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

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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

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

Although much has been learned about the genetics of fitness traits (e.g., size, lifespan), mainly from studies of large-effect mutants and transgenes in yeast, *C. elegans*, *Drosophila* and the mouse (Finch and Rose 1995; Oldham and Hafen 2003; Tatar et al. 2003; Fielenbach and Antebi 2008; Kenyon 2010), 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). 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; Kolaczkowski et al. 2011; Fabian et al. 2012; Adrion et al. 2015; Cogni et al. 2017). Clinal trait

differentiation has been found, for instance, for body size, fecundity, reproductive dormancy, stress resistance and lifespan, typically in a parallel fashion on multiple continents, suggesting that these patterns are adaptive (Coyne 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 adaptive 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 clinally varying SNPs mapping to loci involved in the insulin/insulin-like growth factor signaling (IIS)/target of rapamycin (TOR), ecdysone, torso, EGFR, TGFβ/BMP, JAK/STAT, lipid metabolism, immunity and circadian rhythm pathways (Fabian et al. 2012). Many of the identified variants also exhibit parallel differentiation in Australia (Fabian et al. 2012; Kapun et al. 2016b; also cf. Kolaczkowski et al. 2011; Reinhardt et al. 2014; Machado et al. 2016), thereby strengthening the case for clinal adaptation. However, while many clinal variants might be shaped by selection, some of the observed differentiation might be due to non-adaptive factors, including population structure, demography, admixture or hitchhiking with causative sites (Endler 1977; Duchen et al. 2013; Kao et al. 2015; Bergland et al. 2016). Unambiguously identifying adaptive clinal variants as causal 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 and experimental confirmations of clinal polymorphisms that are potentially subject to spatially varying selection remain scarce (for some exceptions see e.g. Schmidt et al. 2008; Paaby et al. 2010, 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 functional genetic studies to be implicated in the physiological regulation of life history (Tatar et al. 2003; Fielenbach and Antebi 2008; Flatt and Heyland 2011; Flatt et al. 2013). 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 *14-3-3*ε, *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 affect adaptation, natural variation in adult reproductive dormancy in *D. melanogaster* has been connected to the *Pi3K* gene (Williams et al. 2006), and work in *Caenorhabditis remanei* has identified a global selective sweep in the *Caenorhabditis* homolog of *Pi3K*, *age-1* (Jovelin et al. 2014). Multiple lines of evidence also indicate that insulin-like growth factor-1 (IGF-1) signaling mediates physiological life history variation in vertebrate populations (Dantzer 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.

Here we investigate the life history effects of a clinal polymorphism in the forkhead box-O transcription factor gene *foxo* of *D. melanogaster* (Fig. 1), a major regulator of IIS that is homologous to *C. elegans daf-16* and mammalian *FOXO3A*. Molecular studies – mainly in the fly and nematode – have shown that FOXO plays a key role in regulating growth, lifespan and resistance to starvation and oxidative stress (Jünger et al. 2003; Puig et al. 2003; Libina et al. 2003; Murphy et al. 2003; Kramer et al. 2003; 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 (Flachsbart et al. 2009; Willcox et al. 2008). Natural *foxo* variants thus represent promising candidates for mediating life history variation in natural populations.

From our genomic data (Fabian et al. 2012) we identified two strongly clinally varying alternative *foxo* alleles, as defined by 2 focal SNPs, whose frequencies change across latitude by ~60% between Florida and Maine (this paper; also see analyses in Betancourt et al. 2018). To characterize the effects of these genotypes we measured several fitness-related traits (egg-to-adult survival, proxies of size, starvation resistance, fat content) on replicate populations of the two alternative alleles.

Since temperature gradients are thought to underpin – at least partly – latitudinal clines (de Jong and Bochdanovits 2003; Kapun et al. 2016b; and references therein), and because both diet and temperature modulate IIS (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 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. Investigating phenotypic plasticity and genotype-by-environment interactions (G × E) for this variant is of interest since little is known about the relative importance of clinality versus plasticity and their interplay (van Heerwaarden and Sgrò 2017), with most previous work having focused on gene expression, not whole-organism traits (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). For example, *D. melanogaster* feeds and breeds on various kinds of rotting fruit, with 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 and G × E affect traits in a

clinal context is not well understood. We give predictions for the expected phenotypic effects of the *foxo* variant in the Materials and Methods section below.

We find that the *foxo* polymorphism affects multiple clinally varying life history traits, thus indicating that it might be a target of spatially varying selection. Both alternative alleles respond plastically to changes in temperature and diet, but overall there is little evidence for G × E interactions.

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; cf. Betancourt et al. 2018). The A/G polymorphism is a synonymous coding SNP, predicted to be located in the PEST region of the FOXO protein, which serves as a protein degradation signal (analysis with ExPASy [Artimo et al. 2012]; Fig. S2, Supporting Information). The T/G SNP is located in the first intron of *foxo*, with no biological function attributed to this position (Attrill et al. 2016). While our initial identification of these SNPs was based on only three populations (Florida, Pennsylvania, and Maine; Fabian et al. 2012), both SNPs are also strongly clinal in a more comprehensive dataset based on 10 populations along the cline (cf. 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). Since the two *foxo* SNPs are relatively close together (~2 kb apart; Fig. S1A, Supporting Information), we decided to study them experimentally in combination, as alternative 2-SNP alleles. 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). Importantly, this *foxo* polymorphism exhibits stronger clinality than the majority of variants at the *foxo* locus and seems to be maintained non-neutrally by spatially varying selection (see Betancourt et al. 2018).

To isolate the two alternative 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) 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 for another example of 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).

By analyzing the genomes of the DGRP lines used to set up experimental populations we confirmed that sets A and B versus sets C and D were completely fixed ($F_{ST} = 1$) for the HL and LL alleles, respectively; this analysis also showed that there was no major genomic differentiation, as measured by F_{ST}, in the genome-wide

background of the two focal SNPs (also cf. Betancourt et al. 2018). Figure S1B (Supporting Information) shows that the two focal SNPs are in perfect linkage disequilibrium (LD; $r^2 = 1$), without any significant LD in-between the two sites.

