

# **A clinal polymorphism in the insulin signaling transcription factor**

## ***foxo* contributes to life-history adaptation in *Drosophila***

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Running head: Adaptive Clinal Polymorphism in *Drosophila*

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# Abstract

A fundamental aim of adaptation genomics is to identify polymorphisms that underpin variation in fitness traits. In *D. melanogaster* latitudinal life-history clines exist on multiple continents and make an excellent system for dissecting the genetics of adaptation. We have previously identified numerous clinal SNPs in insulin/insulin-like growth factor signaling (IIS), a pathway known from mutant studies to affect life history. However, the effects of natural variants in this pathway remain poorly understood. Here we investigate how two clinal alternative alleles at *foxo*, a transcriptional effector of IIS, affect fitness components (viability, size, starvation resistance, fat content). We assessed this polymorphism from the North American cline by reconstituting outbred populations, fixed for either the low- or high-latitude allele, from inbred DGRP lines. Since diet and temperature modulate IIS, we phenotyped alleles across two temperatures (18°C, 25°C) and two diets differing in sugar source and content. Consistent with clinal expectations, the high-latitude allele conferred larger body size and reduced wing loading. Alleles also differed in starvation resistance and expression of *InR*, a transcriptional target of FOXO. Allelic reaction norms were mostly parallel, with few GxE interactions. Together, our results suggest that variation in IIS makes a major contribution to clinal life-history adaptation.

**KEY WORDS:** cline, life history, adaptation, insulin signaling, pleiotropy, plasticity

# Introduction

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 and Rose 1995; Oldham and Hafen 2003; Tatar et al. 2003; Fielenbach and Antebi 2008; Kenyon 2010; Flatt and Partridge 2018), but loci identified in such laboratory analyses do not necessarily harbor segregating alleles that would contribute to genetic variance for traits in natural populations (Flatt 2004; Flatt and Schmidt 2009; Vonesch et al. 2016; Birney 2016; Fabian et al. 2018). In particular, the identity and presumably subtle effects of naturally occurring life-history polymorphisms are poorly known (Flatt and Schmidt 2009; Paaby and Schmidt 2009; Flatt and 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 and Hoekstra 2011; Turner 2014; Flatt 2016; Siddiq et al. 2017). 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 and 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 ~15,000 years ago and subsequently colonized the rest of  
the world (David and Bocquet 1975; David and Capy 1988; de Jong and  
Bochdanovits 2003; Hoffmann and 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 and Capy  
1988; de Jong and Bochdanovits 2003; Paaby and 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 and Bochdanovits 2003; Schmidt et al. 2005a,  
2005b; Hoffmann and Weeks 2007; Schmidt and Paaby 2008; Kolaczowski 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 likely adaptive (Coyne and Beecham  
1987; Schmidt et al. 2000; Weeks et al. 2002; de Jong and Bochdanovits 2003;

Schmidt et al. 2005a, 2005b; Hoffmann and Weeks 2007; Schmidt and Paaby 2008; Adrion et al. 2015; Fabian et al. 2015; Kapun et al. 2016a).

To begin to identify the genetic basis of life-history clines in *D. melanogaster*, we have previously performed genome-wide analyses of latitudinal differentiation along the North American cline (Fabian et al. 2012; Kapun et al. 2016b) (also see Turner et al. 2008; Bergland et al. 2014; Reinhardt et al. 2014; Machado et al. 2018). Our analysis based on SNP  $F_{ST}$  outliers uncovered pervasive genome-wide patterns of clinality, with hundreds of clinal SNPs mapping to loci involved in the insulin/insulin-like growth factor signaling (IIS)/target of rapamycin (TOR), ecdysone, torso, EGFR, TGF $\beta$ /BMP, JAK/STAT, lipid metabolism, immunity and circadian rhythm pathways (Fabian et al. 2012). Many of the identified variants also exhibit parallel differentiation in Australia (Fabian et al. 2012; Kapun et al. 2016b; also cf. Kolaczowski 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; Duchon et al. 2013; Kao et al. 2015; Bergland et al. 2016). Identifying adaptive clinal variants as targets of selection thus requires comparing clinal patterns against neutral expectations and – optimally – functional genetic testing (Barrett and Hoekstra 2011; Kapun et al. 2016a, 2016b; Flatt 2016). To date, however, functional analyses of clinal polymorphisms that are potentially subject to spatially varying selection remain scarce (for some exceptions see e.g. Schmidt et al. 2008; McKechnie et al. 2010; Paaby et al. 2010; Lee et al. 2013; Paaby et al. 2014; Kapun et al. 2016a; Durmaz et al. 2018; Svetec et al. 2018).

Interestingly, many of the pathways that harbor clinal loci are known from molecular studies to be implicated in the physiological regulation of life history in organisms such as *C. elegans*, *Drosophila* and the mouse (see Tatar et al. 2003; Fielenbach and Antebi 2008; Flatt and Heyland 2011; Flatt et al. 2013; and references therein). In particular, we found strongly clinal SNPs in multiple components of the IIS/TOR pathway, including SNPs in *Drosophila insulin-like peptide* genes *dilp3* and *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)*, 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 and Yin 2001; Clancy et al. 2001; Kenyon 2001; Oldham et al. 2002; Oldham and Hafen 2003; Holzenberger et al. 2003; Tatar et al. 2001; 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 and 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 contribute to 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* (Jovelín 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 and Swanson 2011; Swanson and Dantzer 2014). Together, these findings suggest that allelic variation in IIS/TOR might profoundly affect life-history adaptation, but experimental evidence remains scarce (Flatt et al. 2013; Flatt and Partridge 2018).

Here we provide a comprehensive examination of the life-history effects of a clinally varying 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; Kramer et al. 2008; Hwangbo et al. 2004; Puig and Tijan 2005; Fielenbach and 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 (Flachsbar et al. 2009; Willcox et al. 2008). Natural *foxo* variants thus represent promising candidates for mediating life-history variation in natural populations.

From our previous population genomic data based on three population along the North American cline (Fabian et al. 2012) we identified two strongly clinally varying alternative *foxo* alleles, as defined by 2 focal SNPs, whose frequencies change across latitude by ~60% between Florida and Maine. Here we characterize the effects of these clinal *foxo* genotypes on several fitness-related traits (egg-to-adult

survival, proxies of size, starvation resistance, fat content) by measuring phenotypes on replicate populations of the two alternative alleles under different environmental assay conditions in the laboratory. Since temperature gradients are thought to underpin – at least partly – latitudinal clines (e.g., de Jong and Bochdanovits 2003; Kapun et al. 2016b; and references therein), and because both diet and temperature modulate IIS (e.g., Britton et al. 2002; Kramer et al. 2003; Puig and Tijan 2005; Giannakou et al. 2008; Teleman 2010; Puig and Mattila 2011; Li and Gong 2015; Zhang et al. 2015), we phenotyped replicated population cage cultures of the alternative alleles at two temperatures (18°C, 25°C) and on two commonly used diets that differ mainly in their sugar source (sucrose vs. molasses) and content.

Measuring reaction norms to assess phenotypic plasticity and genotype-by-environment interactions ( $G \times E$ ) for this variant is interesting because still relatively little is known about whether and how clinality and plasticity interact (James et al. 1997; de Jong and 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 and Schmidt 2017; van Heerwaarden and Sgrò 2017). For example, *D. melanogaster* feeds and breeds on various kinds of rotting fruit, with the protein:carbohydrate (P:C) ratios exhibiting spatiotemporal variation (Lachaise et al. 1988; Hoffmann and McKechnie 1991; Markow et al. 1999; Keller 2007), but how dietary plasticity affects traits in a clinal context remains largely unclear. Similarly, the interplay between thermal plasticity and adaptation is incompletely understood (e.g., de Jong and Bochdanovits 2003; Overgaard et al. 2011; Mathur and Schmidt 2017; van Heerwaarden and Sgrò 2017).

We give predictions for the expected phenotypic effects of the *foxo* variant in terms of clinality, plasticity and the physiology of IIS in the Methods section. Our results



show that the *foxo* polymorphism affects multiple fitness components according to these predictions and suggest that it contributes to clinal life-history adaptation; we also find that the alternative alleles respond plastically to temperature and diet but with little evidence for G x E.

## Methods

### IDENTIFICATION AND ISOLATION OF THE *FOXO* POLYMORPHISM

We identified two strongly clinal SNPs in *foxo* in the genomic data of Fabian et al. (2012) by using an  $F_{ST}$  outlier approach: an A/G polymorphism at position 3R: 9892517 (position in the *D. melanogaster* reference genome v.5.0;  $F_{ST}$  = 0.48 between Florida and Maine) and a T/G polymorphism at position 3R: 9894559 ( $F_{ST}$  = 0.42 between Florida and Maine) (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, Maine; Fabian et al. 2012), both SNPs are also strongly clinal in a more comprehensive dataset from 10 populations along the cline (Betancourt et al. 2018), collected by the *Drosophila* Real Time Evolution Consortium (DrosRTEC; Bergland et al. 2014; Kapun et al. 2016b; Machado et al. 2018). 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). Because

the two *foxo* SNPs are located relatively closely to each other (~2 kb apart; Fig. S1A, Supporting Information), we decided to study them experimentally in combination, as alternative 2-SNP alleles. Indeed, as shown in Fig. S1B (Supporting Information), the two focal *foxo* SNPs are in perfect linkage disequilibrium (LD;  $r^2 = 1$ ), without any significant LD in-between the two sites. Although these SNPs do not fall outside the empirical neutral distribution (based on 20,000 SNPs in short introns), they do fall in the tails of the distribution (Betancourt et al. 2018); further work will thus be required to assess whether the allele frequency observations are consistent with neutral demographic history or if a model of spatially varying or balancing selection needs to be invoked.

To isolate the two alternative *foxo* alleles 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. This 'reconstituted or recombinant outbred population' (ROP) or 'Mendelian randomization' approach produces populations that are consistently and completely fixed for the two alternative allelic states to be compared, with the rest of the genetic background being randomized (see Behrman et al. 2018 and Lafuente et al. 2018 for examples using this method). 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 population cages (Fig. S3, Table S1, Supporting Information). ROP cages were established from the DGRP lines by Betancourt et al. (2018); F2 flies were transferred to Lausanne for establishing cages at our laboratory.

Genomic analysis of the DGRP lines used to set up the ROP cages showed that sets A and B versus sets C and D were completely fixed ( $F_{ST} = 1$ ) for the HL and LL

alleles, respectively; this also showed that, although there exist other SNPs that are strongly differentiated ( $F_{ST} > 0.5$ ) between the HL and LL populations, the majority of them are different between the independently replicated sets (blocks) of DGRP lines used to make the HL vs. LL contrast (Betancourt et al. 2018). Such SNPs, which are specific to a given set of lines, do not make a consistent contribution to the overall HL vs. LL contrast.

The most parsimonious interpretation of our results is therefore that the effects reported below are caused by the two *foxo* SNPs which we have studied. However, we cannot completely rule out that other (causative) sites are potentially in long-range LD with our focal SNPs (see Fig. S1B, Supporting Information). A conservative interpretation of our results is thus to view the two focal *foxo* SNPs as representing 'tags' or markers for functionally significant variants segregating at the *foxo* locus that are in LD with the causative site(s), similar to those used in genome-wide association studies (GWAS; e.g., Wang et al. 2010).

## POPULATION CAGES

Population cages were maintained at 25°C, 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 the phenotypic assays population cages were kept for 10 generations to allow for free recombination among lines within each cage and allelic state and to homogenize (randomize) differences in genomic background between the two allelic states to be compared. Before setting up assays, we kept cages for 2 generations under common garden conditions (room

temperature: ~22°C, ~10:14 h light:dark, ~50% humidity). Thus, phenotypes were measured after a total of 12 generations of recombination.

## **PHENOTYPE ASSAYS**

Assays reported here were performed in our laboratory in Lausanne; independent assays were performed under constant environmental conditions by Betancourt et al. (2018), thus allowing us to assess the reproducibility of our results and to identify potential differences in assay conditions between laboratories (cf. Ackermann et al. 2001).

In generation 13 (see above) 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 commonly used diets that differ mainly in their sugar source (sucrose [cornmeal-agar-yeast-sucrose] vs. molasses [cornmeal-agar-yeast-molasses] diet and their protein:carbohydrate ratio (P:C ~1:3.6 vs. ~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<sub>2</sub> 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  $\times$  8 cages  $\times$  4 conditions = 160 vials).

Body size was examined by measuring three proxies: wing area, thorax length and femur length ( $N = 26$ -30 wings, 9-15 thoraxes, 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<sup>2</sup>). 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). 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 (but not wing area) measurements were repeated three times per individual (see below for estimates of 'repeatability'). 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); reduced wing loading (i.e., increased wing dimensions relative to body size) can improve flight performance at low temperature (Frazier et al. 2008).

To measure starvation resistance (i.e., survival upon starvation) we placed flies into vials containing 0.5% agar/water medium and scored the duration of survival (h) upon starvation every 6 h until all flies had died ( $N = 5 \text{ vials} \times 10 \text{ flies per vial} \times 2 \text{ sexes} \times 8 \text{ cages} \times 4 \text{ conditions} = 320 \text{ vials or } 3200 \text{ flies}$ ).

Since there is typically a positive correlation between starvation resistance and lipid content (Hoffmann and Harshman 1999), we also determined whole-body triacylglyceride (TAG) content (in  $\mu\text{g}$  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 ( $8 \text{ cages} \times 4 \text{ conditions} \times 2 \text{ treatments} \times 10 \text{ replicates} = 640 \text{ homogenates}$ ). 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  $\mu\text{g}$ ).

## 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* mRNA (Puig et al. 2003; Puig and 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  $\times$  4 conditions  $\times$  3 biological replicates  $\times$  3 technical replicates = 288 samples). Relative transcript abundance was normalized by using *Actin5C* 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  $\Delta CT$  method. We used the following primer sequences (Casas-Tinto et al. 2007; Ponton et al. 2011): *Actin5C forward*, 5'-GCGTCGGTCAATTCAATCTT-3'; *Actin5C reverse*, 5'-AAGCTGCAACCTCTTCGTCA-3'; *InR forward*, 5'-CACAAGCTGGAAAGAAAGTGC-3'; *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), sex (*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:  $y = A + T + D + A \times T + A \times D + T \times D + A \times T \times D + S(A) + C(A,S)$ , where *y* denotes the response variable (trait). For simplicity, the sexes were analyzed separately (i.e., to reduce the number of higher-order interactions).

For starvation resistance we measured age at death from multiple individuals per replicate vial; we thus estimated and accounted for the random effect of vial (*V*),

nested within the combination of *A*, *S* and *C*, using restricted maximum likelihood (REML) (see Supporting Information for these estimates).

Viability (proportion) data were arcsine square-root transformed prior to analysis. ANOVA on thorax and femur length data was performed using means across 3 measures per individual. From the repeat measurements of these traits on the same individuals, we estimated the ‘repeatability’ of our measurements (i.e., the intraclass correlation; see Whitlock and Schluter 2009) by performing random-effects ANOVAs with REML. Overall, repeatability was very high for femur length (~91.9% for females; 94.4% for males) but less so for thorax length (~29.9% for females; 36.6% for males) (details not shown). Because wings and thoraces were measured on separate individuals, analysis of wing:thorax ratio was performed 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.,  $A \times Tr$ ;  $A \times D \times Tr$ ) test for allelic differences in fat loss upon starvation. For simplicity, this analysis was performed separately for the two rearing temperatures.

To estimate the magnitude of the allelic effects of the *foxo* polymorphism upon the assayed fitness components we calculated Cohen's *d* (Table S3, Supporting Information), a standardized measure of effect size (i.e., a signal to noise ratio, defined as the difference between two means divided by their pooled standard deviation) (Cohen 1988; Sawilowsky 2009). Low values of Cohen's *d* (e.g., 0.01) are commonly interpreted as representing very small effect sizes, whereas effect sizes >0.8 are interpreted as being qualitatively large to very large (Sawilowsky 2009).

