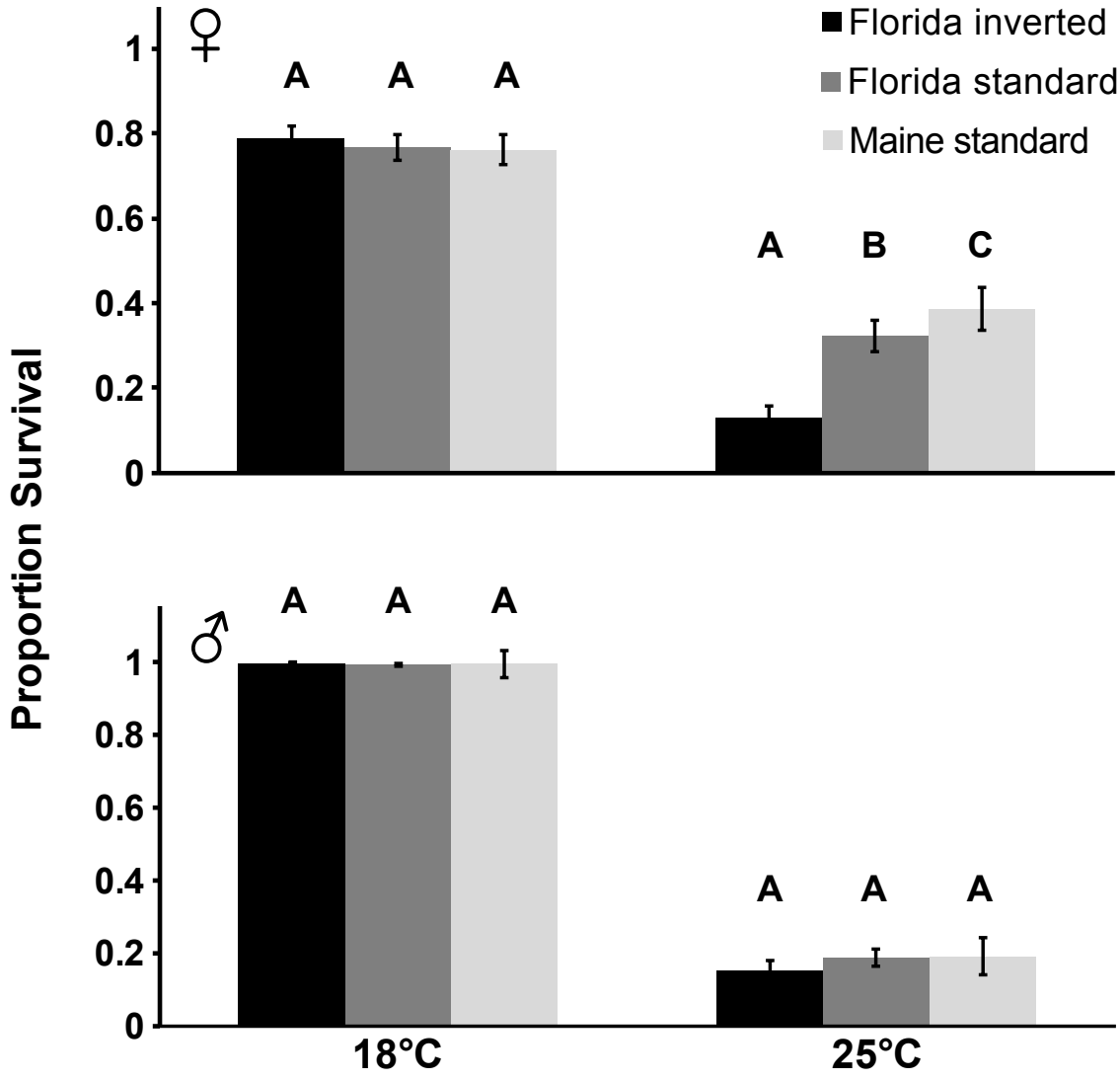

Florida inverted
Florida standard
Maine standard
Age at death (hr)
♀
♂
A
B
C
A
B
C
A
A
B
A
B
C
130
120
110
100
90
80
70
100
90
80
70
60
50
40
18°C
25°C

Figure 3


♀
♂
Florida inverted
Florida standard
Maine standard
Proportion Survival
0
0.2
0.4
0.6
0.8
1
A A A
A B C
A A A
A A A
18°C
25°C

Supporting Information

Supporting Figure Legends

Figure S1. Survival curves as a function of *In(3R)P* karyotype and temperature. Effects of *In(3R)P* and temperature (18°C vs. 25°C) on the proportion adult survival in females and males. The different curves represent Florida inverted (black), Florida standard (red), and Maine standard (blue). See Results, Fig. 1, and Table 1 for details.

Figure S2. Starvation survival curves as a function of *In(3R)P* and temperature. . Effects of *In(3R)P* and temperature (18°C vs. 25°C) on the proportion adult survival upon starvation in females and males. The different curves show Florida inverted (black), Florida standard (red), Maine standard (blue). See Results, Fig. 2 and Table 2 for details.