The most parsimonious interpretation of our approach is that the effects reported below are caused by the *foxo* SNPs that we have set out to study. However, we cannot 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 SNPs as representing 'tags' or markers for functionally significant allelic variation segregating at the *foxo* locus, similar to those used in genome-wide association studies (GWAS), which are in LD with the causative site(s) (e.g., Wang et al. 2010).

POPULATION CAGES

Population cages were maintained at 25ºC, 12:12 h light:dark, 60% relative air humidity and controlled larval density. Larval density was kept constant via egg collections (200-300 eggs per bottle [6 oz. = 177 mL]; 10 bottles per cage), with eclosing adults being released into cages (17.5 x 17.5 x 17.5 cm; BugDorm®) at a density of ~2000-2500 adults per cage. Prior to assays the population cages were kept for 10 generations to allow for recombination among lines within each cage (and allelic state) and to further 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: ~22ºC, ~10:14 h light:dark, ~50% humidity). Thus, phenotypes were measured after a total of 12 generations of recombination.

PHENOTYPE ASSAYS

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_2 anesthesia 4-6 days post-eclosion, and transferred them to fresh vials 24 h prior to assays. Flies used for size assays were stored at -20°C until measurement.

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

Body size was examined by measuring three proxies: wing area, thorax length and femur length (*N* = 26-30 wings, 10-15 thoraces, and 19-21 femurs per cage, treatment, and sex). Right wings and femurs were mounted on slides with

CC/Mount™ tissue mounting medium (Sigma Aldrich) and slides sealed with cover slips. Thorax length was defined as the lateral distance between the upper tip of the thorax and the end of the scutellar plate (*N* = 10-15 individuals per cage, treatment, and sex). Images for morphometric measurements were taken with a digital camera (Leica DFC 290) attached to a stereo dissecting microscope (Leica MZ 125; Leica Microsystems GmbH, Wetzlar, Germany). We used ImageJ software (v.1.47) to measure femur and thorax length (mm) and to define landmarks for calculating wing area (mm^2). To measure wing area we defined 12 landmarks located at various vein intersections along the wing; the total area encompassed by these landmarks was estimated using a custom-made Python script (available upon request). In brief, we split the polygon defined by the landmarks up into triangles and summed across their areas (Fig. S4, Supporting Information). Thorax and femur measurements were repeated three times per individual. From these data, we calculated the ratio of wing area:thorax length, which is inversely related to 'wing loading' (Azevedo et al. 1998; Gilchrist et al. 2000).

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 vials × 10 flies per vial × 2 sexes × 8 cages × 4 conditions = 320 vials or 3200 flies).

Since there is typically a positive correlation between starvation resistance and lipid content (Hoffmann and Harshman 1999), we also determined whole-body triacylglyceride (TAG) content (in μg per fly) using a serum triglyceride determination kit (Sigma Aldrich; Tennessen et al. 2014). For each cage and treatment, triglyceride content was estimated from 5-7-day-old females, either kept under fed or starved (24

h) conditions, by preparing 10 replicate homogenates, each made from 2 flies (8 cages × 4 conditions × 2 treatments × 10 replicates = 640 homogenates). To estimate fat loss upon starvation we calculated the difference between fat content under fed versus starved conditions, using treatment (fed vs. starved) means from each population cage (mean fat loss per fly, in μg).

QRT-PCR ANALYSIS OF INSULIN SIGNALING STATE

A well-established transcriptional read-out of FOXO signaling is the insulin-like receptor InR: under conditions of high insulin (e.g., after a meal), InR synthesis is repressed by a feedback mechanism controlled by FOXO; conversely, under conditions of low insulin, activation of FOXO leads to upregulation of *InR* 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 × 4 conditions × 3 biological replicates × 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 Δ*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), set (*S*; independent blocks of DGRP lines) nested within *A*, replicate cage (*C*) nested within the combination of *A* and *S*, and all 2- and 3-way interactions: $y = A + T + D + A \times T + A \times D + T \times D + A \times T \times D + S(A) + C(A,S)$, where *y* denotes the response variable (trait). For simplicity, the sexes were analyzed separately (i.e., to reduce the number of higher-order interactions).

Whenever measuring multiple individuals from vials, we estimated the random effect of vial (*V*), nested within the combination of *A*, *S* and *C*, using restricted maximum likelihood (REML); since the estimates of this variance component are not of primary biological interest we do not report them.

Viability (proportion) data were arcsine square-root transformed prior to analysis. Analysis of thorax and femur length was performed on means across 3 measures per

individual; because wings and thoraces were measured on separate individuals, analysis of wing:thorax ratio was done on population (cage) means. For fat content, we included the fixed effect of starvation treatment (*Tr*; fed vs. starved); interactions involving *A* and *Tr* (i.e., $A \times Tr$; $A \times D \times Tr$) test for allelic differences in fat loss upon starvation. For simplicity, this analysis was performed separately for the two rearing temperatures.

PREDICTIONS

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

(1) Latitudinal clinality. Traits known to covary positively with latitude include faster development, lower egg-to-adult survival (viability), increased body size, reduced wing loading, reduced fecundity, prolonged lifespan, and increased resistance to starvation, cold and heat stress (Coyne 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; for contrasting predictions for viability see van ‘t Land et al. 1999, and for starvation resistance cf. Karan et al. 1998, Robinson et al. 2002; Hoffmann et al. 2005; Goenaga et al. 2013). We thus expect that the effects of the high- and low-latitude *foxo* alleles should agree with the overall phenotypic patterns along the cline, unless the alleles exhibit countergradient effects on phenotype (Paaby et al. 2014).

(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); effects of IIS or *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 resemble those of flies with low versus high IIS, respectively (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 are co-expressed in IIS mutants; however, flies from high-latitude populations are larger than low-latitude flies, yet reduced IIS causes smaller size.