We also estimated the relative contribution of the *foxo* polymorphism assayed in our laboratory to the overall phenotypic cline for wing area, recently measured on flies from 6 populations along the North American east coast (Betancourt et al. 2018;



using flies assayed on molasses diet at 25°C). We calculated the proportional contribution of the polymorphism to the overall cline as follows:  $\Delta_{foxo} \times \Delta_{frequency} / \Delta_{cline}$ , where  $\Delta_{foxo}$  is the difference in mean wing area between the HL and LL alleles,  $\Delta_{frequency}$  is the allele frequency gradient for the polymorphism between cline ends (Maine vs. Florida, ~60%) and  $\Delta_{cline}$  is the difference in mean wing area between cline ends.

## PREDICTIONS

Here we make some 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,  $G \times E$ , and local adaptation. We compare our results to these predictions in the Results section below.

(1) Latitudinal clinality. Traits which have been found to covary positively with latitude include, for example, 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 (e.g., Coyne and Beecham 1987; Azevedo et al. 1998; Bochdanovits and de Jong 2003a; de Jong and Bochdanovits 2003; Schmidt et al. 2005a, 2005b; Folguera et al. 2008; Schmidt and Paaby 2008; Bhan et al. 2014; Mathur and Schmidt 2017; Durmaz et al. 2018). For some traits clinal patterns have been observed in a parallel fashion on multiple continents, but there can also be major differences among continents (e.g., see discussion in Fabian et al. 2015); for example, contrasting predictions have been made for viability (van 't Land et al. 1999), starvation resistance (Karan et al. 1998, Robinson et al. 2002; Hoffmann et al. 2005; Goenaga et al. 2013) and heat tolerance (Hoffmann et al. 2002; Sgrò et al. 2010).

In general, we would expect that the effects of the high- and low-latitude *foxo* alleles agree with the overall phenotypic patterns across latitude, especially for those traits that have previously been examined along the North American cline (e.g., Coyne and Beecham 1987; Schmidt and Paaby 2008; Paaby et al. 2014; Kapun et al. 2016a; Mathur and Schmidt 2017; Durmaz et al. 2018).

(2) IIS. Traits that are associated 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 and Hafen 2003; Broughton et al. 2005; Teleman 2010). For example, loss-of-function (LOF) 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; Giannakou et al. 2008; Slack et al. 2011); the effects of IIS (or of *foxo*) on viability are, however, not well understood. Conversely, overexpression of *foxo* has opposite effects on most of these traits (e.g., increased lifespan), yet – like LOF alleles – 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 naturally occurring *foxo* alleles tested here differ consistently along this IIS/*foxo* axis of trait covariation. Notably, traits observed in flies from high- versus low-latitude populations in North America resemble those of flies with low versus high IIS, respectively (e.g., de Jong and 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 tend to be 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,  $G \times E$ , and local adaptation. With regard to thermal effects, we would 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, 1994b; James and Partridge 1995; Bochdanovits and de Jong 2003b; Trotta et al. 2006; Folguera et al. 2008; Klepsatel et al. 2013, 2014; Mathur and Schmidt 2017; cf. Hoffmann et al. 2005 for a contrasting prediction for starvation survival).

With respect to dietary effects, higher P:C ratios, for instance, might be expected to cause increased viability, larger size but reduced starvation resistance (Lee and Jang 2014; Lihoreau et al. 2016; Reis 2016). In terms of  $G \times E$ , 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 and Schmidt 2017).

## Results

The clinal *foxo* polymorphism examined here (or causative SNPs in LD with it; see caveat in the Methods section) impacted all fitness components assayed (Table 1; Tables S3 and S4, Supporting Information), including significant effects on egg-to-adult survival (viability) (qualitatively moderate to large effects, as measured by Cohen's *d*), femur length (very small to medium), wing area (medium), thorax length (very small to very large), starvation resistance (very small to medium), and lipid content (very small to large effects).

## ALLELIC VARIATION AT *FOXO* AFFECTS VIABILITY

The *foxo* polymorphism significantly affected 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 had an effect, with viability being higher on sucrose than on molasses diet (Fig. 2; Table 1). We did not find any evidence for  $G \times E$  interactions affecting this trait.

### CLINAL FOXO ALLELES DIFFER IN BODY SIZE

Since both latitude and IIS affect size (de Jong and 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 S4, Supporting Information), and wing:thorax ratio than the LL allele (Fig. 4; Table 1; for thorax data see Fig. S6; Table S4, Supporting Information). These results are consistent with the positive size cline in North America (Coyne and Beecham 1987) and with reduced wing loading at high latitude (Azevedo et al. 1998; Bhan et al. 2014). Remarkably, with regard to wing area, we estimate that the *foxo* polymorphism makes a proportional contribution of ~14% to the total cline for wing area (females:  $\Delta_{foxo} \times \Delta_{frequency} / \Delta_{cline} \approx 0.017 \times 0.6 / 0.074 \approx 0.138$ ; males:  $\Delta_{foxo} \times \Delta_{frequency} / \Delta_{cline} \approx 0.019 \times 0.6 / 0.083 \approx 0.137$ ) – this represents a major contribution to the wing size cline along the North American east coast (Coyne and Beecham 1987; Betancourt et al. 2018).

For all size traits, females were larger than males (Fig. 3; Fig. 4; Table 1; Fig. S5; Fig. S6; Table S4, Supporting Information), as is typically observed. 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 S4, Supporting Information), as is 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 S4, Supporting Information), perhaps in line with the observation that more carbohydrate-rich diets cause smaller size (Reis 2016); however, wing area and wing:thorax ratio were larger on molasses than on sucrose diet (Fig. S5; Table S4, Supporting Information; and Fig. 4; Table 1). Although we found a few  $G \times E$  interactions for size traits (Fig. 4; Fig. 5; Table 1; Fig. S5; Fig. S6; Table S4, Supporting Information), the allelic reaction norms were overall remarkably parallel across environmental conditions.

## **POLYMORPHISM AT *FOXO* IMPACTS STARVATION AND FAT CATABOLISM**

The *foxo* alleles also differed in their effects on resistance to (survival of) starvation in females (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 (e.g., Schmidt and Paaby 2008; Mathur and Schmidt 2017), LL females were more resistant than HL females (Fig. 5; Table 1), suggesting a countergradient effect; in males, there were no allelic differences in resistance (Fig. S7; Table S4, Supporting Information; for estimates of the variance components of the random effect of vial see Table S5, Supporting Information). Overall females were more resistant than males (Fig. 5; Table 1; Fig. S7; Table S4, 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 S4, Supporting Information), as previously reported (Mathur and Schmidt 2017). Flies

raised on molasses diet were more resistant than those raised on sucrose diet (Fig. 5; Table 1; Fig. S7; Table S4, Supporting Information), potentially in support of the finding that lower P:C ratios favor higher resistance (Chippindale et al. 1993; Lee and 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 S4, Supporting Information).

To further examine the physiological basis of starvation resistance 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, 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 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 and 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 S6, 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 S6, Supporting Information).

## Discussion

### CONNECTING ADAPTIVE CLINAL PHENOTYPES TO GENOTYPES

Here we have studied the life-history effects of a strongly clinally varying, presumably adaptive polymorphism in the IIS gene *foxo*, a naturally segregating variant identified from our previous genomic analysis of the North American latitudinal cline (Fabian et al. 2012).

As hypothesized by de Jong and Bochdanovits (2003), genes of the IIS/TOR pathway might represent particularly promising candidates underlying clinal life-history adaptation in *D. melanogaster*: (1) laboratory mutants in this pathway often mirror life-history traits and trade-offs observed in natural populations (de Jong and 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; Flatt and Partridge 2018); (2) reproductive dormancy in response to cool temperature and short photoperiod, a genetically variable and clinal trait (Williams and Sokolowski 1993; Schmidt et al. 2005a; Schmidt and Conde 2006; Schmidt et al. 2005b; Schmidt and 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; Andreatta et al. 2018); (3) genomic analyses of clinal differentiation has identified many clinal SNPs in the IIS/TOR pathway presumably shaped by spatially varying selection (Fig. 1;

Kolaczkowski et al. 2011; Fabian et al. 2012; Kapun et al. 2016b); and (4) genome-wide analyses of variation in size-related traits have identified novel regulators of growth, several of which interact with the IIS/TOR pathway (Vonesch et al. 2016; Strassburger et al. 2017). For example, in support of the idea that variation in IIS contributes to clinal adaptation in *D. melanogaster*, Paaby and colleagues have identified a clinal indel polymorphism in *InR* with pleiotropic effects on development, body size, fecundity, lifespan, oxidative stress resistance, chill coma recovery, and insulin signaling (Paaby et al. 2010, 2014). Our results on *foxo* lend further support to the hypothesis of de Jong and 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 yet 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 therefore 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 not predict with certainty the directionality and/or the degree of pleiotropy of the allelic effects *a priori*, we found that the *foxo* polymorphism



differentially affects several size-related traits and starvation resistance, phenotypes known to be affected by the *foxo* locus. With regard to growth and size, our findings from natural variants agree well with functional genetic studies showing that genetic manipulations of the *foxo* locus affect 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 yet 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 and Tjian 2005). Indeed, the LL allele had higher *InR* mRNA 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, 1994b; Lee and Jang 2014; Lihoreau et al. 2016; Mathur and Schmidt 2017), yet we found very little evidence for allele by environment ( $G \times E$ ) interactions.

While our experimental design does not allow us to disentangle the contribution of the 2 individual SNPs to the total effects seen for the *foxo* polymorphism, our results suggest that the naturally occurring alternative alleles at *foxo* we have examined here – and which are defined by only two linked SNP positions – can apparently have quite strong pleiotropic (or, via LD, correlational) effects upon multiple complex life-history traits, including on viability, several proxies of size and on starvation resistance (for estimates of allelic effect sizes see Table S4, Supporting Information). This is

consistent with the pleiotropic effects seen in *foxo* loss-of-function mutant alleles (see references above) and might support the idea that the architecture of life-history traits, which are connected via multiple trade-offs, is inherently pleiotropic (Williams 1957; Finch and Rose 1995; Flatt et al. 2005; Flatt and Promislow 2007; Flatt and Schmidt 2009; Flatt et al. 2013; Paaby et al. 2014); it also provides a contrast to the model from evo-devo which posits that most evolutionarily relevant mutations should exhibit little or no pleiotropy (Stern 2011). In particular, the pleiotropic effects of the *foxo* variant might explain why this polymorphism might be maintained, through some form of balancing selection, in natural populations along the cline.

## **INSULIN SIGNALING, CLINALITY, AND COUNTERGRADIENT VARIATION**

How does the *foxo* variant contribute to phenotypic clines observed across latitude? High-latitude flies tend to be characterized, for example, by larger body size, decreased fecundity, longer lifespan and improved stress resistance as compared to low-latitude flies, and this differentiation is genetically based (Coyne and Beecham 1987; Schmidt et al. 2005a, 2005b; Schmidt and Paaby 2008; Mathur and Schmidt 2017; Durmaz et al. 2018). Do the allelic effects go in the same direction as the latitudinal gradient, representing cogradients, or do certain allelic effects run counter to the cline, representing countergradient variation (Levins 1968; Conover and Schultz 1995)? Cogradients occur 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 and Schultz 1995; Marcil et al. 2006).

Consistent with clinal expectation, the HL allele confers larger size (Coyne and Beecham 1987; de Jong and 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; Frazier et al. 2008; 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, the *foxo* variant contributes to the observed phenotypic cline in the predicted direction (gradient or cogradient variation) and appears to be 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). Importantly, our results for size-related traits are consistent with independent assays under constant environmental conditions (Betancourt et al. 2018) and suggest a major contribution of the *foxo* polymorphism to the clinality of body size .

For starvation resistance, we found – contrary to clinal predictions – that the HL allele is less resistant than the LL allele, consistent with countergradient variation. Interestingly, a similar countergradient effect (on body size) was found for the *InR* polymorphism mentioned above: the high-latitude *InR<sup>short</sup>* allele confers smaller size, even though flies from high-latitude populations are normally larger (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). However, as mentioned in the methods, we can of course not completely rule out potentially confounding LD effects that might account for this unexpected result with regard to starvation resistance.

In terms of the physiological effects of IIS, 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 (de Jong and

Bochdanovits 2003). 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.

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 clinal selection and the genotype-phenotype map underlying clinal adaptation (Lee et al. 2013; Paaby et al. 2014; Flatt 2016).

With regard to starvation resistance, a caveat is that we found the LL allele to be more resistant, while Betancourt et al. (2018) found the HL allele to be more resistant. This discrepancy might be due to differences in assay protocols: Betancourt et al. (2018) did not use agar which could impose some desiccation in addition to starvation stress. Interestingly, desiccation resistance is known to vary latitudinally along the North America east coast (Rajpurohit et al. 2018), but whether the *foxo* polymorphism examined here affects survival upon desiccation remains unknown and awaits future study.

## **GROWING EVIDENCE FOR A ROLE OF IIS IN LIFE-HISTORY ADAPTATION**

The IIS pathway provides an excellent example of how mechanistic and evolutionary insights might be combined to gain a more complete understanding of the ultimate and proximate determinants of life-history adaptation (Finch and Rose 1995; Houle

2001; Flatt and 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 and Gems 2002; Tatar et al. 2003; Flatt et al. 2005; Flatt and Heyland 2011; Flatt et al. 2013). In particular, these studies have revealed that the IIS pathway plays an evolutionarily conserved role in the physiological regulation of longevity (Partridge and Gems 2002; Tatar et al. 2003); they have also given us some of the clearest examples of alleles exhibiting antagonistic pleiotropy (Williams 1957; Flatt and Promislow 2007; and references above).

The functional characterization of this pathway therefore promised an opportunity for evolutionary geneticists to identify natural variants involved in life-history evolution (de Jong and Bochdanovits 2003). Yet, ‘life history loci’ identified via functional genetic analysis need not necessarily contribute to standing variation for these traits in the wild (Flatt 2004; Flatt and Schmidt 2009; Fabian et al. 2018). For some time, it thus remained unclear whether natural variation in this pathway impacts variation in fitness-related traits in natural populations (see Reznick 2005; Fabian et al. 2018).

Today, we have growing evidence that variation in IIS indeed can make an important contribution to life-history variation in flies and other insects, worms, fish, reptiles and mammals, including effects on longevity in humans (e.g., de Jong and Bochdanovits 2003; Williams et al. 2006; Flachsbarth 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 and Page 2010; Dantzer and Swanson 2012; Jovelín et al. 2014; Paaby et al. 2014; Swanson and Dantzer 2014; McGaugh et al. 2015; Schwartz and

Bronikowski 2016; Zhao et al. 2016; and references therein). On the other hand, 'evolve and resequence' studies of *Drosophila* longevity have failed to find a major contribution of standing variation in IIS to evolved changes in life history and lifespan, perhaps suggesting that the IIS pathway might be selectively constrained, at least with regard to the evolution of certain traits (e.g., Remolina et al. 2012; Fabian et al. 2018; Flatt and Partridge 2018). In sum, this body of work illustrates how one might be able to connect genotypes to molecular mechanisms to components of fitness by studying a fundamentally important physiological pathway from multiple angles (Finch and Rose 1995; Houle 2001; Flatt and Heyland 2011; Flatt et al. 2013).

## Conclusions

Here we have found that a strongly clinal polymorphism at the *foxo* locus (which might be viewed as a marker for functionally significant alleles) has effects on several fitness components known to vary across latitude, including viability, size-related traits, starvation resistance and fat content. The directionality of most of these effects matches overall phenotypic clines observed along the North American east coast, especially with regard to size (e.g., Coyne and Beecham 1987; Schmidt et al. 2005a, 2005b; Schmidt and Paaby 2008; Durmaz et al. 2018). Together, our results thus suggest that standing variation in the IIS pathway makes an important and – at least partly – predictable contribution to life-history clines in *Drosophila*.