Figure S1

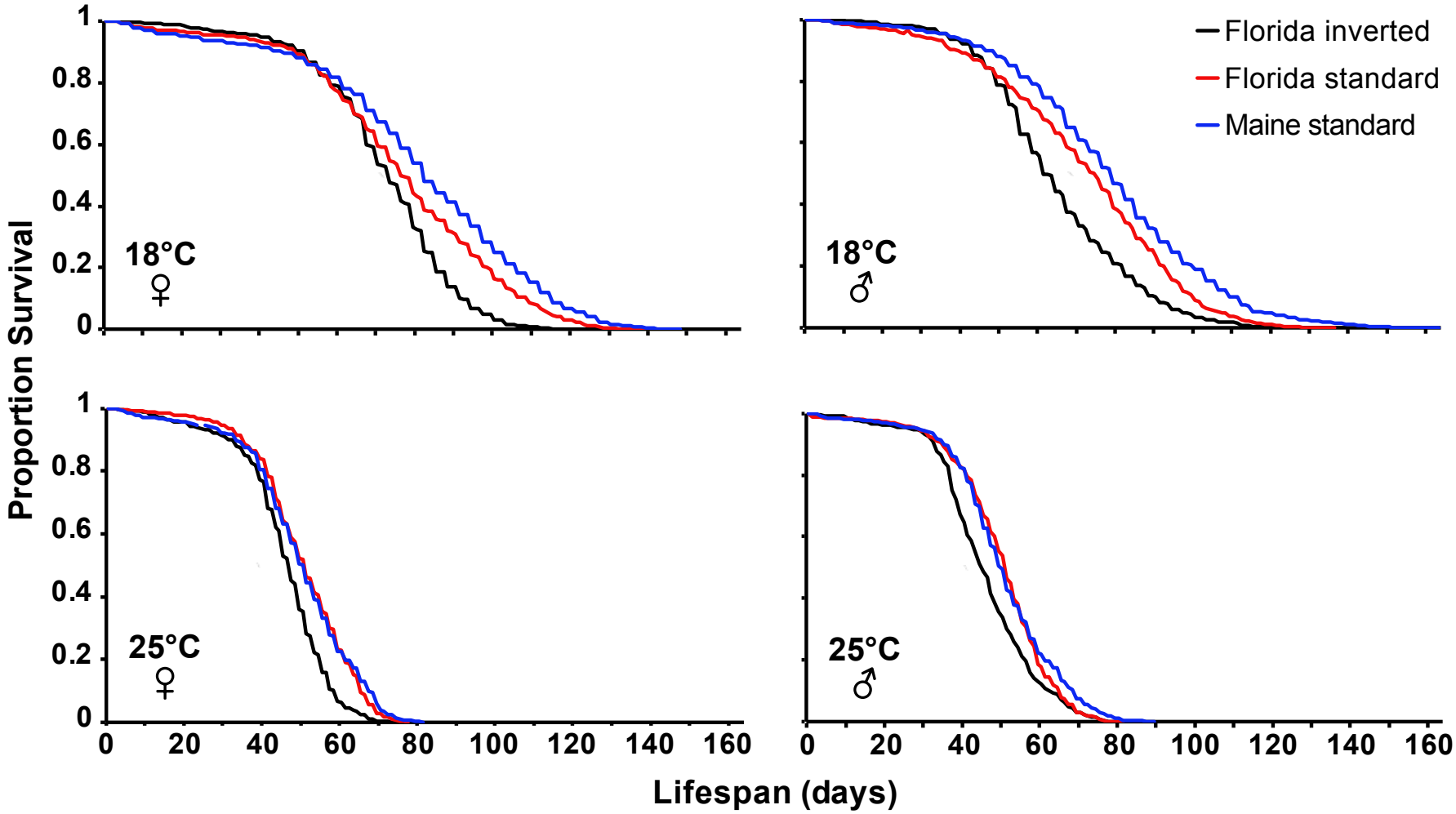
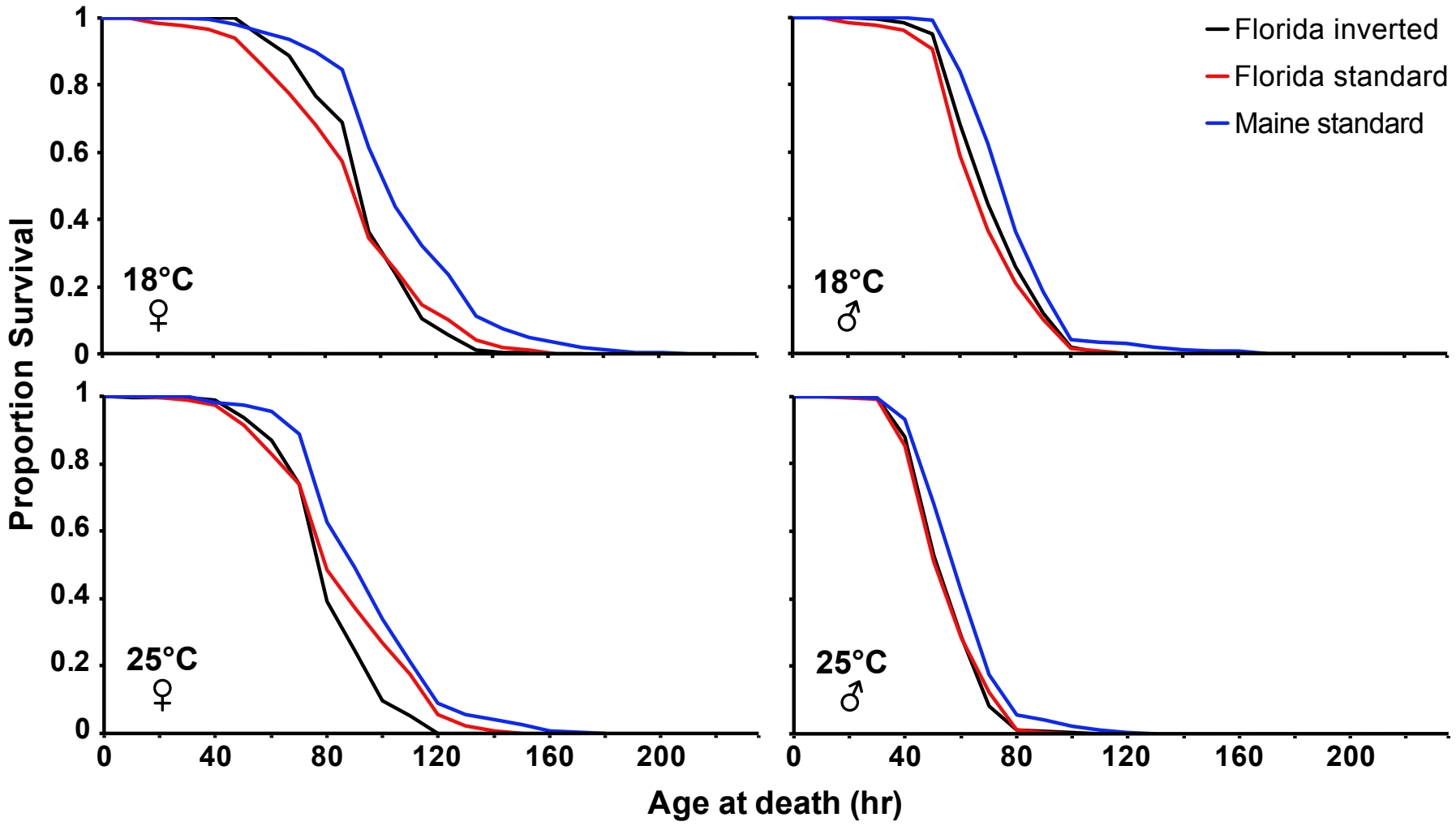