(3) Plasticity, G $\times$ E, and local adaptation. With regard to thermal effects, we expect flies raised at lower temperature to exhibit prolonged development, reduced viability, larger size, reduced wing loading, lower fecundity, increased lifespan, and improved starvation resistance (David et al. 1994; Partridge et al. 1994a, 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, are 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 × 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

POLYMORPHISM AT *FOXO* AFFECTS VIABILITY

The *foxo* polymorphism had a significant effect on egg-to-adult survival (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), maybe consistent with a recent study reporting that viability is higher at lower P:C ratios (Lihoreau et al. 2016). We did not find any evidence for G × 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 S3, Supporting Information), and wing:thorax ratio than the LL allele (Fig. 4; Table 1; for thorax data see Fig. S6; Table S3, Supporting Information). These results are consistent with the positive size cline in North America (Coyne and Beecham 1987; Betancourt et al. 2018) and with reduced wing loading at high latitude (Azevedo et al. 1998; Bhan et al. 2014). Since *foxo* overexpression can reduce overall size (Jünger et al. 2003), these results suggest that the LL allele might exhibit increased FOXO function as compared to the HL allele (see below). For all size traits, females were larger than males (Fig. 3; Fig. 4; Table 1; Fig. S5; Fig. S6; Table S3, 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 S3, 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 S3, 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 S3, Supporting Information; and Fig. 4; Table 1). Although we found a few G × E interactions for size traits (Fig. 4; Fig. 5; Table 1; Fig. S5; Fig. S6; Table S3, Supporting Information), the allelic reaction norms were overall remarkably parallel across environmental conditions.

POLYMORPHISM AT *FOXO* INFLUENCES STARVATION RESISTANCE AND FAT CATABOLISM

The *foxo* alleles also differed in their effects on female starvation resistance (Fig. 5; Table 1), as might be expected based on the observation that *foxo* mutants are more starvation sensitive than wildtype (Jünger et al. 2003; Kramer et al. 2003, 2008). However, contrary to clinal predictions (Schmidt and Paaby 2008; Mathur and Schmidt 2017; Betancourt et al. 2018), 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 S3, Supporting Information). Overall females were more resistant than males (Fig. 5; Table 1; Fig. S7; Table S3, Supporting Information), consistent with some but not other studies (Goenaga et al. 2010; but see Matzkin et al. 2009). For both females and males, starvation resistance was higher at 18°C than at 25°C (Fig. 5; Table 1; Fig. S7; Table S3, Supporting Information), as previously reported (Mathur and Schmidt 2017). Flies raised on molasses diet were more resistant than those raised on sucrose diet (Fig. 5; Table 1; Fig. S7; Table S3, Supporting Information), 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 S3, 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 S4, Supporting Information). Dietary conditions also affected *InR* levels, with flies raised on molasses producing more *InR* than flies raised on sucrose diet (Fig. S8; Table S4, Supporting Information).

Discussion

CONNECTING ADAPTIVE CLINAL PHENOTYPES TO GENOTYPES

Here we have studied the life history effects of a strongyl clinally varying and presumably adaptive polymorphism in the IIS gene *foxo*, a naturally segregating

variant that we have identified from our genomic analysis of the North American latitudinal cline (Fabian et al. 2012; also see Betancourt et al. 2018).

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); (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; Kolaczkowksi et al. 2011; Fabian et al. 2012; Kapun et al. 2016b); and (4) genome-wide analyses of variation in size traits have identified novel regulators of growth, several of which interact with the IIS/TOR pathway (Vonesch et al. 2016; Strassburger et al. 2017). 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 thus be highly pleiotropic, whereas 'evolutionarily relevant' mutations or alleles might have much more subtle effects, with little or no pleiotropy (Stern 2000). Based on our knowledge of the traits affected by *foxo* in null mutants and transgenes (Jünger et al. 2003; Kramer et al. 2003, 2008; Slack et al. 2011), we measured how the clinal 2-SNP variant affects size traits and starvation resistance.

Although we could not predict with certainty the directionality or the degree of pleiotropy of the allelic effects *a priori*, we found that the *foxo* polymorphism differentially affects size-related traits and starvation resistance, two traits 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 little evidence for allele by environment interactions.

While our experimental design does not allow us to disentangle the contribution of the 2 individual SNPs to the total effects seen 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 – apparently have strong pleiotropic (or at least correlated) effects on multiple complex life history traits, including on viability, several proxies of size and on starvation resistance. 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, 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 is maintained 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; Azevedo et al. 1998; Schmidt et al. 2005a, 2005b; Folguera et al. 2008; Schmidt and Paaby 2008; Mathur and Schmidt 2017). Do the allelic effects go in the same direction as the latitudinal gradient, representing cogradient variation, or do certain allelic effects run counter to the cline, representing countergradient variation (Levins 1968; Conover and Schultz 1995)? Cogradient variation occurs when diversifying selection favors different traits in different environments, as expected from selection along a cline, whereas countergradient variation occurs when stabilizing selection favors similar traits in different environments (Conover 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; 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).

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

For IIS itself, 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).

LIMITS TO OUR REDUCTIONIST UNDERSTANDING OF ADAPTATION?

Some of the above considerations illustrate the limitations of using a reductionist approach to establish adaptive effects of individual alleles (Rockman 2012). In his famous 1974 book *The Genetic Basis of Evolutionary Change* Richard Lewontin writes: '*Even if it were possible to randomize the alleles at a single locus with respect to the rest of the genome and then to measure the marginal fitnesses of the alternative genotypes at that locus to an arbitrary level of accuracy, it would be a useless occupation. Genes in populations do not exist in random combinations with other genes. The alleles at a locus are segregating in a context that includes a great deal of correlation with the segregation of other genes at nearby loci... Context and interaction are of the essence.'* (Lewontin 1974, p. 318).