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## LITERATURE CITED

- Ackermann, M., Bijlsma, R., James, A. C., Partridge, L., Zwaan, B. J., and S. C. Stearns 2001. Effects of assay conditions in life-history experiments with *Drosophila melanogaster*. J. Evol. Biol. 14:199-209.
- Adrion, J.R., Hahn, M. W., and B. S. Cooper. 2015. Revisiting classic clines in *Drosophila melanogaster* in the age of genomics. Trends Genet. 31:434-444.
- Alvarez-Ponce, D., Aguadé, M., and J. Rozas. 2009. Network-level molecular evolutionary analysis of the insulin/TOR signal transduction pathway across 12 *Drosophila* genomes. Genome Res. 19:234-242.
- Andreatta, G., Kyriacou, C. P., Flatt, T., and R. Costa. 2018. Aminergic Signaling Controls Ovarian Dormancy in *Drosophila*. Sci. Rep. 8:2030.
- Artimo, P., Jonnalagedda, M., Arnold, K., Baratin, D., Csardi, G., de Castro, E., Duvaud, S., Flegel, V., Fortier, A., Gasteiger, E., Grosdidier, A., Hernandez, C.,

775 Ioannidis, V., Kuznetsov, D., Liechti, R., Moretti, S., Mostaguir, K., Redaschi, N.,  
776 Rossier, G., Xenarios, I., and H. Stockinger. 2012. ExPASy: SIB bioinformatics  
777 resource portal. *Nucleic Acids Res.* 40:W597-W603.

778 Attrill, H., Falls, K., Goodman, J. L., Millburn, G. H., Antonazzo, G., Rey, A. J.,  
779 Marygold, S. J., and the FlyBase Consortium. 2016. FlyBase: establishing a Gene  
780 Group resource for *Drosophila melanogaster*. *Nucleic Acids Res.* 44:D786-D792.

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

784 Barrett, R. D. H., and H. E. Hoekstra. 2011. Molecular spandrels: tests of adaptation  
785 at the genetic level. *Nature Rev. Genet.* 12:767-780.

786 Barson, N. J., Aykanat, T., Hindar, K., Baranski, M., Bolstad, G. H., Fiske, P., Jacq,  
787 C., Jensen, A. J., Johnston, S. E., Karlsson, S., Kent, M., Moen, T., Niemelä, E.,  
788 Nome, T., Næsje, T. F., Orell, P., Romakkaniemi, A., Sægrov, H., Urdal, K.,  
789 Erkinaro, J., Lien, S., and C. R. Primmer. 2015. Sex-dependent dominance at a  
790 single locus maintains variation in age at maturity in salmon. *Nature* 528:405-408.

791 Bergland, A. O., Behrman, E. L., O'Brien, K. R., Schmidt, P. S., and D. A. Petrov.  
792 2014. Genomic evidence of rapid and stable adaptive oscillations over seasonal  
793 time scales in *Drosophila*. *PLoS Genet.* 10:e1004775.

794 Bergland, A. O., Tobler, R., González, J., Schmidt, P., and D. A. Petrov. 2016.  
795 Secondary contact and local adaptation contribute to genome-wide patterns of  
796 clinal variation in *Drosophila melanogaster*. *Mol. Ecol.* 25:1157-1174.

797 Behrman, E. L., Howick, V. M., Kapun, M., Staubach, F., Bergland, A. O., Petrov, D.  
798 A., Lazzaro, B. P., and P. S. Schmidt. 2018. Rapid seasonal evolution in innate



799 immunity of wild *Drosophila melanogaster*. Proc. Roy. Soc. London B  
800 285:20172599.

801 Betancourt, N. J., Rajpuorhit, S., Durmaz, E., Kapun, M., Fabian, D.K., Flatt, T., and  
802 P. S. Schmidt. 2018. Allelic polymorphism at *foxo* contributes to local adaptation in  
803 *Drosophila melanogaster*. Preprint, bioRxiv, doi: <https://doi.org/10.1101/471565>

804 Bhan, V., Parkash, R., and D. D. Aggarwal. 2014. Effects of body-size variation on  
805 flight-related traits in latitudinal populations of *Drosophila melanogaster*. J. Genet.  
806 93:103-112.

807 Birney, E. 2016. The Mighty Fruit Fly Moves into Outbred Genetics. PLoS Genet. 12:  
808 e1006388.

809 Bochdanovits, Z., and G. de Jong. 2003a. Temperature dependence of fitness  
810 components in geographical populations of *Drosophila melanogaster*: changing the  
811 association between size and fitness. Biol. J. Linnean Soc. 80:717-725.

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

815 Böhni, R., Riesgo-Escovar, J., Oldham, S., Brogiolo, W., Stocker, H., Andruss, B. F.,  
816 Beckingham, K., and E. Hafen. 1999. Autonomous control of cell and organ size by  
817 CHICO, a *Drosophila* homolog of vertebrate IRS1-4. Cell 97:865-875.

818 Britton, J., Lockwood, W., Li, L., Cohen, S. M., and B. A. Edgar. 2002. *Drosophila's*  
819 insulin/PI3-kinase pathway coordinates cellular metabolism with nutritional  
820 conditions. Dev. Cell 2:239-249.

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

824 Broughton, S. J., Piper, M. D. W., Ikeya T., Bass, T. M., Jacobson, J., Driege, Y.,  
825 Martinez, P., Hafen, E., Withers, D. J., Leivers, S. J., and L. Partridge. 2005.  
826 Longer lifespan, altered metabolism, and stress resistance in *Drosophila* from  
827 ablation of cells making insulin-like ligands. Proc. Natl. Acad. Sci. U.S.A. 102:3105-  
828 3110.

829 Casas-Tinto, S., Marr II, M. T., Andreu, P., and O. Puig. 2007. Characterization of the  
830 *Drosophila* insulin receptor promoter. Biochim. Biophys. Acta 1769:236-243.

831 Catalán, A., Glaser-Schmitt, A., Argyridou, E., Duchen, P., and J. Parsch. 2016. An  
832 Indel Polymorphism in the *MtnA* 3' Untranslated Region Is Associated with Gene  
833 Expression Variation and Local Adaptation in *Drosophila melanogaster*. PLoS  
834 Genet. 12:e1005987.

835 Chen, Y., Lee, S. F., Blanc, E., Reuter, C., Wertheim, B., Martinez-Diaz, P.,  
836 Hofmann, A. A., and L. Partridge. 2012. Genome-Wide Transcription Analysis of  
837 Clinal Genetic Variation in *Drosophila*. PLoS ONE 7:e34620.

838 Chippindale, A. K., Leroi, A. M., Kim, S. B., and M. R. Rose. 1993. Phenotypic  
839 plasticity and selection in *Drosophila* life-history evolution. I. Nutrition and the cost  
840 of reproduction. J. Evol. Biol. 6:171-193.

841 Clancy, D. J., Gems, D., Harshman, L. G., Oldham, S., Stocker, H., Hafen, E.,  
842 Leivers, S. J., and L. Partridge. 2001. Extension of life-span by loss of CHICO, a  
843 *Drosophila* insulin receptor substrate protein. Science 292:104-106.

844 Clemson, A. S., Sgrò, C. M., and M. Telonis-Scott. 2016. Thermal plasticity in  
845 *Drosophila melanogaster* populations from eastern Australia: quantitative traits to  
846 transcripts. J. Evol. Biol. 29:2447-2463.

847 Cogni, R., Kuczynski, K., Koury, S., Lavington, E., Behrman, E. L., O'Brien, K. R.,  
848 Schmidt, P. S., and W. F. Eanes. 2017. On the Long-term Stability of Clines in  
849 Some Metabolic Genes in *Drosophila melanogaster*. Sci. Rep. 7:42766.

850 Cohen, J. 1988. Statistical power analysis for the behavioral sciences. 2nd edition.  
851 Lawrence Earlbaum, Hillsdale (NJ).

852 Conover, D. O., and E. T. Schultz. 1995. Phenotypic similarity and the evolutionary  
853 significance of countergradient variation. Trends Ecol. Evol. 10:248-252.

854 Cooper, B. S., Tharp II, J. M., Jernberg I. I., and M. J. Angilletta Jr. 2012.  
855 Developmental plasticity of thermal tolerances in temperate and subtropical  
856 populations of *Drosophila melanogaster*. J. Therm. Biol. 37:211-216.

857 Coyne, J. A., and E. Beecham. 1987. Heritability of Two Morphological Characters  
858 Within and Among Natural Populations of *Drosophila melanogaster*. Genetics 117:  
859 727-737.

860 Dantzer, B., and E. M. Swanson. 2011. Mediation of vertebrate life histories via  
861 insulin-like growth factor-1. Biol. Rev. 87:414-429.

862 David, J. R., and C. Bocquet. 1975. Evolution in a cosmopolitan species: genetic  
863 latitudinal clines in *Drosophila melanogaster* wild populations. Experientia 31:164-  
864 166.

865 David, J. R., and P. Capi. 1988. Genetic variation of *Drosophila melanogaster*  
866 natural populations. Trends Genet. 4:106-111.

867 David, J. R., Moreteau, B., Gauthier, J. P., Pétavy, G., Stockel, A., and A. G.  
868 Imasheva. 1994. Reaction Norms of Size Characters in Relation to Growth  
869 Temperature in *Drosophila melanogaster* - an Isofemale Lines Analysis. Genet. Sel.  
870 Evol. 26:229-251.

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

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

877 Durmaz, E., Benson, C., Kapun, M., Schmidt, P., and T. Flatt. 2018. An Inversion  
878 Supergene in *Drosophila* Underpins Latitudinal Clines in Survival Traits. J. Evol.  
879 Biol. 31:1354-1364.

880 Endler, J. A. 1977. Geographic Variation, Speciation and Clines. Princeton Univ.  
881 Press, Princeton, NJ.

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

885 Fabian, D. K., Lack, J. B., Mathur, V., Schlötterer, C., Schmidt, P. S., Pool, J. E., and  
886 T. Flatt. 2015. Spatially varying selection shapes life-history clines among  
887 populations of *Drosophila melanogaster* from sub-Saharan Africa. J. Evol. Biol.  
888 28:826-840.

889 Fabian, D.K., Garschall, K., Klepsatel, P., Santos-Matos, G., Sucena, E., Kapun, M.,  
890 Lemaitre, B., Schlötterer, C., Arking, R., and T. Flatt. 2018. Evolution of longevity  
891 improves immunity in *Drosophila*. Evol. Lett. 2:567-579.

892 Fielenbach, N., and A. Antebi. 2008. *C. elegans* dauer formation and the molecular  
893 basis of plasticity. Genes Dev. 22:2149-2165.

894 Finch, C. E., and M. R. Rose. 1995. Hormones and the physiological architecture of  
895 life history evolution. Quart. Rev. Biol. 70:1-52.

896 Flachsbarth, F., Caliebe, A., Kleindorff, R., Blanché, H., von Eller-Eberstein, H.,  
897 Nikolaus, S., Schreiber, S., and A. Nebel. 2009. Association of FOXO3A variation  
898 with human longevity confirmed in German centenarians. Proc. Natl. Acad. Sci.  
899 U.S.A. 106:2700-2705.

900 Flatt, T. 2004. Assessing natural variation in genes affecting *Drosophila* lifespan.  
901 Mech. Ageing Dev. 125:155-159.

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

904 Flatt, T., and A. Heyland. 2011. Mechanisms of Life History Evolution. Oxford  
905 University Press, Oxford.

906 Flatt, T., and L. Partridge. 2018. Horizons in the evolution of aging. BMC Biol.  
907 16(1):93.

908 Flatt, T., and D. E. L. Promislow. 2007. Still pondering an age-old question. Science  
909 318:1255-1256.

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

912 Flatt, T., Tu, M.-P., and M. Tatar. 2005. Hormonal pleiotropy and the juvenile  
913 hormone regulation of *Drosophila* development and life history. BioEssays 27:999-  
914 1010.

915 Flatt, T., Amdam, G. V., Kirkwood, T. B. L., and S. W. Omholt. 2013. Life-History  
916 Evolution and the Polyphenic Regulation of Somatic Maintenance and Survival.  
917 Quart. Rev. Biol. 88:185-218.

918 Folguera, G., Ceballos, S., Spezzi, L., Fanara, J. J., and E. Hasson. 2008. Clinal  
919 variation in developmental time and viability, and the response to thermal  
920 treatments in two species of *Drosophila*. Biol. J. Linnean Soc. 95:233-245.

921 Frazier, M. R., Harrison, J. F., Kirkton, S. D., and S. P. Roberts. 2008. Cold rearing  
922 improves cold-flight performance in *Drosophila* via changes in wing morphology. *J.*  
923 *Exp. Biol.* 211: 2116-2122.

924 Gems, D., Sutton, A. J., Sundermeyer, M. L., Albert, P. S., King, K. V., Edgley, M. L.,  
925 Larsen, P. L., and D. L. Riddle. 1998. Two pleiotropic classes of *daf-2* mutation  
926 affect larval arrest, adult behavior, reproduction and longevity in *Caenorhabditis*  
927 *elegans*. *Genetics* 150:129-155.

928 Giannakou, M. E., and L. Partridge. 2007. Role of insulin-like signalling in *Drosophila*  
929 lifespan. *Trends Biochem. Sci.* 32:180-188.

930 Giannakou, M. E., Goss, M., Jünger, M. A., Hafen, E., Leivers, S. J., and L.  
931 Partridge. 2004. Long-lived *Drosophila* with overexpressed dFOXO in adult fat  
932 body. *Science* 305:361.

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

936 Gilchrist, A. S., Azevedo, R. B. R., Partridge, L., and P. O'Higgins. 2000. Adaptation  
937 and constraint in the evolution of *Drosophila melanogaster* wing shape. *Evol. Dev.*  
938 2:114-124

939 Goenaga, J., Fanara, J. J., and E. Hasson. 2010. A quantitative genetic study of  
940 starvation resistance at different geographic scales in natural populations of  
941 *Drosophila melanogaster*. *Genet. Res.* 92:253-259.

942 Goenaga, J., Fanara, J. J., and E. Hasson. 2013. Latitudinal Variation in Starvation  
943 Resistance is Explained by Lipid Content in Natural Populations of *Drosophila*  
944 *melanogaster*. *Evol. Biol.* 40:601-612.

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

948 Hoffmann, A. A., and S. W. McKechnie. 1991. Heritable Variation in Resource  
 949 Utilization and Response in a Winery Population of *Drosophila melanogaster*.  
 950 *Evolution* 45:1000-1015.

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

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

956 Hoffmann, A. A., Shirriffs, J., and M. Scott. 2005. Relative importance of plastic vs  
 957 genetic factors in adaptive differentiation: geographical variation for stress  
 958 resistance in *Drosophila melanogaster* from eastern Australia. *Func. Ecol.* 19:222-  
 959 227.

960 Holzenberger, M., Dupont, J., Ducos, B., Leneuve, P., G  lo  n, A., Even, P. C.,  
 961 Cervera, P., and Y. Le Bouc. 2003. IGF-1 receptor regulates lifespan and  
 962 resistance to oxidative stress in mice. *Nature* 421:182-187.

963 Houle, D. 2001. Characters as the Units of Evolutionary Change. Pp. 109-140 in G.  
 964 P. Wagner, ed. *The Character Concept in Evolutionary Biology*. Academic Press,  
 965 San Diego, CA.

966 Hwangbo, D. S., Gersham, B., Tu, M.-P., Palmer, M., and M. Tatar. 2004. *Drosophila*  
 967 dFOXO controls lifespan and regulates insulin signalling in brain and fat body.  
 968 *Nature* 429:562-566.

969 James, A. C., and L. Partridge. 1995. Thermal evolution of rate of larval development  
970 in *Drosophila melanogaster* in laboratory and field populations. J. Evol. Biol. 8:315-  
971 330.

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

975 Johnston, S. E., Gratten, J., Berenos, C., Pilkington, J. G., Clutton-Brock, T. H.,  
976 Pemberton, J. M., and J. Slate. 2013. Life history trade-offs at a single locus  
977 maintain sexually selected genetic variation. Nature 502:93-95.

978 Jones, F. C., Grabherr, M. G., Chan, Y. F., Russell, P., Mauceli, E., Johnson, J.,  
979 Swofford, R., Pirun, M., Zody, M. C., White, S., Birney, E., Searle, S., Schmutz, J.,  
980 Grimwood, J., Dickson, M. C., Myers, R. M., Miller, C. T., Summers, B. R., Knecht,  
981 A. K., Brady, S. D., Zhang, H., Pollen, A. A., Howes, T., Amemiya, C., Broad  
982 Institute Genome Sequencing Platform & Whole Genome Assembly Team,  
983 Baldwin, J., Bloom, T., Jaffe, D. B., Nicol, R., Wilkinson, J., Lander, E. S., Di Palma,  
984 F., Lindblad-Toh, K., and D. M. Kingsley. 2012. The genomic basis of adaptive  
985 evolution in threespine sticklebacks. Nature 484:55-61.