Florida inverted
Florida standard
Maine standard
Proportion Survival
18°C ♀
18°C ♂
25°C ♀
25°C ♂
0
0.2
0.4
0.6
0.8
1
0
20
40
60
80
100
120
140
160
Lifespan (days)

Figure S2


Florida inverted
Florida standard
Maine standard
Proportion Survival
1
0.8
0.6
0.4
0.2
0
18°C
♀
18°C
♂
25°C
♀
25°C
♂
0
40
80
120
160
200
Age at death (hr)

Chapter 7

General Discussion and Perspectives

The Main Objective of this Dissertation

Adaptation across environmental gradients, for example across latitude or altitude, typically results in the evolution of clines, i.e. patterns of gradual, systematic change or differentiation in genotype frequencies and/or phenotypic traits along such gradients, presumably by spatial differences in temperature and/or seasonality. In my Ph.D. thesis, I have used the *Drosophila melanogaster* model system to examine the effects of two strongly clinally varying polymorphisms on life-history traits in order to gain a better understanding of the genetics of adaptation.

Brief Recap of the Chapters in this Thesis

My thesis consists of 7 chapters. In **Chapter 1**, I have provided a brief overview of life-history variation in *D. melanogaster*, this species' demographic and colonization history, and the genetic basis of clinal adaptation. In **Chapters 2, 3 and 4**, I have presented results of phenotypic analyses of a clinally varying, presumably adaptive polymorphism in the insulin signaling transcription factor *foxo*, aimed at examining the role of this variant in affecting life history and in contributing to previously observed phenotypic clines across latitude. In **Chapters 5 and 6**, I have reported life-history effects of a major, cosmopolitan, clinally varying inversion polymorphism, *In(3R)Payne*, which has previously been shown to be maintained by spatially varying (clinal) selection across latitude. Here, in **Chapter 7**, I provide a brief summary and discussion of my main findings; for a more detailed discussion of my results I refer the reader to the detailed discussion sections in the individual chapters.