This is an incisive critique of the kind of experimental approach we have used here. However, adopting the alternative approach, i.e. using a macroscopic, quantitative genetics description, also comes at a cost, namely treating the genetic architecture of adaptive traits as a phenomenological, mechanistic black box (Houle 2001; Stern and Orgogozo 2008; Barrett and Hoekstra 2011; Flatt and Heyland 2011; Rockman, 2012; Flatt et al. 2013; Nunes et al. 2013). The problem is that – in the absence of functional analysis of candidate loci or alleles – neither population nor quantitative genetics can provide an explicit understanding of how causative polymorphisms map to evolutionarily relevant traits (Nunes et al. 2013). Indeed, as Lewontin argued himself (1974, 2000), if an adequate description of evolution is to be

given, evolutionary genetics must tackle the problem of mapping genotypes into phenotypes: '*Much of the past and the present problems of population genetics can be understood only as an attempt to finesse the unsolved problem of an adequate description of development.'* (Lewontin 2000, p. 7).

GROWING EVIDENCE FOR A ROLE OF IIS IN LIFE HISTORY ADAPTATION

The IIS pathway might serve a good example of how mechanistic and evolutionary insights might be combined to gain a more complete understanding of life history evolution (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). In particular, these studies have revealed that IIS 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 thus promised an opportunity for evolutionary geneticists to identify natural variants involved in life history evolution (de Jong and Bochdanovits 2003). On the other hand, '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).

For some time, it thus remained unclear whether natural variation in this pathway impacts variation in fitness-related traits in natural populations (cf. Reznick 2005). This situation has changed in recent years: today we have growing evidence that IIS contributes to life history variation and adaptation in flies and other insects, worms, fish, reptiles and mammals, including effects on longevity in humans (de Jong and Bochdanovits 2003; Williams et al. 2006; Flachsbart et al. 2008; Suh et al. 2008; Willcox et al. 2008; Alvarez-Ponce et al. 2009; Sparkman et al. 2009, 2010; Paaby et al. 2010; Stuart and Page 2010; Dantzer and Swanson 2012; Jovelin et al. 2014; Paaby et al. 2014; Swanson and Dantzer 2014; McGaugh et al. 2015; Schwartz and Bronikowski 2016; Zhao et al. 2016). On the other hand, other studies, for example using 'evolve and resequence' approaches, 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 for the evolution of certain traits (e.g., see Remolina et al. 2012; see disscussion in Flatt and Partridge 2018). In sum, this body of work illustrates how, by studying a candidate pathway from multiple angles, one might be able to connect genotypes to molecular mechanisms to environments and to adaptive phenotypes (cf. Houle 2001; Flatt et al. 2005, 2013).

Conclusions

Here we have found that a clinal polymorphism in the insulin signaling transcription factor gene *foxo* has pleiotropic (or correlated) effects on several major fitness-related traits which are themselves known to be clinally varying, including egg-to-adult survival, several size-related traits, starvation resistance as well as fat content.

The directionality of most of these effects matches the observed phenotypic trait clines (Schmidt et al. 2005a, 2005b; Schmidt and Paaby 2008; Betancourt et al. 2018), thus confirming previous genomic data suggesting that this variant might be shaped by spatially varying selection (Fabian et al. 2012). Our results are also in good agreement with functional genetic studies of the *foxo* locus using mutants and transgenes (Jünger et al. 2003; Kramer et al. 2008; Slack et al. 2011). Together with the results on *InR* by Paaby et al. (2010, 2014), our study demonstrates that standing genetic variation in IIS makes an important and – at least partly – predictable contribution to clinal life history adaptation in *Drosophila*. More generally, our study illustrates how genomic analyses of clinal gradients might be combined with functional assays to validate putatively adaptive effects of candidate polymorphisms.

ACKNOWLEDGEMENTS

We thank the members of the Schmidt and Flatt labs for assistance in the lab, and Fisun Hamaratoglu, Tad Kawecki, Wolf Blanckenhorn and Marc Tatar for helpful discussion and/or comments on a previous version of our manuscript. Our research was supported by the Swiss National Science Foundation (SNSF grants PP00P3_133641; PP00P3_165836; 310030E-164207; 310003A182262 to TF), the Austrian Science Foundation (FWF P21498-B11 to TF), the National Institutes of Health (NIH R01GM100366 to PS), the National Science Foundation (NSF DEB 0921307 to PSS), and the Department of Ecology and Evolution at the University of Lausanne. Parts of this paper were written while TF was a Visiting Professor in the Research Training Group 2200 ‘Evolutionary Processes in Adaptation and Disease’ at the Institute for Evolution and Biodiversity, University of Münster, Germany, and

supported by Mercator Fellowship from the German Research Foundation (DFG) to TF.

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.

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Table 1. Summary of ANOVA results for viability, femur length, wing area:thorax length ratio, and female starvation resistance. 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.

	Fat content	
Factor	**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 14-3-3ε. AKT/PKB antagonizes the activity of the tuberous sclerosis complex 1/2 (TSC1/TSC2); TSC1/2 in turn inactivates RAS homologue enriched in brain (RHEB). The inactivation of RHEB 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.