986 Jovelin, R., Comstock, J. S., Cutter, A. D., and P.C. Phillips. 2014. A Recent Global  
987 Selective Sweep on the *age-1* Phosphatidylinositol 3-OH Kinase Regulator of the  
988 Insulin-Like Signaling Pathway Within *Caenorhabditis remanei*. G3 (Bethesda)  
989 4:1123-1133.

990 Jünger, M. A., Rintelen, F., Stocker, H., Wasserman, J. D., Végh, M., Radimerski, T.,  
991 Greenberg, M. E., and E. Hafen. 2003. The *Drosophila* forkhead transcription factor  
992 FOXO mediates the reduction in cell number associated with reduced insulin  
993 signaling. J. Biol. 2(3):20.



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

998 Kapun, M., Schmidt, C., Durmaz, E., Schmidt, P. S., and T. Flatt. 2016a. Parallel  
 999 effects of the inversion *In(3R)Payne* on body size across the North American and  
 1000 Australian clines in *Drosophila melanogaster*. *J. Evol. Biol.* 29:1059-1072.

1001 Kapun, M., Fabian, D. K., Goudet, J., and T. Flatt. 2016b. Genomic Evidence for  
 1002 Adaptive Inversion Clines in *Drosophila melanogaster*. *Mol. Biol. Evol.* 33:1317-  
 1003 1336.

1004 Karan, D., Dahiya, N., Munjal, A. K., Gibert, P., Moreteau, B., Parkash, R., and J. R.  
 1005 David. 1998. Desiccation and Starvation Tolerance of Adult *Drosophila*: Opposite  
 1006 Latitudinal Clines in Natural Populations of Three Different Species. *Evolution*  
 1007 52:825-831.

1008 Keller, A. 2007. *Drosophila melanogaster's* history as a human commensal. *Curr.*  
 1009 *Biol.* 17:R77-R81.

1010 Kenyon, C. 2001. A conserved regulatory system for aging. *Cell* 105:165-168.

1011 Kenyon, C. J. 2010. The genetics of ageing. *Nature* 464:504-512.

1012 Kenyon, C., Chang, J., Gensch, E., Rudner, A., and R. Tabtiang. 1993. A *C. elegans*  
 1013 mutant that lives twice as long as wild type. *Nature* 366:461-464.

1014 Klepsatel, P., Gálíková, M., De Maio, N., Huber, C. D., Schlötterer, C., and T. Flatt.  
 1015 2013. Variation in Thermal Performance and Reaction Norms Among Populations  
 1016 of *Drosophila melanogaster*. *Evolution* 67:3573-3587.

1017 Klepsatel, P., Gálíková, M., Huber, C. D., and T. Flatt. 2014. Similarities and  
1018 differences in altitudinal versus latitudinal variation for morphological traits in  
1019 *Drosophila melanogaster*. *Evolution* 68:1385-1398.

1020 Kolaczowski, B., Kern, A. D., Holloway, A. K., and D. J. Begun. 2011. Genomic  
1021 differentiation between temperate and tropical Australian populations of *Drosophila*  
1022 *melanogaster*. *Genetics* 187:245–260.

1023 Kramer, J. M., Davidge, J. T., Lockyer, J. M., and B. E. Staveley. 2003. Expression of  
1024 *Drosophila* FOXO regulates growth and can phenocopy starvation. *BMC Dev. Biol.*  
1025 3:5.

1026 Kramer, J. M., Slade, J. D., and B. E. Staveley. 2008. *foxo* is required for resistance  
1027 to amino acid starvation in *Drosophila*. *Genome* 51:668-672.

1028 Kubrak, O. I., Kučerová, L., Theopold, U., and D. R. Nässel. 2014. The Sleeping  
1029 Beauty: How Reproductive Diapause Affects Hormone Signaling, Metabolism,  
1030 Immune Response and Somatic Maintenance in *Drosophila melanogaster*. *PLoS*  
1031 *ONE* 9:e113051.

1032 Lachaise, D., Cariou, M.-L., David J. R., Lemeunier, F., Tsacas, L., and M.  
1033 Ashburner. 1988. Historical biogeography of the *Drosophila melanogaster* species  
1034 subgroup. *Evol. Biol.* 22:159-225.

1035 Lafuente, E., Duneau, D., and P. Beldade. 2018. Genetic basis of thermal plasticity  
1036 variation in *Drosophila melanogaster* body size. *PLOS Genetics* 14: e1007686.

1037 Lee, K. P., and T. Jang. 2014. Exploring the nutritional basis of starvation resistance  
1038 in *Drosophila melanogaster*. *Func. Ecol.* 28:1144-1155.

1039 Lee, S. F., Eyre-Walker, Y. C., Rane, R. V., Reuter, C., Vinti, G., Rako, L., Partridge,  
1040 L., and A. A. Hoffmann. 2013. Polymorphism in the *neurofibromin* gene, *Nf1*, is

1041 associated with antagonistic selection on wing size and development time in  
1042 *Drosophila melanogaster*. Mol. Ecol. 22:2716-2725.

1043 Levine, M. T., Eckert, M. L., and D. J. Begun. 2011. Whole-Genome Expression  
1044 Plasticity across Tropical and Temperate *Drosophila melanogaster* Populations  
1045 from Eastern Australia. Mol. Biol. Evol. 28:249-256.

1046 Levins, R. 1968. Evolution in Changing in Environments. Princeton Univ. Press,  
1047 Princeton, NJ.

1048 Levins, R. 1969. Thermal Acclimation and Heat Resistance in *Drosophila* Species.  
1049 Am. Nat. 103:483-499.

1050 Li, Q., and Z. Gong. 2015. Cold-sensing regulates *Drosophila* growth through insulin-  
1051 producing cells. Nat. Comm. 6:10083.

1052 Libina, N., Berman, J. R., and C. Kenyon. 2003. Tissue-specific activities of C.  
1053 *elegans* DAF-16 in the regulation of lifespan. Cell 115:489-502.

1054 Lihoreau, M., Poissonnier, L.-A., Isabel, G., and A. Dussutour. 2016. *Drosophila*  
1055 females trade off good nutrition with high-quality oviposition sites when choosing  
1056 foods. J. Exp. Biol. 219:2514-2524.

1057 Machado, H. E., Bergland, A. O., O'Brien, K. R., Behrman, E. L., Schmidt, P. S., and  
1058 D. A. Petrov. 2016. Comparative population genomics of latitudinal variation in  
1059 *Drosophila simulans* and *Drosophila melanogaster*. Mol. Ecol. 25:723-740.

1060 Machado, H. E., Bergland, A. O., Taylor, R., Tilk, S., Behrman, E. L., Dyer, K.,  
1061 Fabian, D. K., Flatt, T., González, J., Karasov, T.L., Kozeretska, I., Lazzaro, B. P.,  
1062 Merritt, T. J. S., Pool, J. E., O'Brien, K., Rajpurohit, S., Roy, P. R., Schaeffer, S. W.,  
1063 Serga, S., Schmidt, P., and D. Petrov. 2018. Broad geographic sampling reveals  
1064 predictable and pervasive seasonal adaptation in *Drosophila*. bioRxiv doi:  
1065 <https://doi.org/10.1101/337543>.

1066 Mackay, T. F., Stone, E. A., and J. F. Ayroles. 2009. The genetics of quantitative  
1067 traits: challenges and prospects. *Nat. Rev. Genet.* 10:565-577.

1068 Mackay, T. F. C., Richards, S., Stone E. A., Barbadilla, A., Ayroles, J. F., Zhu, D.,  
1069 Casillas, S., Han, Y., Magwire, M. M., Cridland, J. M., Richardson, M. F., Anholt, R.  
1070 R., Barrón, M., Bess, C., Blankenburg, K. P., Carbone, M. A., Castellano, D.,  
1071 Chaboub, L., Duncan, L., Harris, Z., Javaid, M., Jayaseelan, J. C., Jhangiani, S. N.,  
1072 Jordan, K. W., Lara, F., Lawrence, F., Lee, S. L., Librado, P., Linheiro, R. S.,  
1073 Lyman, R. F., Mackey, A. J., Munidasa, M., Muzny, D. M., Nazareth, L., Newsham,  
1074 I., Perales, L., Pu, L. L., Qu, C., Ràmia, M., Reid, J. G., Rollmann, S. M., Rozas, J.,  
1075 Saada, N., Turlapati, L., Worley, K. C., Wu, Y. Q., Yamamoto, A., Zhu, Y.,  
1076 Bergman, C. M., Thornton, K. R., Mittelman, D., and R. A. Gibbs. 2012. The  
1077 *Drosophila melanogaster* Genetic Reference Panel. *Nature* 482:173-178.

1078 Marcil, J., Swain, D. P., and J. A. Hutchings. 2006. Countergradient variation in body  
1079 shape between two populations of Atlantic cod (*Gadus morhua*). *Proc. Roy. Soc.*  
1080 *London B* 273:217-223.

1081 Markow, T. A., Raphael, B., Dobberfuhl, D., Breitmeyer, C. M., Elser, J. J., and E.  
1082 Pfeiler. 1999. Elemental stoichiometry of *Drosophila* and their hosts. *Func. Ecol.*  
1083 13:78-84.

1084 Mathur, V., and P.S. Schmidt. 2017. Adaptive patterns of phenotypic plasticity in  
1085 laboratory and field environments in *Drosophila melanogaster*. *Evolution* 71:465-  
1086 474.

1087 Mattila, J., Bremer, A., Ahonen, L., Kostianen, R., and O. Puig. 2009. *Drosophila*  
1088 FoxO Regulates Organism Size and Stress Resistance through an Adenylate  
1089 Cyclase. *Mol. Cell Biol.* 29:5357-5365.

1090 Matzkin, L. M., Watts, T. D., and T. A. Markow. 2009. Evolution of stress resistance  
1091 in *Drosophila*: interspecific variation in tolerance to desiccation and starvation.  
1092 Func. Ecol. 23:521-527.

1093 McGaugh, S. E., Bronikowski, A. M., Kuo, C.-H., Reding, D. M., Addis, E. A., Flagel,  
1094 L. E., Janzen, F. J., and T. S. Schwartz. 2015. Rapid molecular evolution across  
1095 amniotes of the IIS/TOR network. Proc. Natl. Acad. Sci. U.S.A. 112:7055-7060.

1096 McKechnie, S. W., Blacket, M. J., Song, S. V., Rako, L., Carroll, X., Johnson, T. K.,  
1097 Jensen, L. T., Lee, S. F., Wee, C. W., and A. A. Hoffmann. 2010. A clinally varying  
1098 promoter polymorphism associated with adaptive variation in wing size in  
1099 *Drosophila*. Mol. Ecol. 19:775-784.

1100 Méndez-Vigo, B., Martínez-Zapater, J. M., and C. Alonso-Blanco. 2013. The  
1101 Flowering Repressor *SVP* Underlies a Novel *Arabidopsis thaliana* QTL Interacting  
1102 with the Genetic Background. PLoS Genet. 9:e1003289.

1103 Murphy, C. T., McCarroll, S. A., Bargmann, C. I., Frasser, A., Kamath, R. S.,  
1104 Ahringer, J., Li, H., and C. Kenyon. 2003. Genes that act downstream of DAF-16 to  
1105 influence the lifespan of *Caenorhabditis elegans*. Nature 424:277-283.

1106 Oldham, S., and E. Hafen. 2003. Insulin/IGF and target of rapamycin signaling: a  
1107 TOR de force in growth control. Trends Cell Biol. 13:79-85.

1108 Oldham, S., Stocker, H., Laffargue, M., Wittwer, F., Wymann, M., and E. Hafen.  
1109 2002. The *Drosophila* insulin/IGF receptor controls growth and size by modulating  
1110 PtdIns P3 levels. Development 129:4103-4109.

1111 Overgaard, J., Kristensen, T. N., Mitchell, K. A., and A. A. Hoffmann. 2011. Thermal  
1112 Tolerance in Widespread and Tropical *Drosophila* Species: Does Phenotypic  
1113 Plasticity Increase with Latitude? Am. Nat. 178:S80-S96.

1114 Paaby, A. B., and P. S. Schmidt. 2009. Dissecting the genetics of longevity in  
1115 *Drosophila melanogaster*. *Fly* (Austin) 3:1-10.

1116 Paaby, A. B., Bergland, A. O., Behrman, E. L., and P. S. Schmidt. 2014. A highly  
1117 pleiotropic amino acid polymorphism in the *Drosophila* insulin receptor contributes  
1118 to life-history adaptation. *Evolution* 68:3395–3409.

1119 Paaby, A. B., Blacket, M. J., Hoffmann, A. A., and P. S. Schmidt. 2010. Identification  
1120 of a candidate adaptive polymorphism for *Drosophila* life history by parallel  
1121 independent clines on two continents. *Mol. Ecol.* 19:760-774.

1122 Partridge, L., and D. Gems. 2002. Mechanisms of ageing: public or private? *Nat.*  
1123 *Rev. Genet.* 3:165-175.

1124 Partridge, L., Barrie, B., Fowler, K., and V. French. 1994a. Evolution and  
1125 development of body size and cell size in *Drosophila melanogaster* in response to  
1126 temperature. *Evolution* 48:1269-1276.

1127 Partridge, L., Barrie, B., Fowler, K., and V. French, V. 1994b. Thermal Evolution of  
1128 Pre-Adult Life-History Traits in *Drosophila melanogaster*. *J. Evol. Biol.* 7:645-663.

1129 Partridge, L., Gems, D., and D. J. Withers, D. J. 2005. Sex and Death: What Is the  
1130 Connection? *Cell* 120:461-472.

1131 Ponton, F., Chapuis, M.-P., Pernice, M., Sword, G. A., and S. J. Simpson. 2011.  
1132 Evaluation of potential reference genes for reverse transcription-qPCR studies of  
1133 physiological responses in *Drosophila melanogaster*. *J. Insect Physiol.* 57:840-850.

1134 Puig, O., and J. Mattila. 2011. Understanding Forkhead Box Class O Function:  
1135 Lessons from *Drosophila melanogaster*. *Antiox. Redox Sign.* 14:635-647.

1136 Puig, O., and R. Tjian. 2005. Transcriptional feedback control of insulin receptor by  
1137 dFOXO / FOXO1. *Genes Dev.* 19:2435-2446.

1138 Puig, O., Marr, M. T., Ruhf, M. L., and R. Tjian. 2003. Control of cell number by  
1139 *Drosophila* FOXO: downstream and feedback regulation of the insulin receptor  
1140 pathway. *Genes Dev.* 17:2006-2020.

1141 Rajpurohit, S., Gefen, E., Bergland, A.O., Petrov, D.A., Gibbs, A.G., and P. S.  
1142 Schmidt. 2018. Spatiotemporal dynamics and genome-wide association genome-  
1143 wide association analysis of desiccation tolerance in *Drosophila melanogaster*. *Mol.*  
1144 *Ecol.* 27:3525-3540.

1145 Reinhardt, J. A., Kolaczowski, B., Jones, C. D., Begun, D. J., and A. D. Kern. 2014.  
1146 Parallel Geographic Variation in *Drosophila melanogaster*. *Genetics* 197:361-373.

1147 Reis, T. 2016. Effects of Synthetic Diets Enriched in Specific Nutrients on *Drosophila*  
1148 Development, Body Fat, and Lifespan. *PLoS ONE* 11:e0146758.

1149 Remolina, S.C., Chang, P.L., Leips, J., Nuzhdin, S.V., and K. A. Hughes. 2012.  
1150 Genomic Basis of Aging and Life History Evolution in *Drosophila melanogaster*.  
1151 *Evolution* 66:3390-3403.

1152 Reznick, D. N. 2005. The genetic basis of aging: an evolutionary biologist's  
1153 perspective. *Sci. Aging Knowl. Env. (SAGE KE)* 11:pe7.