The Genomic Basis of Life-History Adaptation in *Drosophila*

The fruit (or vinegar) fly *Drosophila melanogaster* is an ancestrally tropical insect that has originated in sub-Saharan Africa and subsequently become cosmopolitan, having evolved major adaptations to novel temperate and seasonal habitats during its range expansion (David & Capy 1988; De Jong & Bochdanovits 2003; Kolaczkowski *et al.* 2011; Fabian *et al.* 2012; Adrion *et al.* 2015; Kapun *et al.* 2016a). For example, as a manifestation of this adaptation to new climates and habitats, natural populations of this species exhibit major patterns of clinal differentiation both at phenotypic and genetic level, often exhibiting parallel clines on multiple continents, thus representing a naturally replicated system of convergent adaptive evolution (Paaby *et al.* 2010; Adrion *et al.* 2015; Schrider *et al.* 2016; Kapun *et al.* 2016b; a). However, with a few exceptions, still very little is known about the identity and function of naturally occurring polymorphisms that might causally underpin adaptive clinal life-history differentiation. Contributing to an improved understanding of the genetic basis of clinal life-history adaptation in the *Drosophila* model was the overarching aim of my dissertation research work. Specifically, my thesis research deals with two related questions: (1) Does natural, clinal variation at loci of the insulin/insulin-like growth factor signaling (IIS) pathway contribute to clinal life-history adaptation and, if so, how?, and, similarly, (2) Does a well-known, clinally varying chromosomal inversion polymorphism, called *In(3R)Payne*, contribute to clinal life-history adaptation and, and if yes, how?

Does Variation in Insulin Signaling Contribute to Clinal Life-History Adaptation?

Since loss-of-function mutants in the evolutionarily conserved insulin/insulin-like growth factor signaling (IIS) / target of rapamycin (TOR) pathway have been shown to affect several major fitness-related traits (e.g., growth, size, ovarian development, fecundity, stress

resistance, and lifespan, it has been hypothesized that this pathway might be an important determinant of life-history adaptation (Brogiolo *et al.* 2001; Barbieri *et al.* 2003; Swanson & Dantzer 2014; Das & Arur 2017).

Interestingly, in previous work from our group, Fabian *et al*. (2012) have performed the first genome-wide analysis of latitudinal differentiation along the North American east coast and identified numerous strongly clinally varying single nucleotide polymorphisms (SNPs) in this pathway. Based on functional genetic studies of this pathway, and based on the observation of parallel clinal differentiation of many IIS components on multiple continents, IIS has been speculated to be one of the main drivers of life-history clinality (De Jong & Bochdanovits 2003; Paaby *et al.* 2010; Fabian *et al.* 2012; Kapun *et al.* 2016b).

One of the most important components of this is the forkhead box-O transcription factor *foxo*, a gene involved in regulating growth, size, stress resistance and lifespan (Jünger *et al.* 2003; Barthel *et al.* 2005; Zheng *et al.* 2007; Flatt *et al.* 2008; Kramer *et al.* 2008; Yamamoto & Tatar 2011; Alic *et al.* 2014). Given the central role of this transcription factor, we set out to investigate the phenotypic effects of a strongly clinal and potentially adaptive 2-SNP variant in this gene by performing functional phenotypic assays across two independent laboratories.

Our analyses in Chapters 2 and 3 reveal that this *foxo* polymorphism has pleiotropic effects on multiple life-history traits, including egg-to-adult survival, several proxies of body size and on starvation resistance (Chapters 2 and 3). Our findings for this naturally occurring variant are consistent with functional genetic studies of large-effect mutants or transgenes showing that *foxo* affects, amongst other traits, body size and starvation resistance (Jünger *et al.* 2003; Kramer *et al.* 2003; 2008; Slack *et al.* 2011; Tang *et al.* 2011) as well as with clinal predictions for latitudinally varying traits (Robinson *et al.* 2000; De Jong & Bochdanovits 2003; Wadgymar *et al.* 2017).

Specifically, our results in Chapter 2 show that in North American populations the high-latitude allele, as compared to the low-latitude allele, confers decreased egg-to-adult survival (viability), larger body size and increased insulin signaling (as measured by effects on expression of the *insulin-like receptor* (*InR*), a major transcriptional target of *foxo*; see Puig 2003; Puig & Tjian 2014). Moreover, we found that both alleles exhibited plastic responses to temperature and diet in the direction predicted based on previous studies of thermal and dietary plasticity, however, we found little evidence for genotype by environment interactions (GxE). Thus, even though we did not find strong patterns of GxE that might be indicative of local adaptation, our experiments provide robust evidence that this natural variant contributes to clinal adaptation across latitude.