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

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

Figure 1

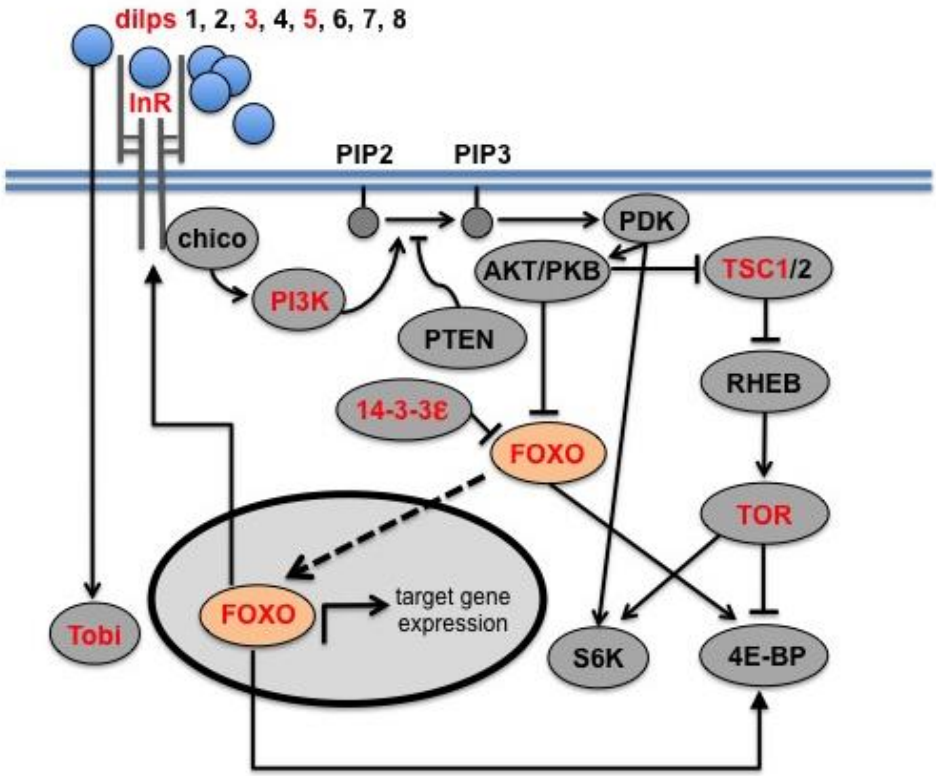

dilps 1, 2, 3, 4, 5, 6, 7, 8
InR
PIP2
PIP3
chico
PI3K
PTEN
PDK
AKT/PKB
TSC1/2
RHEB
14-3-3ε
FOXO
TOR
FOXO
target gene expression
Tobi
S6K
4E-BP

Figure 2

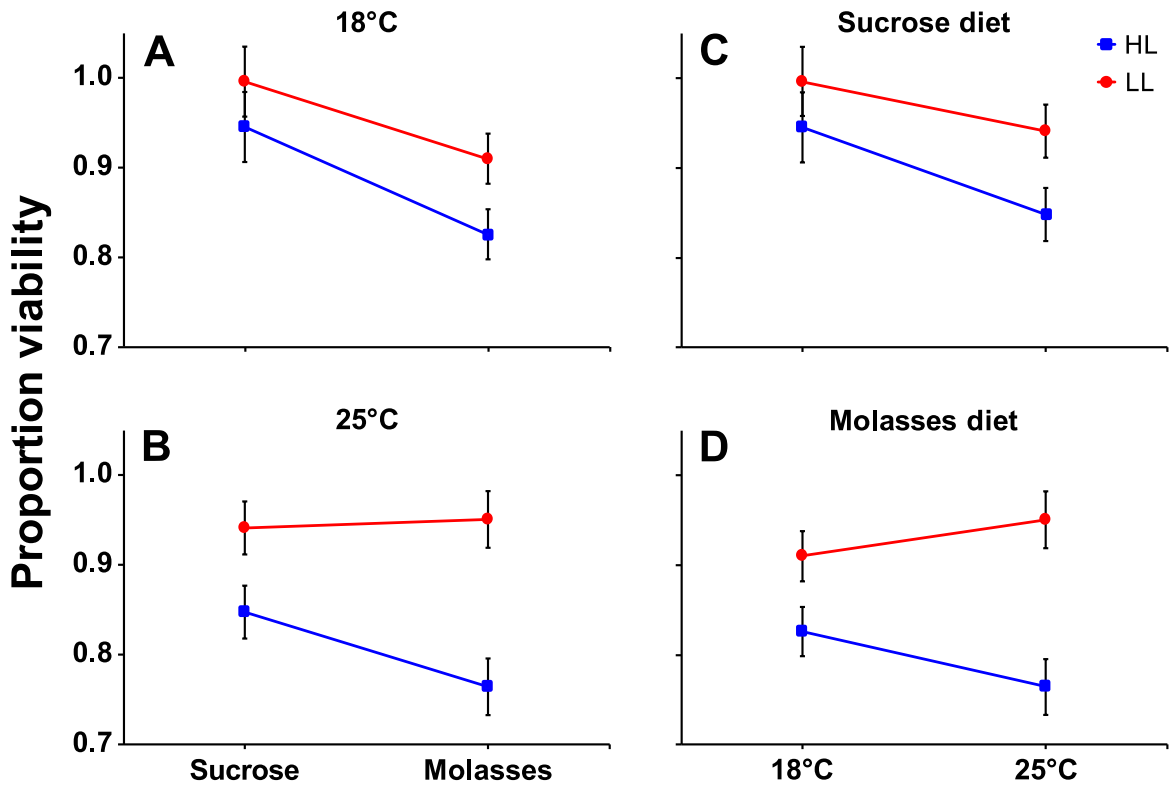

A
18°C
B
25°C
C
Sucrose diet
D
Molasses diet
HL
LL
Proportion viability
1.0
0.9
0.8
0.7
Sucrose
Molasses
18°C
25°C