1154 Robinson, S. J. W., Zwaan, B., and L. Partridge. 2000. Starvation Resistance and  
1155 Adult Body Composition in a Latitudinal Cline of *Drosophila melanogaster*.  
1156 *Evolution* 54:1819-1824.

1157 Rockman, M. V. 2012. The QTN program and the alleles that matter for evolution: all  
1158 that's gold does not glitter. *Evolution* 66:1-17.

1159 Sawilowsky, S. 2009. New effect size rules of thumb. *J. Mod. Appl. Stat. Meth.* 8:467-  
1160 474.

1161 Schiesari, L., Andreatta, G., Kyriacou, C. P., O'Connor, M. B., and R. Costa. 2016.  
1162 The Insulin-Like Proteins dILPs-2/5 Determine Diapause Inducibility in *Drosophila*.  
1163 PLoS ONE 11:e0163680.

1164 Schluter, D., Price, T. D., and L. Rowe. 1991. Conflicting selection pressures and life  
1165 history trade-offs. Proc. Roy. Soc. London B 246:11-17.

1166 Schmidt, P. S., and D. R. Conde. 2006. Environmental Heterogeneity and the  
1167 Maintenance of Genetic Variation for Reproductive Diapause in *Drosophila*  
1168 *melanogaster*. Evolution 60:1602-1611.

1169 Schmidt, P. S., and A. B. Paaby. 2008. Reproductive Diapause and Life-History  
1170 Clines in North American Populations of *Drosophila melanogaster*. Evolution  
1171 62:1204-1215.

1172 Schmidt, P. S., Duvernell, D. D., and W. F. Eanes. 2000. Adaptive evolution of a  
1173 candidate gene for aging in *Drosophila*. Proc. Natl. Acad. Sci. U.S.A. 97:10861-  
1174 10865.

1175 Schmidt, P. S., Matzkin, L., Ippolito, M., and W. F. Eanes. 2005a. Geographic  
1176 Variation in Diapause Incidence, Life-History Traits, and Climatic Adaptation in  
1177 *Drosophila melanogaster*. Evolution 59:1721-1732.

1178 Schmidt, P. S., Paaby, A. B., and M. S. Heschel. 2005b. Genetic variance for  
1179 diapause expression and associated life histories in *Drosophila melanogaster*.  
1180 Evolution 59:2616-2625.

1181 Schmidt, P. S., Zhu, C-T., Das, J., Batavia, M., Yang, L., and W. F. Eanes. 2008. An  
1182 amino acid polymorphism in the *couch potato* gene forms the basis for climatic  
1183 adaptation in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U.S.A. 105:16207-  
1184 16211.



1185 Schwartz, T. S., and A. M. Bronikowski. 2016. Evolution and Function of the Insulin  
1186 and Insulin-like Signaling Network in Ectothermic Reptiles: Some Answers and  
1187 More Questions. *Integr. Comp. Biol.* 56:171-184.

1188 Sgrò, C. M., Overgaard, J., Kristensen, T. N., Mitchell, K. A., Cockerell, F. E., and A.  
1189 A. Hoffmann. 2010. A comprehensive assessment of geographic variation in heat  
1190 tolerance and hardening capacity in populations of *Drosophila melanogaster* from  
1191 eastern Australia. *J. Evol. Biol.* 23: 2484-2493.

1192 Siddiq, M. A., Loehlin, D. W., Montooth, K. L., and J. W. Thornton. 2017.  
1193 Experimental test and refutation of a classic case of molecular adaptation in  
1194 *Drosophila melanogaster*. *Nat. Ecol. Evol.* 1(2):0025.

1195 Slack, C., Giannakou, M. E., Foley, A., Goss, M., and L. Partridge. 2011. dFOXO-  
1196 independent effects of reduced insulin-like signaling in *Drosophila*. *Aging Cell* 10:  
1197 735-748.

1198 Sparkman, A. M., Vleck, C. M., and A. M. Bronikowski. 2009. Evolutionary ecology of  
1199 endocrine-mediated life-history variation in the garter snake *Thamnophis elegans*.  
1200 *Ecology* 90:720-728.

1201 Sparkman, A. M., Byars, D., Ford, N. B., and A. M. Bronikowski. 2010. The role of  
1202 insulin-like growth factor-1 (IGF-1) in growth and reproduction in female brown  
1203 house snakes (*Lamprophis fuliginosus*). *Gen. Comp. Endocrinol.* 168:408-414.

1204 Stalker, H. D. 1980. Chromosome-Studies in Wild Populations of *Drosophila*  
1205 *melanogaster*. II. Relationship of Inversion Frequencies to Latitude, Season, Wing-  
1206 Loading and Flight Activity. *Genetics* 95(1):211-223.

1207 Stern, D. L. 2000. Perspective: evolutionary developmental biology and the problem  
1208 of variation. *Evolution* 54:1079-1091.

1209 Stern, D. L., 2011. Evolution, Development, & the Predictable Genome. Roberts &  
1210 Co. Publishers, Greenwood Village, CO.

1211 Strassburger, K., Zoeller, T., Sandmann, T., Leible, S., Kerr, G., Boutros, M., and A.  
1212 A. Teleman. 2017. Sorting & Sequencing Flies by Size: Identification of Novel TOR  
1213 Regulators and Parameters for Successful Sorting. bioRxiv doi:  
1214 <https://doi.org/10.1101/119719>

1215 Stuart, J. A., and M. M. Page. 2010. Plasma IGF-1 is negatively correlated with body  
1216 mass in a comparison of 36 mammalian species. Mech. Ageing Dev. 131:591-598.

1217 Suh, Y., Atzmon, G., Cho, M.-O., Hwang, D., Liu, B., Leahy, D. J., Barzilai, N., and P.  
1218 Cohen. 2008. Functionally significant insulin-like growth factor I receptor mutations  
1219 in centenarians. Proc. Natl. Acad. Sci. U.S.A. 105:3438-3442.

1220 Svetec, N., Saelao, P., Cridland, J. M., Hoffmann, A. A., and D. J. Begun. 2018.  
1221 Functional Analysis of a Putative Target of Spatially Varying Selection in the  
1222 *Menin1* Gene of *Drosophila melanogaster*. G3 (Bethesda), in press.

1223 Swanson, E. M., and B. Dantzer. 2014. Insulin-like growth factor-1 is associated with  
1224 life-history variation across Mammalia. Proc. Roy. Soc. London B 281:20132458.

1225 Tang, H. Y., Smith-Caldas, M. S. B., Driscoll, M. V., Salhadar, S., and A. W.  
1226 Shingleton. 2011. FOXO regulates organ-specific phenotypic plasticity in  
1227 *Drosophila*. PLoS Genet. 7(11):e1002373.

1228 Tatar, M., and C.-M. Yin. 2001. Slow aging during insect reproductive diapause: why  
1229 butterflies, grasshoppers and flies are like forms. Exp. Gerontol. 36:723-738.

1230 Tatar, M., Bartke, A., and A. Antebi. 2003. The Endocrine Regulation of Aging by  
1231 Insulin-like Signals. Science 299:1346-1351.

1232 Tatar, M., Kopelman, A., Epstein, D., Tu, M.-P., Yin, C.-M., and R. S. Garofalo. 2001.  
1233 A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs  
1234 neuroendocrine function. *Science* 292:107-110.

1235 Teleman, A. A. 2010. Molecular mechanisms of metabolic regulation by insulin in  
1236 *Drosophila*. *Biochem. J.* 425:13-26.

1237 Tennessen, J. M., Barry, W. E., Cox, J., and C. S. Thummel. 2014. Methods for  
1238 studying metabolism in *Drosophila*. *Methods* 68:105-115.

1239 Trotta, V., Calboli, F. C. F., Ziosi, M., Guerra, D., Pezzoli, M. C., David, J. R., and S.  
1240 Cavicchi. 2006. Thermal plasticity in *Drosophila melanogaster*: A comparison of  
1241 geographic populations. *BMC Evol. Biol.* 6:67.

1242 Turner, T. L. 2014. Fine-mapping natural alleles: quantitative complementation to the  
1243 rescue. *Mol. Ecol.* 23:2377-2382.

1244 Turner, T. L., Levine, M. T., Eckert, M. L., and D. J. Begun. 2008. Genomic Analysis  
1245 of Adaptive Differentiation in *Drosophila melanogaster*. *Genetics* 179:455-473.

1246 van Heerwaarden, B., and C. M. Sgrò. 2017. The quantitative genetic basis of clinal  
1247 divergence in phenotypic plasticity. *Evolution* 71:2618-2633.

1248 van 't Land, J., van Putten, P., Zwaan, B., Kamping A., and W. van Delden. 1999.  
1249 Latitudinal variation in wild populations of *Drosophila melanogaster*: heritabilities  
1250 and reaction norms. *J. Evol. Biol.* 12:222-232.

1251 Vonesch, S. C., Lamparter, D., Mackay, T. F. C., Bergmann, S., and E. Hafen. 2016.  
1252 Genome-Wide Analysis Reveals Novel Regulators of Growth in *Drosophila*  
1253 *melanogaster*. *PLoS Genet.* 12:e1005616.

1254 Wang, K., Dickson, S. P., Stolle, C. A., Krantz, I. D., Goldstein, D. B., and H.  
1255 Hakonarson. 2010. Interpretation of Association Signals and Identification of Causal  
1256 Variants from Genome-wide Association Studies. *Am. J. Hum. Genet.* 86:730-742.

1257 Weeks, A. R., McKechnie, S. W., and A. A. Hoffmann. 2002. Dissecting adaptive  
1258 clinal variation: markers, inversions and size/stress associations in *Drosophila*  
1259 *melanogaster* from a central field population. Ecol. Lett. 5:756-763.

1260 Whitlock, M. C., and D. Schluter. 2009. The Analysis of Biological Data. Roberts and  
1261 Company Publishers, Greenwood Village, CO.

1262 Willcox, B. J., Donlon, T. A., He, Q., Chen, R., Grove, J. S., Yano, K., Masaki, K. H.,  
1263 Willcox, D. C., Rodriguez, B., and J. D. Curb. 2008. FOXO3A genotype is strongly  
1264 associated with human longevity. Proc. Natl. Acad. Sci. U.S.A. 105:13987-1399.

1265 Williams, G. C. 1957. Pleiotropy, natural selection, and the evolution of senescence.  
1266 Evolution 11:398-411.

1267 Williams, K. D., Busto, M., Suster, M. L., So, M. L., So, A. K.-C., Ben-Shahar, Y.,  
1268 Leivers, S. J., and M. B. Sokolowski. 2006. Natural variation in *Drosophila*  
1269 *melanogaster* diapause due to the insulin-regulated PI3-kinase. Proc. Natl. Acad.  
1270 Sci. U.S.A. 103:15911-15915.

1271 Williams, K. D., and M. B. Sokolowski. 1993. Diapause in *Drosophila melanogaster*  
1272 females: a genetic analysis. Heredity 71:312-317.

1273 Zhang, B., Xiao, R., Ronan, E. A., He, Y., Hsu, A-L., Liu, J., and X. Z. Xu. 2015.  
1274 Environmental Temperature Differentially Modulates *C. elegans* Longevity through  
1275 a Thermosensitive TRP Channel. Cell Rep. 11:1414-1424.

1276 Zhao, L., Wit, J., Svetec, N., and D. J. Begun. 2015. Parallel Gene Expression  
1277 Differences between Low and High Latitude Populations of *Drosophila*  
1278 *melanogaster* and *D. simulans*. PLoS Genet. 11:e1005184.

1279 Zhao, X., Bergland, A. O., Behrman, E. L., Gregory, B. D., Petrov, D. A., and P.S.  
1280 Schmidt. 2016. Global Transcriptional Profiling of Diapause and Climatic Adaptation  
1281 in *Drosophila melanogaster*. Mol. Biol. Evol. 33:707-720.

## DATA ACCESSIBILITY

Phenotypic raw data are available from Dryad at [doi to be added upon acceptance](#).

## AUTHOR CONTRIBUTIONS

T.F. and P.S. conceived the project. D.F. and M.K. identified the *foxo* SNPs and performed genomic analyses. T.F., P.S., E.D. and S.R. designed the experiments. SR and NB established reconstituted outbred populations. E.D., S.R. and N.B. performed the experiments. E.D., N.B., P.S. and T.F. analyzed the data. E.D., P.S. and T.F. wrote the paper with input from the other authors.

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## COMPETING INTERESTS

The authors of this manuscript have declared no competing interests.

**Table 1.** Summary of ANOVA results for viability; femur length; wing area:thorax length ratio; female starvation resistance (also cf. Table S5). White and grey cells show results for females and males, respectively. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

Factor	Proportion Viability	Femur Length	Wing Area: Thorax Length Ratio	Starvation Resistance
Allele	$F_{1,32}=20.65^{***}$	$F_{1,32}=16.66^{***}$	$F_{1,4}=46.64^{***}$	$F_{1,32}=23.86^{***}$
		$F_{1,32}=0.16$	$F_{1,4}=82.17^{***}$	
Temperature	$F_{1,114}=3.24$	$F_{1,1923}=1617.80^{***}$	$F_{1,18}=477.45^{***}$	$F_{1,1547}=732.08^{***}$
		$F_{1,1923}=443.60^{***}$	$F_{1,18}=1366.87^{***}$	
Diet	$F_{1,114}=8.43^{**}$	$F_{1,1923}=144.72^{***}$	$F_{1,18}=50.35^{***}$	$F_{1,1547}=129.99^{***}$
		$F_{1,1923}=68.24^{***}$	$F_{1,18}=127.77^{***}$	
Allele x Temperature	$F_{1,114}=2.25$	$F_{1,1923}=0.36$	$F_{1,18}=0.14$	$F_{1,1547}=3.43$
		$F_{1,1923}=1.40$	$F_{1,18}=0.32$	
Temperature x Diet	$F_{1,114}=1.85$	$F_{1,1923}=13.26^{***}$	$F_{1,18}=16.64^{***}$	$F_{1,1547}=14.81^{***}$
		$F_{1,1923}=4.65$	$F_{1,18}=56.36^{***}$	
Allele x Diet	$F_{1,114}=1.71$	$F_{1,1923}=3.28$	$F_{1,18}=0.21$	$F_{1,1547}=16.22^{***}$
		$F_{1,1923}=4.04^*$	$F_{1,18}=2.53$	
Allele x Temperature x Diet	$F_{1,114}=0.39$	$F_{1,1923}=6.41^*$	$F_{1,18}=0$	$F_{1,1547}=1.63$
		$F_{1,1923}=0.95$	$F_{1,18}=8.34^{**}$	
Set(Allele)	$F_{2,32}=2.50$	$F_{2,32}=5.89^{**}$	$F_{2,4}=6.86^{**}$	$F_{2,32}=45.24^{***}$
		$F_{2,32}=0.75$	$F_{2,4}=3.80^*$	
Cage(Set, Allele)	$F_{4,32}=61.25^{***}$	$F_{4,32}=37.43^{***}$	NA	$F_{4,32}=11.17^{***}$
		$F_{4,32}=415.66^{***}$	NA	

**Table 2.** ANOVA results for female fat loss upon starvation. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . The fixed factor ‘Treatment’ has two levels: fed vs. starved; interactions involving the factors ‘Allele’ and ‘Treatment’ test for allelic differences in fat catabolism.

Factor	Fat content	
	18°C	25°C
Allele	$F_{1,32}=0.02$	$F_{1,32}=1.90$
Diet	$F_{1,301}=70.97^{***}$	$F_{1,300}=310.82^{***}$
Treatment	$F_{1,301}=223.48^{***}$	$F_{1,300}=130.68^{***}$
Allele x Diet	$F_{1,301}=20.58^{***}$	$F_{1,300}=6.93^{**}$
Diet x Treatment	$F_{1,301}=25.46^{***}$	$F_{1,300}=21.31^{***}$
Allele x Treatment	$F_{1,301}=7.01^{**}$	$F_{1,300}=1.24$
Allele x Diet x Treatment	$F_{1,301}=0$	$F_{1,300}=7.03^{**}$
Set(Allele)	$F_{2,32}=13.11^{***}$	$F_{2,32}=4.24^{*}$
Cage(Set, Allele)	$F_{4,32}=9.46^{***}$	$F_{4,32}=1.44$

## FIGURE CAPTIONS

**Figure 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 and Hafen 2003; Giannakou and 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 *Drosophila* insulin-like peptides (dilps 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 14-3-3 $\epsilon$ . 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 disinhibits, 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 occurring alleles of the genes in the IIS/TOR pathway remain poorly understood, but clinal polymorphisms in *InR* (Paaby et al. 2010; Paaby et al. 2014) and *foxo* (this study) have pleiotropic effects on life history in *Drosophila*.