In Chapter 3 my collaborators from Paul Schmidt's laboratory at the University of Pennsylvania (Philadelphia, USA) and I directly compared the effects of the *foxo* variant with novel phenotypic data collected across the North American east coastal cline by the Schmidt lab. For the natural populations along the cline, we confirmed the existence of the previously described body size cline but also identified a new cline for starvation resistance, indicating that flies from high latitudes are more starvation resistant than flies from low latitudes. This new finding is consistent with the notion that flies from high-latitude populations are typically more stress resistant and longer-lived than flies from low latitudes (see Chapter 3 for a more detailed discussion). Notably, for body size the patterns of differentiation between the two alternative *foxo* alleles mirrored those observed in natural populations, thus suggesting that the *foxo* variant makes a major contribution to the size cline. For developmental time, we failed to observe a clinal pattern both for natural populations as well as for the *foxo* variant (results of developmental time assays performed in Lausanne also failed to yield a clear allelic difference; data not shown).

One strength of our studies of the *foxo* variant is that we performed independent experiments in two laboratories, here in Lausanne as well as in Philadelphia. On the positive side, our assays under different laboratory conditions mutually confirmed that the clinal *foxo* variant has clear-cut, robust and replicable effects on various size-related traits and that these effects go, as mentioned above, in the direction that is predicted from the overall body size cline along the North American east coast. However, an important caveat is that the same was unfortunately not true for starvation resistance: this fact is reflected in the contrasting results and conclusions in Chapters 2 versus 3. For the presentation of this thesis we have opted to present the two complementary studies as independent stand-alone studies, to be judged based on their individual merit and internal self-consistency. We are currently in the process of trying to get to the bottom of this discrepancy; this will obviously be important before we can submit our two complementary studies for publication. In principle, it is possible that the discrepant results for starvation resistance in Chapters 2 versus 3 are due to subtle differences in laboratory and assay conditions between the laboratories in Lausanne and Philadelphia (see, for example, Ackermann *et al*. 2001). For example, differences in larval density and crowding might affect the expression of starvation resistance (Zwaan *et al.* 1991); indeed, larval density was rather tightly controlled in our assays in Lausanne but a bit more laxly so in the assays in Philadelphia. Similarly, infections with *Drosophila* C virus (which can often go undetected) can lead to gut blockage and nutritional stress which might potentially exacerbate the effects of starvation stress (Zwaan *et al.* 1991; Zinke *et al.* 2002; Chtarbanova *et al.* 2014). We note that during the Philadelphia assays there was indeed a small issue with a viral infection, resulting in the exclusion of two out of three experimental blocks in the starvation assay (see Chapter 3 for details). We can thus not fully exclude the possibility that the third (apparently not infected) block might have suffered from an unnoticed viral infection; yet, even if this would have been the case, it is not easy to see why this would revert the latitudinal

directionality of the allelic effects for starvation resistance (see Chapters 2 and 3). For the time being, we stand by our results and conclusions from our work here in Lausanne: we conclude that the low-latitude allelic state is more starvation resistant than the high-latitude allele. If this is correct, it implies that – in contrast to its effects on body size, the *foxo* variant exhibits a countergradient effect (see discussion in Chapter 2). Whatever the case in terms of the true allelic directionality of these effects, it is important to keep in mind that both studies found significant differences in starvation resistance between the two allelic states that are highly unlikely due to chance.

Importantly, our experiments in Chapters 2 and 3 represent the first evidence for life-history effects of natural alleles at this locus, confirming our previous genomic analysis which suggested that clinally varying SNPs in *foxo* might contribute to clinal adaptation (Fabian *et al.* 2012), similar to previous findings for a clinal (indel) variant at the *InR* locus (Paaby *et al.* 2010; 2014). Together with previous studies (Paaby *et al.* 2010; 2014), our work thus clearly demonstrates that variation in IIS can make an important and – at least partly – predictable contribution to clinal life-history adaptation in *Drosophila*. It will clearly be of great interest to examine other clinally varying loci affecting the IIS pathway.

The "gold standard" for testing natural variants is the manipulation and replacement of naturally occurring alleles in controlled isogenic background by using homologous recombination. While this is technically feasible nowadays, it still technically very challenging, especially when attempting to manipulate single nucleotides. As outlined in Chapter 4, we are currently in the process of using the CRISPR/Cas9 genome editing method to confirm the causative effects of the clinal *foxo* polymorphism in a maximally controlled genetic background (Cong *et al.* 2013). In contrast to an earlier unsuccessful attempt, we are now using a modified, improved protocol; we should hopefully know in a few months whether homologous recombination has successfully worked in our hands. If successful, this

approach will allow us to investigate the alleles at the two *foxo* SNP positions singly as well as in all pairwise combinations, thereby permitting us to investigate additive versus epistatic effects.

The Contribution of a Chromosomal Inversion Polymorphism to Clinal Adaptation

In the second part of my thesis, in Chapters 5 and 6, I investigated the phenotypic effects of an adaptive, clinally varying chromosomal inversion polymorphism, *In(3R)Payne*, along the North American east coast. The *In(3R)Payne* inversion has long been thought to be a major driver or adaptive trait clines, both in North America and Australia (Weeks *et al.* 2002; Kapun *et al.* 2016a).