Figure 3

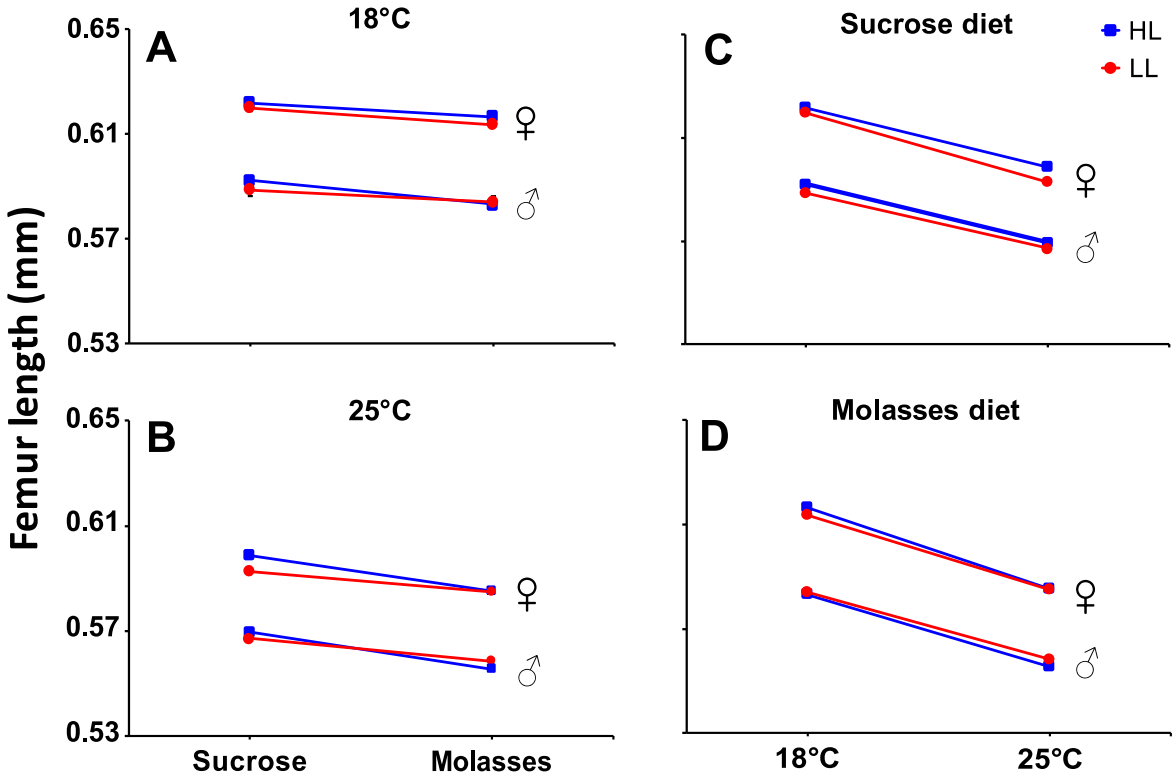

A
18°C
B
25°C
C
Sucrose diet
D
Molasses diet
HL
LL
♀
♂
Femur length (mm)
0.65
0.61
0.57
0.53
Sucrose
Molasses
18°C
25°C

Figure 4

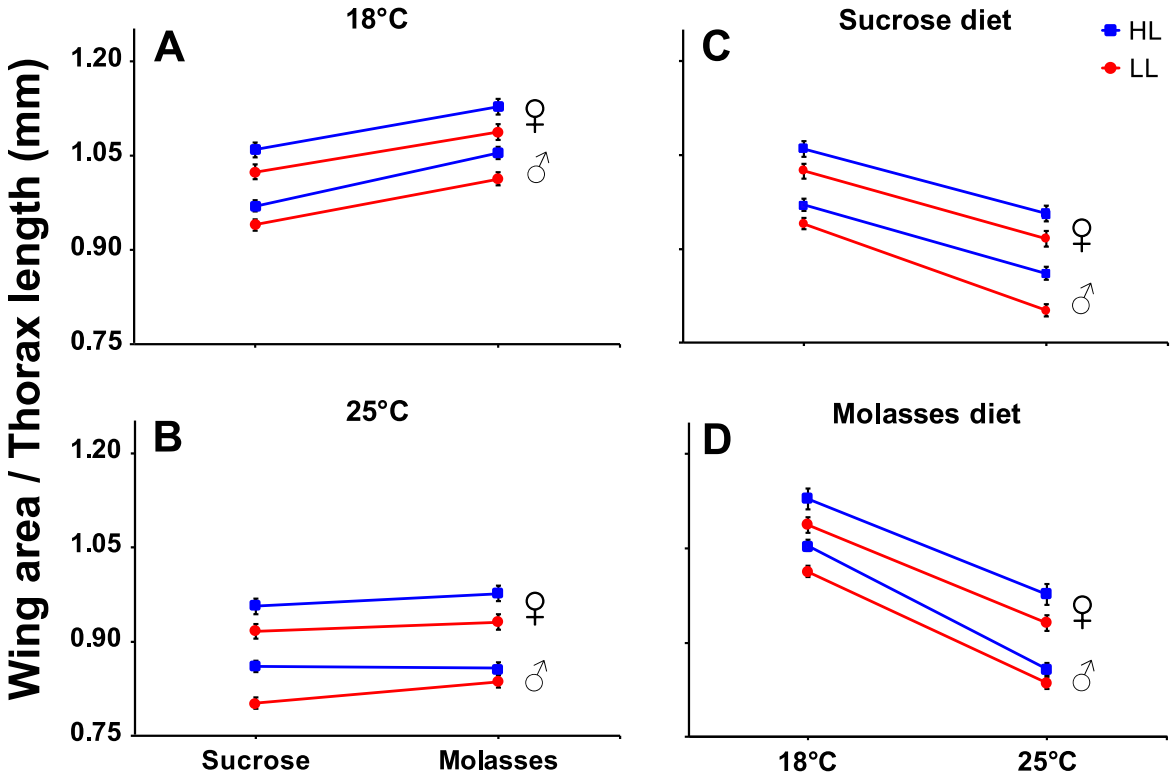

Wing area / Thorax length (mm)
A
18°C
B
25°C
C
Sucrose diet
D
Molasses diet
HL
LL
♀
♂
1.20
1.05
0.90
0.75
Sucrose
Molasses
18°C
25°C