**Figure 2.** Viability (egg-to-adult survival). Effects of the clinal *foxo* variant on the proportion viability (egg-to-adult survival). (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.

**Figure 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.

**Figure 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.

1370

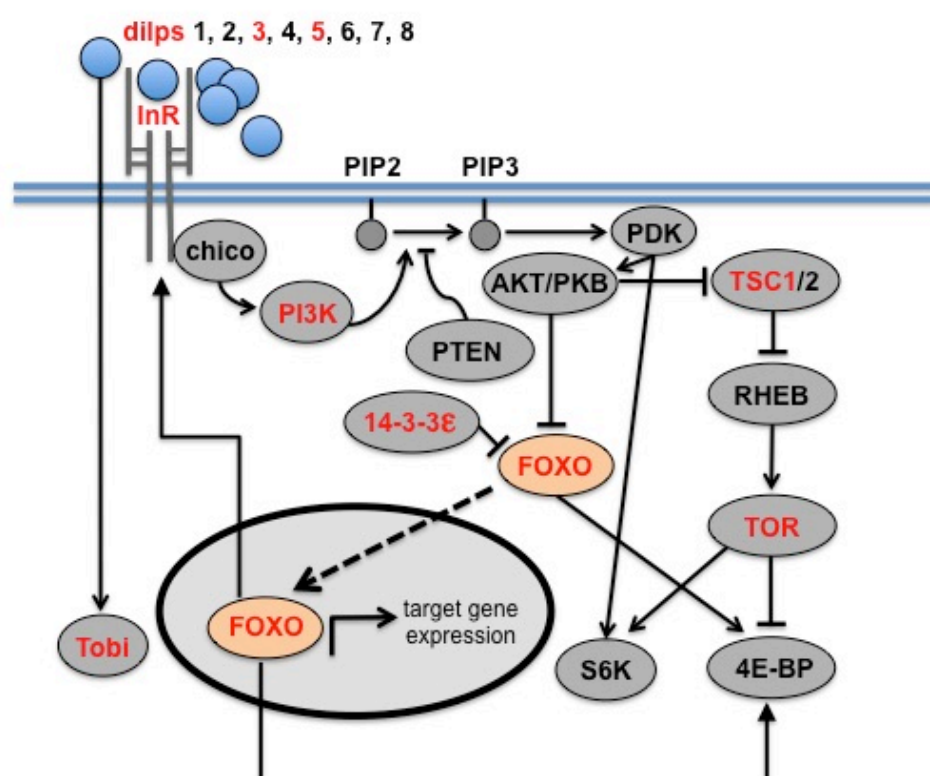
1371 **Figure 5**, Starvation resistance. Effects of the clinal *foxo* polymorphism on the  
 1372 duration of survival (in hrs) upon starvation in females. (A) Dietary reaction norms at  
 1373 18°C. (B) Dietary reaction norms at 25°C. (C) Thermal reaction norms measured on  
 1374 sucrose diet. (D) Thermal reaction norms measured on molasses diet. Data in (A, B)  
 1375 are the same as those shown in (C, D). Shown are means and standard errors. Red  
 1376 lines: low-latitude (LL) allele, blue lines: high-latitude (HL) allele.

1377

1378 **Figure 6**. Fat loss upon starvation. Effects of the clinal *foxo* variant on female  
 1379 triglyceride loss upon starvation ( $\mu g/fly$ ). (A) Dietary reaction norms at 18°C. (B)  
 1380 Dietary reaction norms at 25°C. (C) Thermal reaction norms measured on sucrose  
 1381 diet. (D) Thermal reaction norms measured on molasses diet. Data in (A, B) are the  
 1382 same as those shown in (C, D). Shown are means and (propagated) standard errors.  
 1383 Red lines: low-latitude (LL) allele, blue lines: high-latitude (HL) allele.