In Chapter 5, consistent with previous previous data from Australia showing that this inversion contributes to the body size cline on this continent (Kennington *et al.* 2007; Kapun *et al.* 2016b), we found that *In(3R)Payne* also contributes to the body size cline along the North American east coast (Kapun *et al.* 2016a). Our new data thus reveal for the first time that this inversion has parallel effects on body size along two independent clinal gradients, thus demonstrating that this inversion is maintained, at least partly, by spatially varying selection acting on body size on two continents. As discussed in Chapter 5, an important reason for why *In(3R)Payne* is maintained along latitudinal clines must somehow have to do with spatially varying selection on body size. Moreover, it is worth pointing out that the effect sizes (i.e., Cohen's standardized effect size) were extremely similar between the previous Australian findings and our results for the North American cline. Interestingly, *In(3R)Payne* harbors several loci that are known from developmental genetics studies to be involved in growth regulation and the determination of adult body size (see Chapter 5 for details).

In Chapter 6, we discovered a new role for *In(3R)Payne* in affecting three survival traits that are known to vary clinally across latitude. Consistent with previous clinal findings, we

observed that high-latitude flies are on average longer-lived and more stress resistant than low-latitude flies (Schmidt *et al.* 2000; a; b; Schmidt & Paaby 2008; Mathur & Schmidt 2017). More interestingly, we found that at least part of the latitudinal differentiation for these survival traits is explained by the clinal distribution of *In(3R)Payne* inversion polymorphism itself: overall, standard arrangement flies from Maine and Florida tended to be longer-lived and more stress resistant than inverted arrangement flies from Florida. Thus, our results show that *In(3R)Payne* underpins, at least to some degree, the latitudinal clines in these survival traits. Together with the fact that *In(3R)Payne* harbors many clinally varying SNPs in genes known to affect body size, lifespan and stress resistance (Kapun *et al.* 2016a), our results demonstrate that this inversion affects multiple clinally varying life-history traits, not just body size as previously suspected (Rako *et al.* 2006; Kapun *et al.* 2016b). In terms of plasticity and GxE across two growth temperatures, we found several (relatively minor) karyotype-by-environment interactions. Most interestingly, inversion homokaryons were more starvation resistant than standard homokaryons from Florida, yet this effect was rather small and only observed at 18°C.

While our results clearly establish major effects of this chromosomal inversion on clinally varying life-history traits, they are not sufficient to explain how this inversion polymorphism in maintained in natural populations along the cline (also see Kapun *et al.* 2016a): most of the fitness-related traits examined seem to be negatively affected by the inverted arrangement. For example, as is discussed in more detail in Chapter 6, it would be of great future interest to examine heterokaryons of this inversion and test whether *In(3R)Payne* might be subject to fitness overdominance. Similarly, the climatic (and other environmental) factors, and thus the selective factors, that underlie the latitudinal cline in the frequency of this inversion are incompletely understood (see Kapun *et al.* 2016a). Thus, the exact nature of the selective forces that maintain this inversion polymorphism remain to be elucidated in future work.

Conclusions

Genome-wide studies have identified many candidate genes and alleles that might contribute to life-history adaptation in natural populations (e.g., along putatively adaptive clinal gradients) in *Drosophila* and other organisms, but causative confirmation of such genomic candidates ultimately requires functional testing and experimentation. In my Ph.D. work I have therefore set out to examine two putatively adaptive, clinally varying polymorphisms in *D. melanogaster*, which have been previously identified through genomic analysis (e.g., see Fabian *et al*. 2012), by experimentally investigating their phenotypic effects upon fitness-related traits. In my experiments, I was able to show that both clinal polymorphisms indeed contribute – as predicted – to previously observed phenotypic patterns of clinal differentiation along the North American east coast, thus confirming that these candidate variants are shaped by spatially varying selection. Thus, together with a small handful of previous studies (e.g., Paaby *et al.* 2010, 2014), my experiments represent an important proof-of-principle: it is possible to experimentally isolate and examine clinally varying polymorphisms and to show that they contribute to presumably adaptive phenotypic clines. More specifically, my studies provide the first experimental evidence for life-history effects of a natural polymorphism in *foxo*, a central transcription factor of the insulin signaling pathway, as well as for causative associations between the chromosomal inversion polymorphism *In(3R)Payne*, body size and survival traits along the North American cline. In sum, my Ph.D. dissertation demonstrates that both clinal polymorphisms are very likely to be *bona fide* targets of spatially varying (clinal) selection in *D. melanogaster*.

Outlook

Currently, some efforts are still ongoing in our laboratory to further examine the effects of these clinal polymorphisms, including assays involving CRISPR/Cas9 applied to the *foxo* polymorphism; RNA-sequencing of *In(3R)P* homokaryons; and so forth.