Figure 5

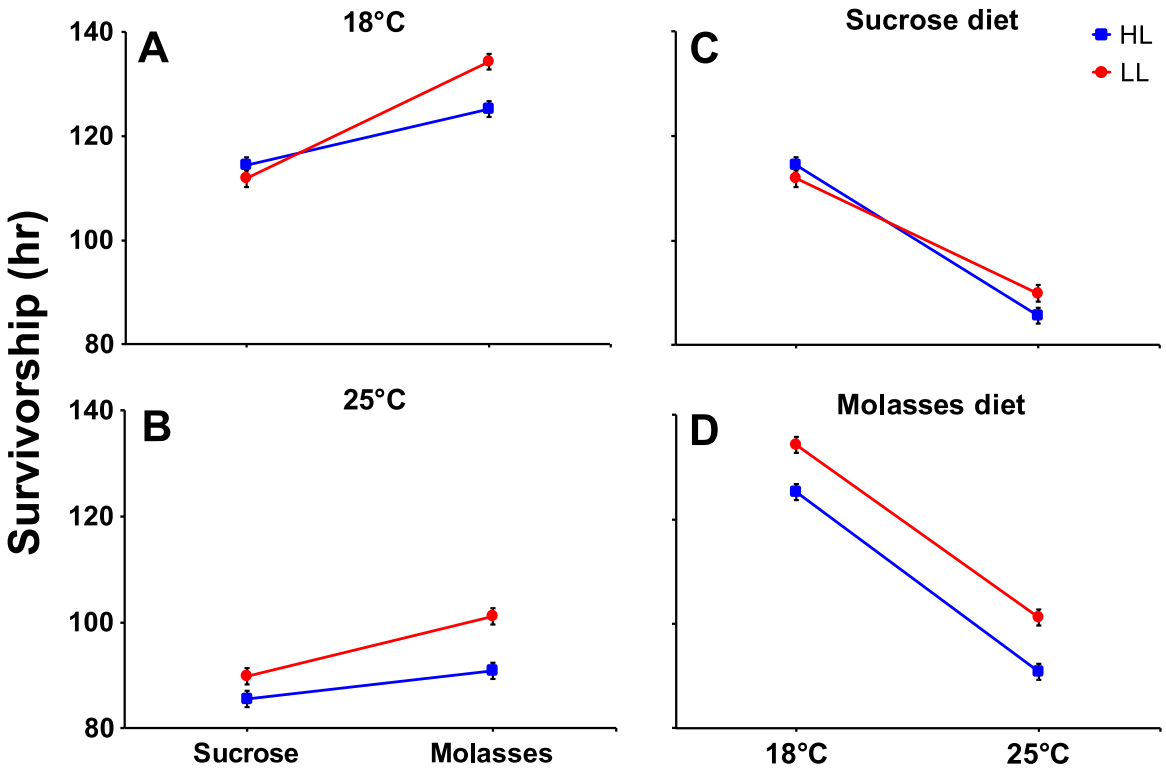
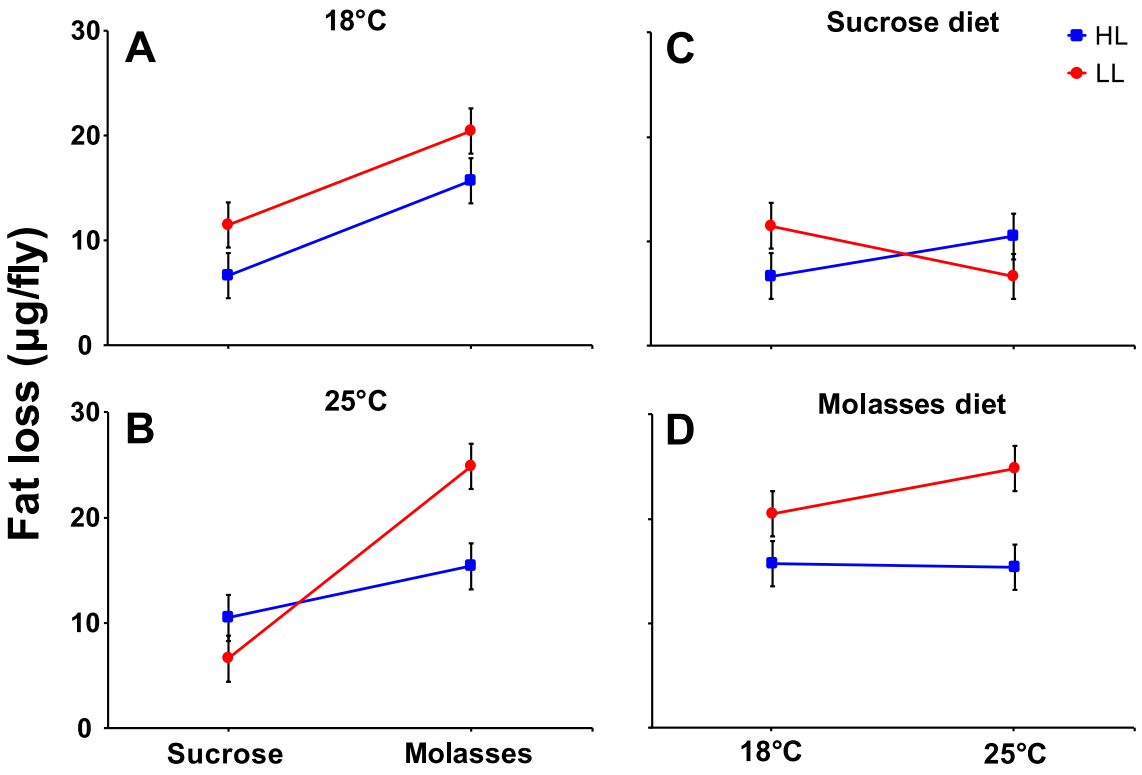

A
18°C
B
25°C
C
Sucrose diet
D
Molasses diet
HL
LL
Survivorship (hr)
140
120
100
80
Sucrose
Molasses
18°C
25°C

Figure 6


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

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 ≥ 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).

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^2) in females and males. (A) Dietary reaction norms at 18°C. (B) Dietary reaction norms at 25°C. (C) Thermal reaction norms on sucrose diet. (D) Thermal reaction norms on molasses diet. Shown are means and standard errors. Red lines: low-latitude (LL) allele, blue lines: high-latitude (HL) allele. See Results section for details.

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

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.