1384

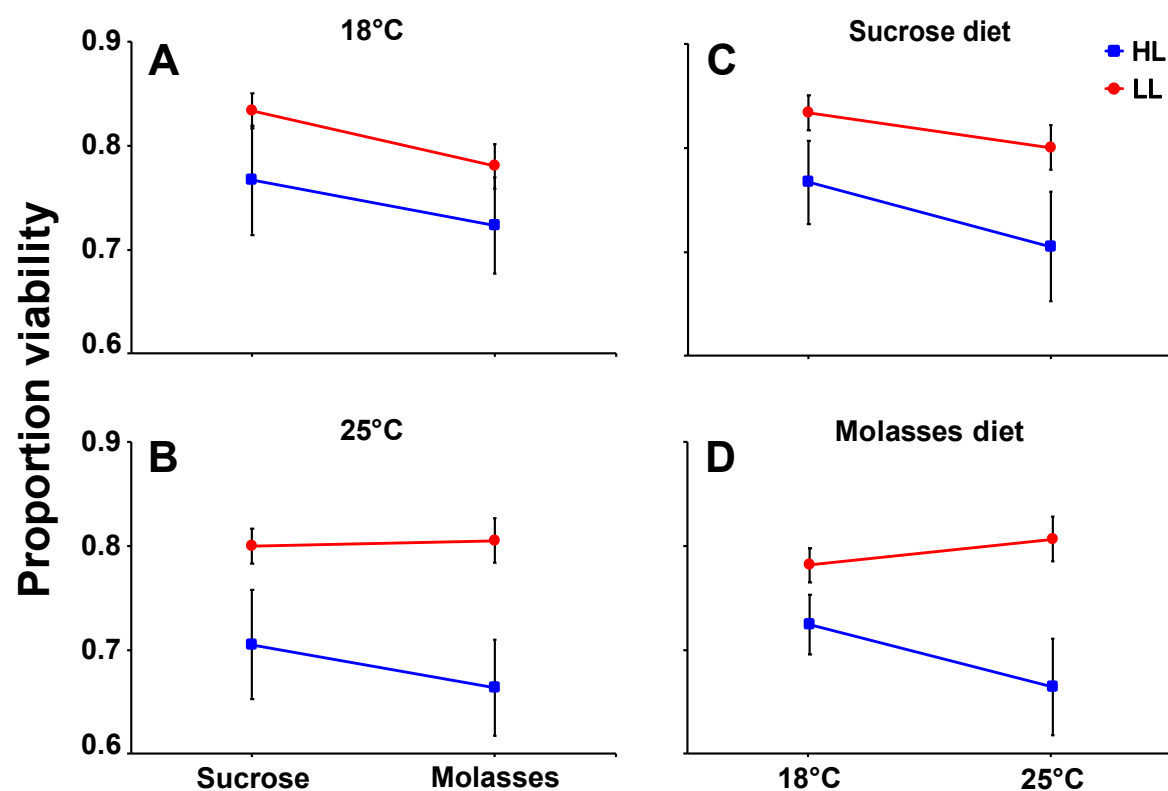
# 1385 Figure 1



1386

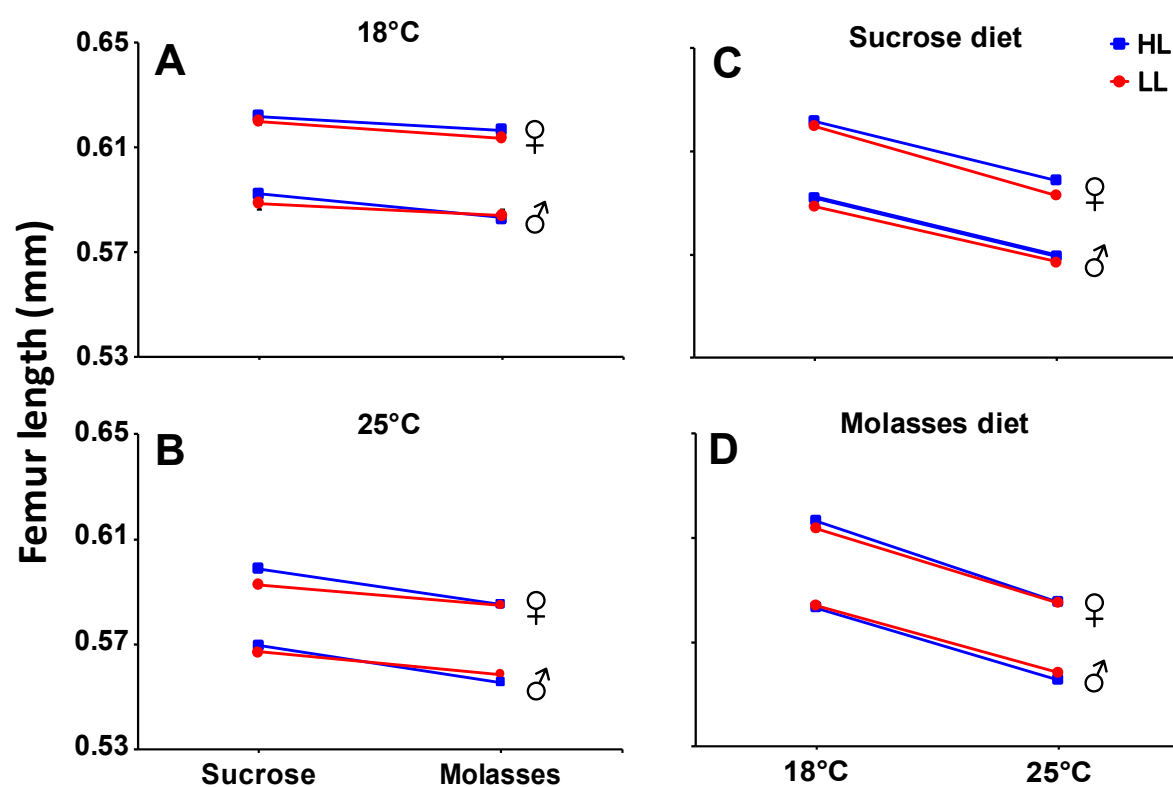
1387

1388 **Figure 2**

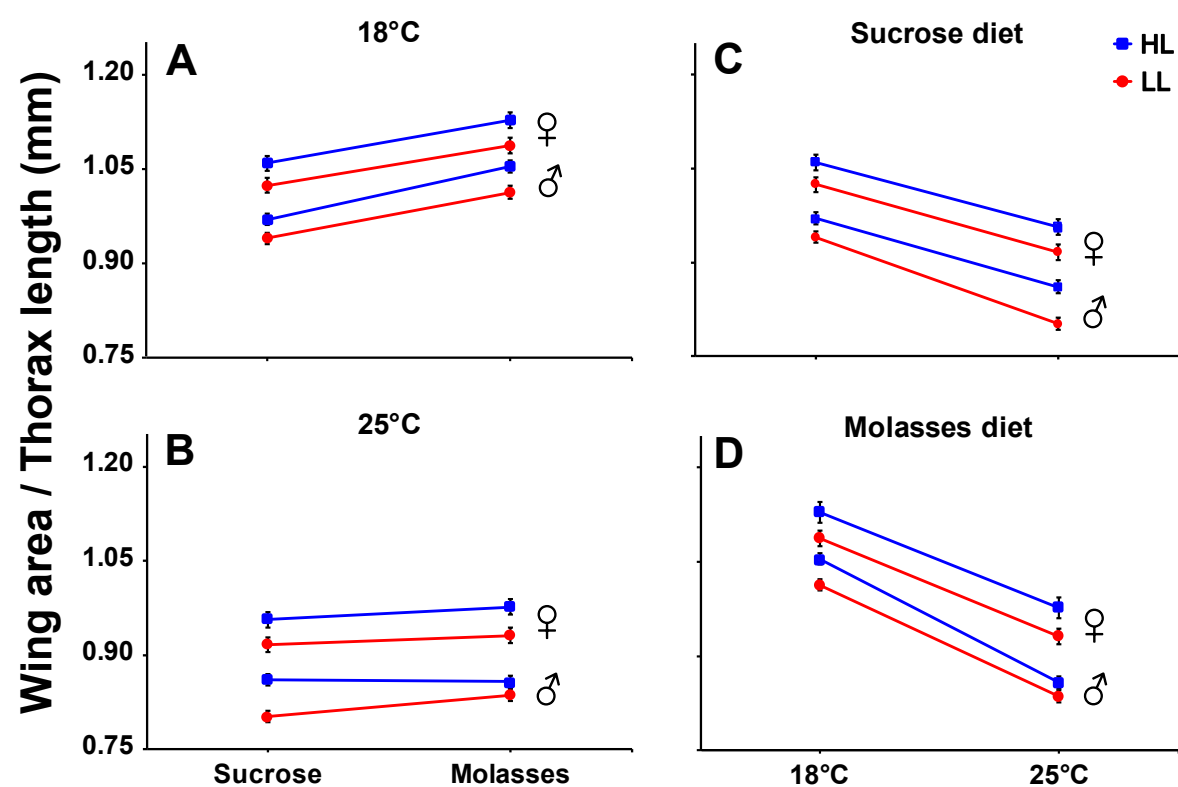


1389

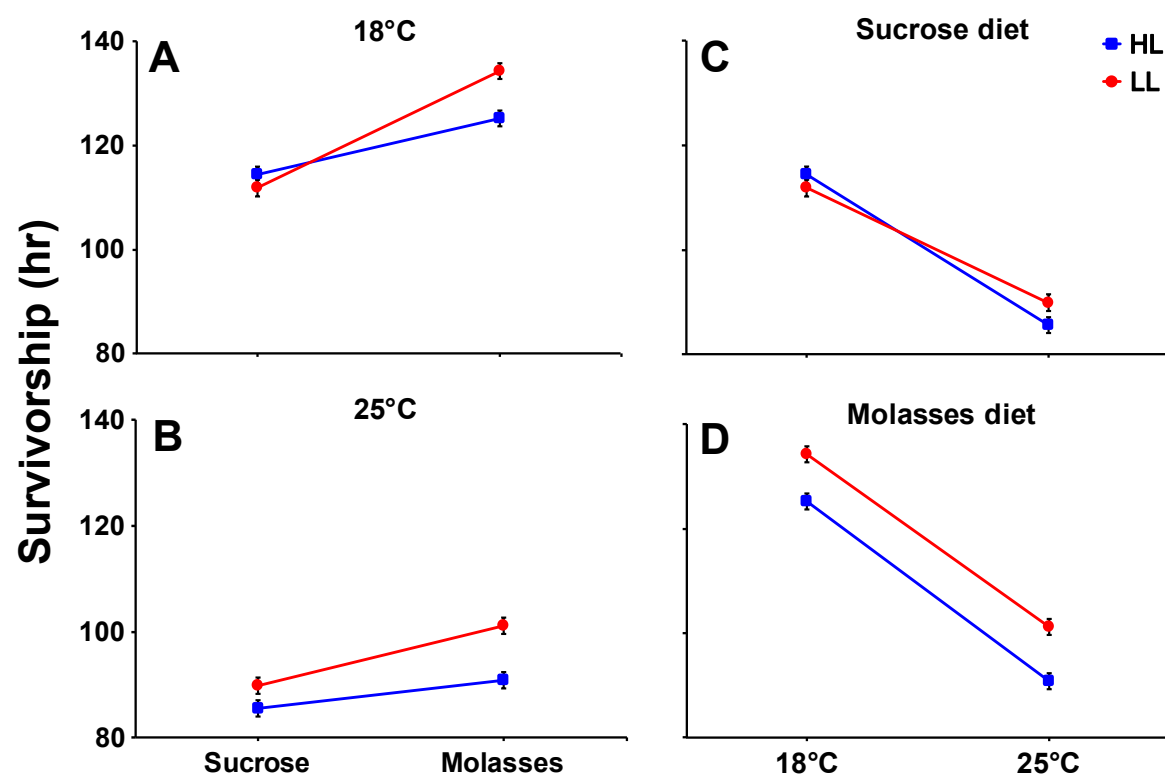
**Figure 3**



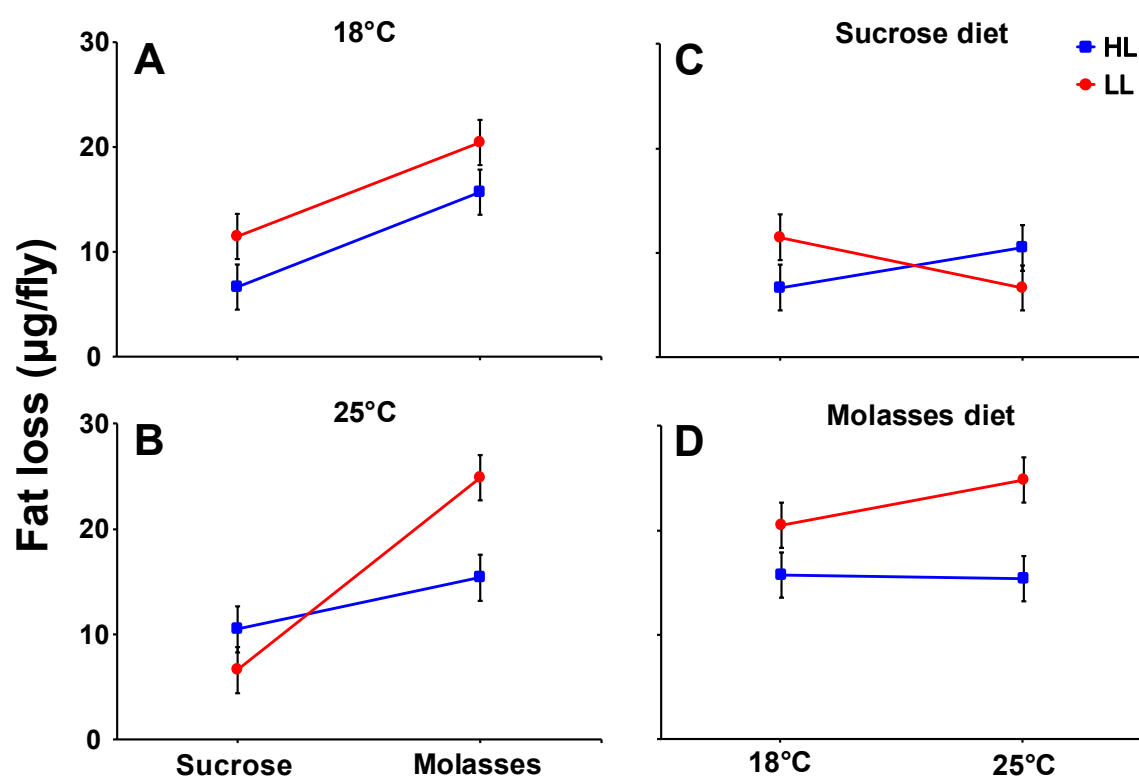
**Figure 4**



**Figure 5**



**Figure 6**





Supporting Information File

## Supporting Information

**Figure 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. Note that the SNP in-between the two focal SNPs is much less strongly clinal, with a much higher frequency in Florida than the 2 candidate SNPs. 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  $\geq 0.1$ ) 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. Nonetheless, we cannot rule out with certainty that other SNPs are in LD with our two focal SNPs; a cautious interpretation would thus be to view our focal SNPs as representing "tag SNPs". Also see Fig. S3; also see analyses in Betancourt et al. (2018).

**Figure S2.** PEST motif prediction for FOXO. The T/G polymorphism in *foxo* at position 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 = glutamic acid).

## Supporting Information File

**Figure 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; also see analyses in Betancourt et al. (2018).

**Figure 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.

**Figure S5.** Effects of the *foxo* variant on total wing area. Effects of the clinal *foxo* variant on wing area (mm<sup>2</sup>) 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.

**Figure 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

# Supporting Information File

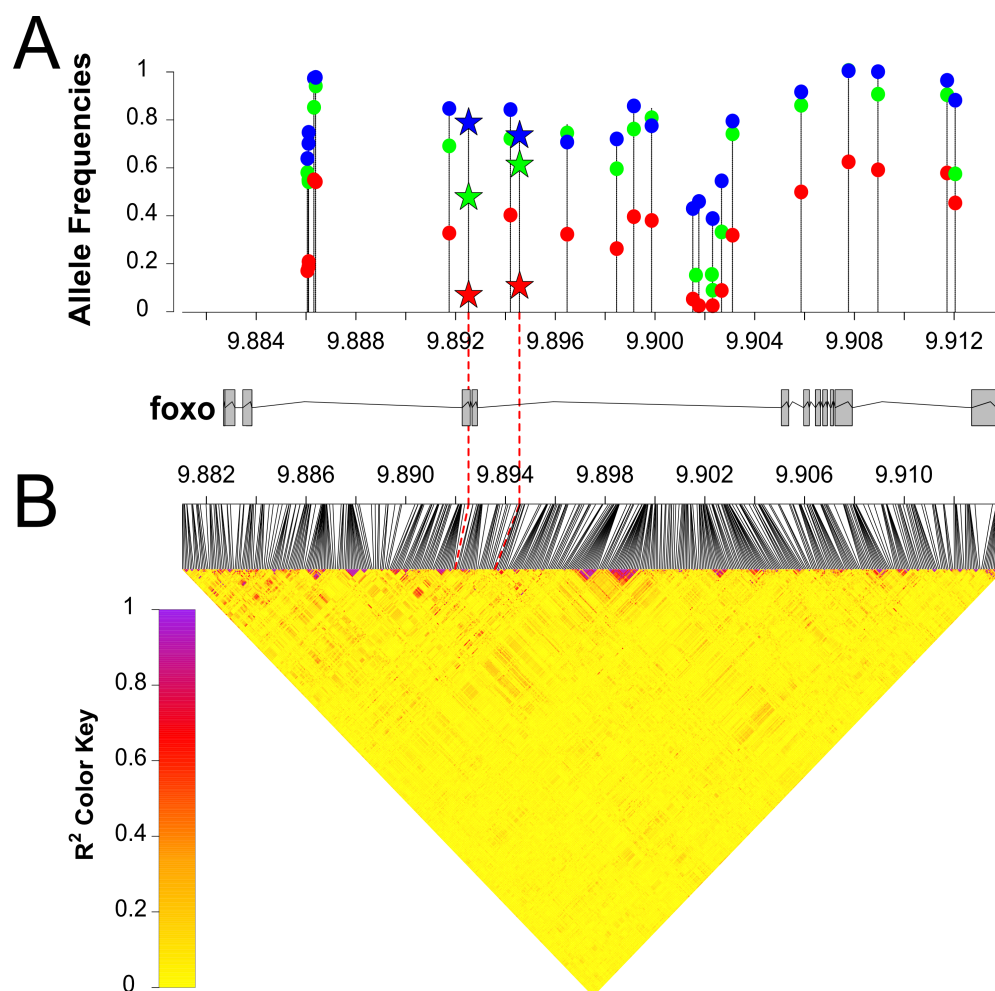
errors. Red lines: low-latitude (LL) allele, blue lines: high-latitude (HL) allele. See Results section for details.

**Figure S7.** Effects of the *foxo* variant on male survival upon starvation. Effects of the clinal *foxo* polymorphism on the duration of survival (in hrs) 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.

**Figure 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.

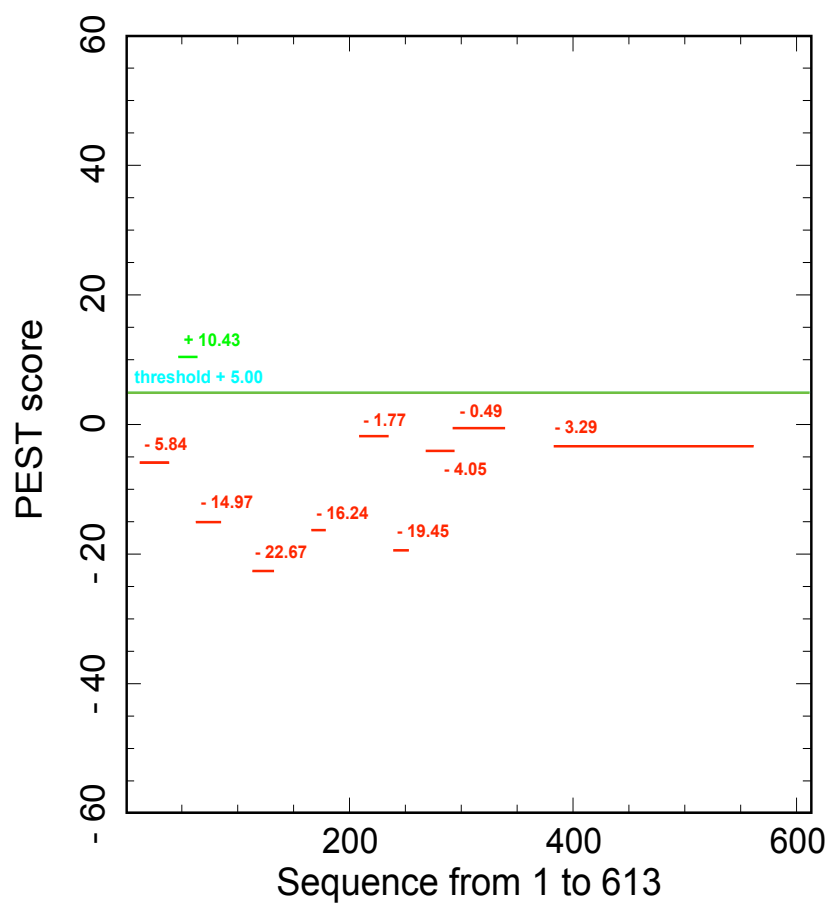
Supporting Information File

**Figure S1**



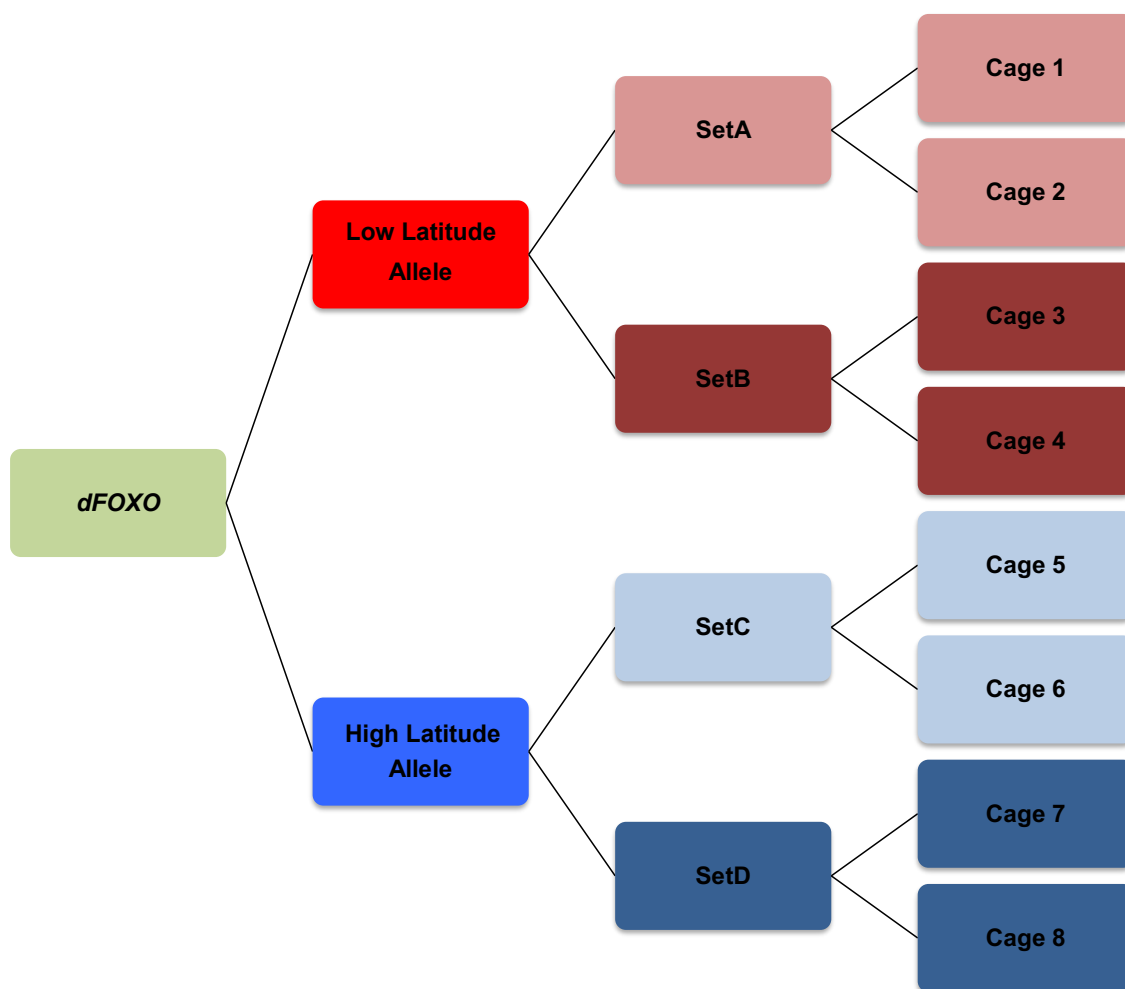
# Supporting Information File

**Figure S2**



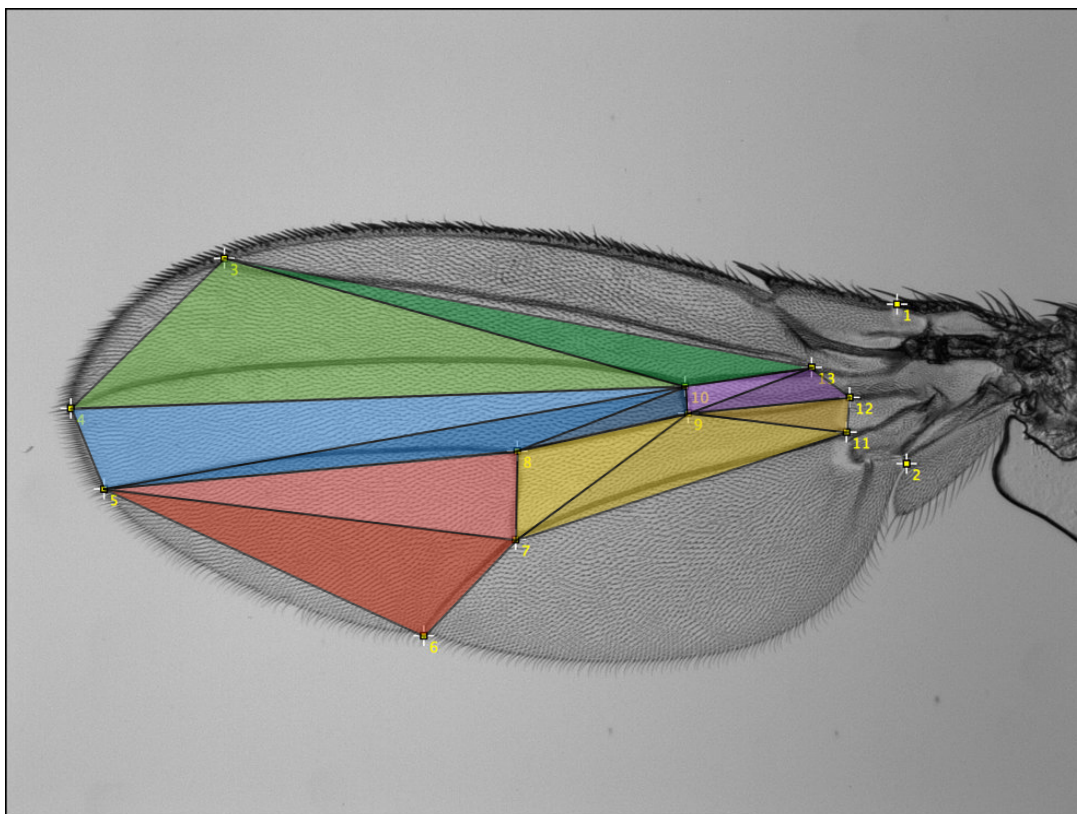
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**Figure S3**