With regard to the inversion, it would for example be important and interesting to learn more about the mode and nature of selection that maintains the *In(3R)Payne* inversion polymorphic, for instance by carrying out population cage experiments to test for balancing and/or frequency-dependent selection, with cages being seeded at different starting proportions of inverted vs. standard homokaryons. Similarly, in my experiments on this inversion I have only used homokaryons, but it will be critically important to examine heterokaryons, for the polymorphism might be maintained by overdominance. In addition, it would be of great interest to use reciprocal transplant experiments in order to test for effects of local adaptation conferred by *In(3R)P*, e.g. using outdoor population cages in Florida versus Maine. This would provide novel insights into the underlying selective forces that maintain this inversion polymorphism.

With regard to the insulin signaling pathway it would for example be tempting to functionally test the effects of other clinally varying SNPs in genes other than *foxo* (i.e., *InR*, *14-3-ε*, *Tobi* and *PI3K*) by reconstituting outbred populations or by using using CRISPR/Cas9. Interestingly, for instance, our laboratory's recent work has identified an extremely strongly differentiated SNP in *InR* that is nearly completely fixed for alternative alleles between inversion vs. standard homokaryons of *In*(*3R*)*P*.

Together, such future experiments would help significantly to further advance our understanding of the genetic basis of clinal adaptation and of spatially varing selection in *Drosophila melanogaster* and beyond.

References

Ackermann M, Bijlsma R, James AC, Partridge L, Zwaan BJ, Stearns SC (2001) Effects of assay conditions in life history experiments with *Drosophila melanogaster*. *Journal of Evolutionary Biology*, **14**, 199-209.

Adrion JR, Hahn MW, Cooper BS (2015) Revisiting classic clines in *Drosophila melanogaster* in the age of genomics. *Trends in Genetics*, **31**, 434–444.

Alic N, Giannakou ME, Papatheodorou I *et al.* (2014) Interplay of *dfoxo* and two ETS-family transcription factors determines lifespan in *Drosophila melanogaster*. (SK Kim, Ed,). *PLoS Genetics*, **10**, e1004619.

Barbieri M, Bonafè M, Franceschi C, Paolisso G (2003) Insulin/IGF-I-signaling pathway: an evolutionarily conserved mechanism of longevity from yeast to humans. *American Journal of Physiology - Endocrinology And Metabolism*, **285**, E1064–71.

Barthel A, Schmoll D, Unterman TG (2005) *Foxo* proteins in insulin action and metabolism. *Trends in Endocrinology & Metabolism*, **16**, 183–189.

Brogiolo W, Stocker H, Ikeya T *et al.* (2001) An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. *Current Biology*, **11**, 213–221.

Chtarbanova S, Lamiable O, Lee K-Z *et al.* (2014) *Drosophila* C virus systemic infection leads to intestinal obstruction. *Journal of virology*, **88**, 14057–14069.

Cong L, Ran FA, Cox D *et al.* (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science*, **339**, 819–823.

Das D, Arur S (2017) Conserved insulin signaling in the regulation of oocyte growth, development, and maturation. *Molecular Reproduction and Development*, 1–52.

David JR, Capy P (1988) Genetic variation of *Drosophila melanogaster* natural populations. *Trends in Genetics*, **4**, 106–111.

De Jong G, Bochdanovits Z (2003) Latitudinal clines in *Drosophila melanogaster*: body size, allozyme frequencies, inversion frequencies, and the insulin-signalling pathway. *Journal of Genetics*, **82**, 207–223.

Fabian DK, Kapun M, Nolte V *et al.* (2012) Genome-wide patterns of latitudinal differentiation among populations of *Drosophila melanogaster* from North America. *Molecular Ecology*, **21**, 4748–4769.

Flatt T, Min KJ, D'Alterio C *et al.* (2008) *Drosophila* germ-line modulation of insulin signaling and lifespan. *Proceedings of the National Academy of Sciences*, **105**, 6368–6373.

Jünger MA, Rintelen F, Stocker H *et al.* (2003) The *Drosophila* forkhead transcription factor *FOXO* mediates the reduction in cell number associated with reduced insulin signaling. *Journal of biology*, **2**, 20.

Kapun M, Fabian DK, Goudet J, Flatt T (2016a) Genomic evidence for adaptive inversion clines in *Drosophila melanogaster*. *Molecular Biology and Evolution*, **33**, 1317–1336.

Kapun M, Schmidt C, Durmaz E, Schmidt PS, Flatt T (2016b) Parallel effects of the inversion *In(3R)Payne* on body size across the North American and Australian clines in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, **29**, 1059–1072.

Kennington WJ, Hoffmann AA, Partridge L (2007) Mapping regions within cosmopolitan inversion *In(3R)Payne* associated with natural variation in body size in *Drosophila melanogaster*. *Genetics*, **177**, 549–556.

Kolaczkowski B, Kern AD, Holloway AK, Begun DJ (2011) Genomic differentiation between temperate and tropical Australian populations of *Drosophila melanogaster*. *Genetics*, **187**, 245–260.

Kramer JM, Davidge JT, Lockyer JM, Staveley BE (2003) Expression of *Drosophila FOXO*

regulates growth and can phenocopy starvation. *BMC developmental biology*, **3**, 5.
Kramer JM, Slade JD, Staveley BE (2008) *foxo* is required for resistance to amino acid starvation in *Drosophila*. *Genome*, **51**, 668–672.
Mathur V, Schmidt PS (2017) Adaptive patterns of phenotypic plasticity in laboratory and field environments in *Drosophila melanogaster*. *Evolution*, **71**, 465–474.
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**, 3395–3409.
Paaby AB, Blacket MJ, Hoffmann AA, Schmidt PS (2010) Identification of a candidate adaptive polymorphism for *Drosophila* life history by parallel independent clines on two continents. *Molecular Ecology*, **19**, 760–774.
Puig O (2003) Control of cell number by *Drosophila FOXO*: downstream and feedback regulation of the insulin receptor pathway. *Genes & Development*, **17**, 2006–2020.
Puig O, Tjian R (2014) Nutrient Availability and Growth: Regulation of Insulin Signaling by *dfoxo*/*FOXO*1. *Cell Cycle*, **5**, 503–505.
Rako L, Anderson AR, Sgrò CM, Stocker AJ, Hoffmann AA (2006) The association between inversion *In(3R)Payne* and clinally varying traits in *Drosophila melanogaster*. *Genetica*, **128**, 373–384.
Robinson SJW, Zwaan B, Partridge L (2000) Starvation resistance and adult body composition in a latitudinal cline of *Drosophila melanogaster*. *Evolution*, **54**, 1819.
Schmidt PS, Paaby AB (2008) Reproductive diapause and life-history clines in North American populations of *Drosophila melanogaster*. *Evolution*, **62**, 1204–1215.
Schmidt PS, Duvernell DD, Eanes WF (2000) Adaptive evolution of a candidate gene for aging in *Drosophila*. *Proceedings of the National Academy of Sciences*, **97**, 10861–10865.
Schmidt PS, Matzkin L, Ippolito M, Eanes WF (2005a) Geographic variation in diapause incidence, life-history traits, and climatic adaptation in *Drosophila melanogaster*. *Evolution*, **59**, 1721–1732.
Schmidt PS, Paaby AB, Heschel MS (2005b) Genetic variance for diapause expression and associated life histories in *Drosophila melanogaster*. *Evolution*, **59**, 2616–2625.
Schrider DR, Hahn MW, Begun DJ (2016) Parallel evolution of copy-number variation across continents in *Drosophila melanogaster*. *Molecular Biology and Evolution*, msw014.
Slack C, Giannakou ME, Foley A, Goss M, Partridge L (2011) *dfoxo*-independent effects of reduced insulin-like signaling in *Drosophila*. *Aging Cell*, **10**, 735–748.
Swanson EM, Dantzer B (2014) Insulin-like growth factor-1 is associated with life-history variation across Mammalia. *Proceedings. Biological sciences / The Royal Society*, **281**, 20132458.
Tang HY, Smith-Caldas MSB, Driscoll MV, Salhadar S, Shingleton AW (2011) *FOXO* regulates organ-specific phenotypic plasticity in *Drosophila* (E Hafen, Ed,). *PLoS Genetics*, **7**, e1002373.
Wadgymar SM, Daws SC, Anderson JT (2017) Integrating viability and fecundity selection to illuminate the adaptive nature of genetic clines. *Evolution Letters*, **1**, 26–39.
Weeks AR, McKechnie SW, Hoffmann AA (2002) Dissecting adaptive clinal variation: markers, inversions and size/stress associations in *Drosophila melanogaster* from a central field population. *Ecology Letters*, **5**, 756–763.
Yamamoto R, Tatar M (2011) Insulin receptor substrate *chico* acts with the transcription factor *FOXO* to extend *Drosophila* lifespan. *Aging Cell*, **10**, 729–732.
Zheng X, Yang Z, Yue Z, Alvarez JD, Sehgal A (2007) *FOXO* and insulin signaling regulate sensitivity of the circadian clock to oxidative stress. *Proceedings of the National Academy of Sciences*, **104**, 15899–15904.

Zinke I, Schütz CS, Katzenberger JD, Bauer M, Pankratz MJ (2002) Nutrient control of gene expression in *Drosophila*: microarray analysis of starvation and sugar-dependent response. *The EMBO Journal*, **21**, 6162–6173.

Zwaan BJ, Bijlsma R, Hoekstra RF (1991) On the developmental theory of ageing. I. starvation resistance and longevity in *Drosophila melanogaster* in relation to pre-adult breeding conditions. *Heredity*, **66**, 29–39.