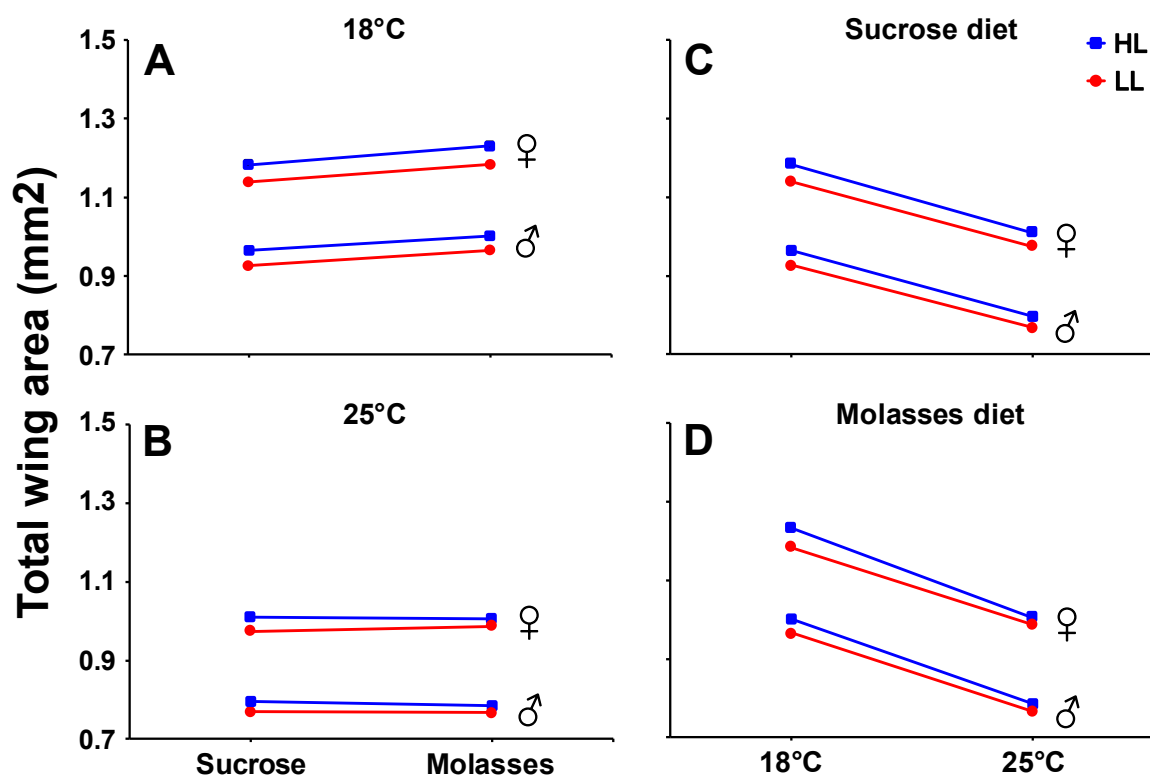
## Supporting Information File

**Figure S4**



Supporting Information File

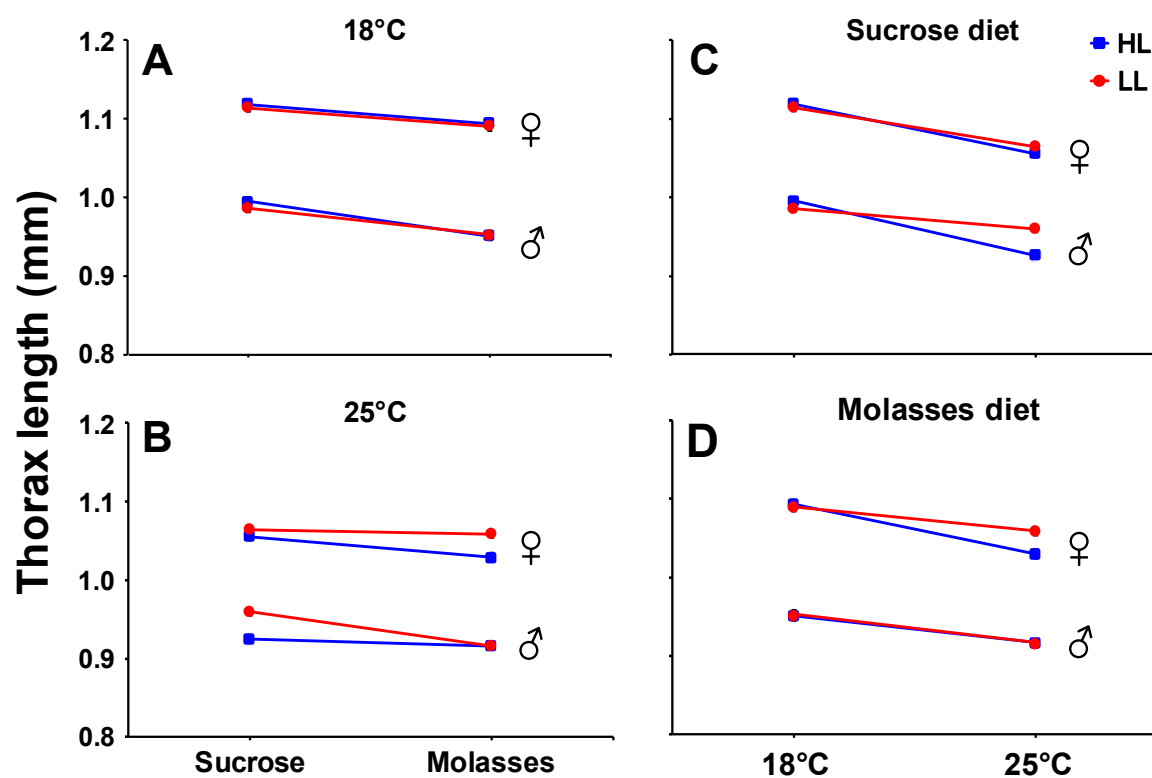
Figure S5





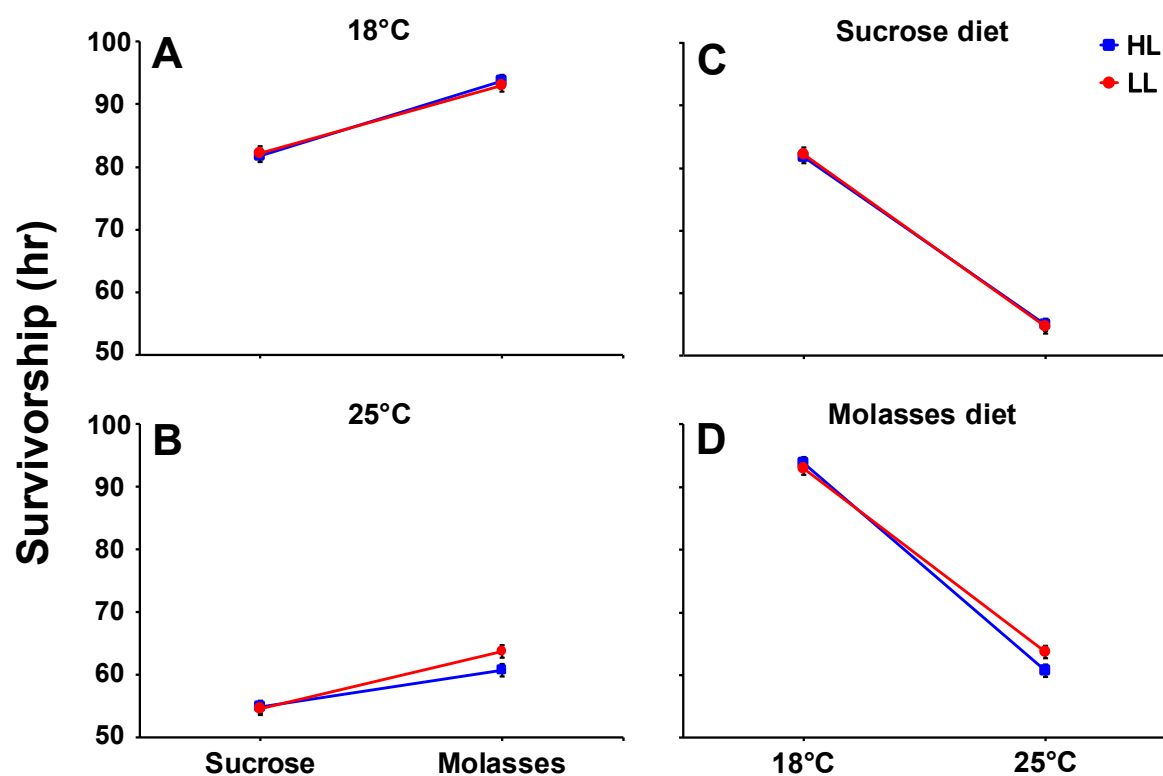
Supporting Information File

Figure S6



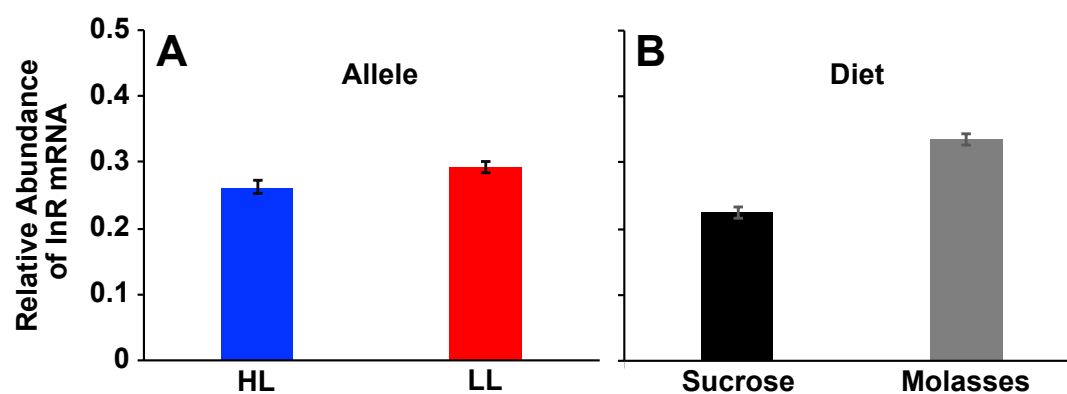
Supporting Information File

Figure S7



Supporting Information File

**Figure S8**



**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

# Supporting Information File

**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. The principal (but not exclusive) differences between the two diets are their carbohydrate source (sucrose vs. molasses) and their protein:carbohydrate (P:C) ratios.

**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) ( <i>Polenta, Migros</i> )	50	61.3
Yeast (g/L) ( <i>Actilife, Migros</i> )	50	12.4
Sugar (g/L) ( <i>Cristal, Migros</i> )	50	0
Molasses (g/L) ( <i>Zuckerrohrmelasse, EM Schweiz</i> )	0	109.6
Agar (g/L) ( <i>Drosophila Agar Type II, Genesee</i> )	7	6
Nipagin 10% (ml/L) ( <i>Sigma Aldrich</i> )	10	14.3
Propionic acid (ml/L) ( <i>Sigma Aldrich</i> )	6	6

**S2c. Nutritional values of sucrose and molasses diets**

	Sucrose	Molasses
Energy (kcal)	527.50	567.77
Protein (g/L)	26.50	10.48
Total carbohydrate (g/L)	94.50	129.42
P:C ratio	~ 1:3.6 ( $\approx 0.28$ )	~ 1:12.3 ( $\approx 0.08$ )

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**Table S3.** Summary of effect size estimates (Cohen's  $d$ ) for viability, femur length, wing area, thorax length, starvation resistance, and fat (TAG) content. White and grey cells show results for females and males, respectively.  $d = 0.01$ , very small;  $d = 0.20$ , small;  $d = 0.50$ , medium;  $d = 0.80$ , large;  $d = 1.20$ , very large.

Factor	18°C Sucrose diet	18°C Molasses diet	25°C Sucrose diet	25°C Molasses diet
Viability	0.49	0.50	0.54	0.89
Femur Length	0.09	0.17	0.49	0.00
	0.25	0.05	0.14	0.20
Wing Area	0.59	0.67	0.66	0.35
	0.68	0.62	0.72	0.48
Thorax Length	0.13	0.08	0.20	0.81
	0.26	0.07	1.15	0.00
Starvation Resistance	0.11	0.34	0.24	0.51
	0.03	0.04	0.03	0.26
TAG content (Fed)	0.19	0.70	0.25	0.72
TAG content (Starved)	0.72	0.24	0.04	0.04

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**Table S4.** Summary of ANOVA results for wing area, thorax length, and male starvation resistance (also cf. Table S5). White and grey cells show the results for females and males, respectively; data for starvation resistance are for males only. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ . See Results section for details.

Factor in ANOVA	Total Wing Area	Thorax Length	Starvation Resistance
Allele	$F_{1,32}=105.39^{***}$	$F_{1,32}=4.33^*$	$F_{1,32}=0.70$
	$F_{1,32}=103.87^{***}$	$F_{1,32}=3.78$	
Temperature	$F_{1,912}=2852.52^{***}$	$F_{1,422}=216.46^{***}$	$F_{1,1553}=1711.77^{***}$
	$F_{1,918}=3962.67^{***}$	$F_{1,381}=145.46^{***}$	
Diet	$F_{1,912}=48.36^{***}$	$F_{1,422}=31.90^{***}$	$F_{1,1553}=176.44^{***}$
	$F_{1,918}=28.15^{***}$	$F_{1,381}=88.62^{***}$	
Allele x Temperature	$F_{1,912}=7.15^{**}$	$F_{1,422}=10.66^{**}$	$F_{1,1553}=0.58$
	$F_{1,918}=5.89^*$	$F_{1,381}=8.72^{**}$	
Temperature x Diet	$F_{1,912}=35.96^{***}$	$F_{1,422}=1.67$	$F_{1,1553}=7.51^{**}$
	$F_{1,918}=56.66^{***}$	$F_{1,381}=3.48$	
Allele x Diet	$F_{1,912}=0.73$	$F_{1,422}=2.44$	$F_{1,1553}=0.58^{***}$
	$F_{1,918}=1.08$	$F_{1,381}=2.46$	
Allele x Temperature x Diet	$F_{1,912}=1.79$	$F_{1,422}=1.89$	$F_{1,1553}=2.48$
	$F_{1,918}=0.22$	$F_{1,381}=11.19^{***}$	
Set (Allele)	$F_{2,32}=53.59^{***}$	$F_{2,32}=8.05^{***}$	$F_{2,32}=1.01$
	$F_{2,32}=30.53^{***}$	$F_{2,32}=7.56^{***}$	
Cage (Set, Allele)	$F_{4,32}=64.45^{***}$	$F_{4,32}=3.41^{**}$	$F_{4,32}=12.78^{***}$
	$F_{4,32}=29.58^{***}$	$F_{4,32}=0.73$	

**Table S5.** Summary of REML variance component estimates for starvation resistance. White and grey cells show results for females and males, respectively.

Random Effect	Variance Ratio	Variance Component	Std Error	95% Lower	95% Upper	Wald <i>p</i> -Value	Percentage of Total
<b>Vial(Cage,Set,Allele)</b>	0.00	-0.19	2.96	-6.00	5.62	0.95	0.00
	0.00	0.13	1.29	-2.39	2.65	0.92	0.07
<b>Residual</b>		474.90	17.08	443.13	510.23		100.00
		199.07	7.14	185.78	213.85		99.93
<b>Total</b>		474.90	17.08	443.13	510.23		100.00
		199.21	7.08	186.03	213.85		100.00



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**Table S6.** 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 <i>InR</i>
Allele	$F_{1,80}=4.54^*$
Temperature	$F_{1,80}=0.90$
Diet	$F_{1,80}=75.99^{***}$
Allele x Temperature	$F_{1,80}=0.05$
Temperature x Diet	$F_{1,80}=0.05$
Allele x Diet	$F_{1,80}=0.41$
Allele x Temperature x Diet	$F_{1,80}=0.08$
Set (Allele)	$F_{2,80}=6.53^{**}$
Cage (Set, Allele)	$F_{4,80}=5.73^{***}$