Figure S1

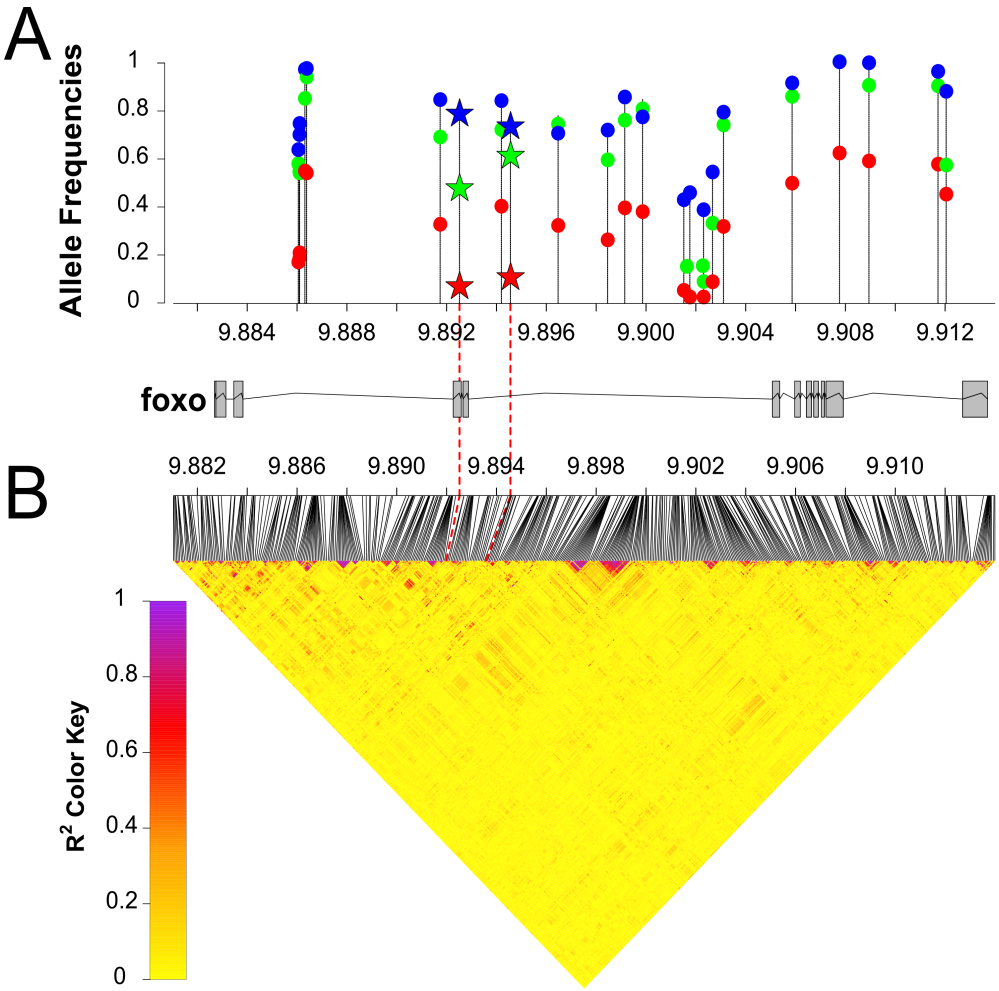

A
Allele Frequencies
1
0.8
0.6
0.4
0.2
0
9.884
9.888
9.892
9.896
9.900
9.904
9.908
9.912
foxo
B
9.882
9.886
9.890
9.894
9.898
9.902
9.906
9.910
R2 Color Key
1
0.8
0.6
0.4
0.2
0

Figure S2

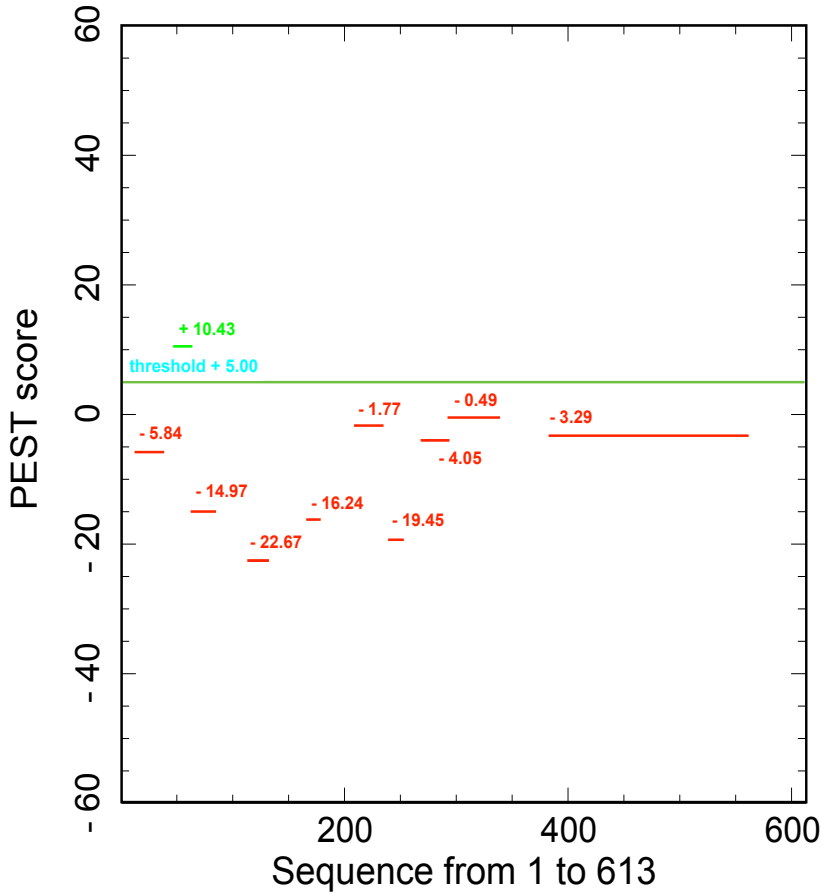

PEST score
60
40
20
0
- 20
- 40
- 60
200
400
600
Sequence from 1 to 613
+ 10.43
threshold + 5.00
- 5.84
- 14.97
- 22.67
- 16.24
- 1.77
- 19.45
- 4.05
- 0.49
- 3.29

Figure S3

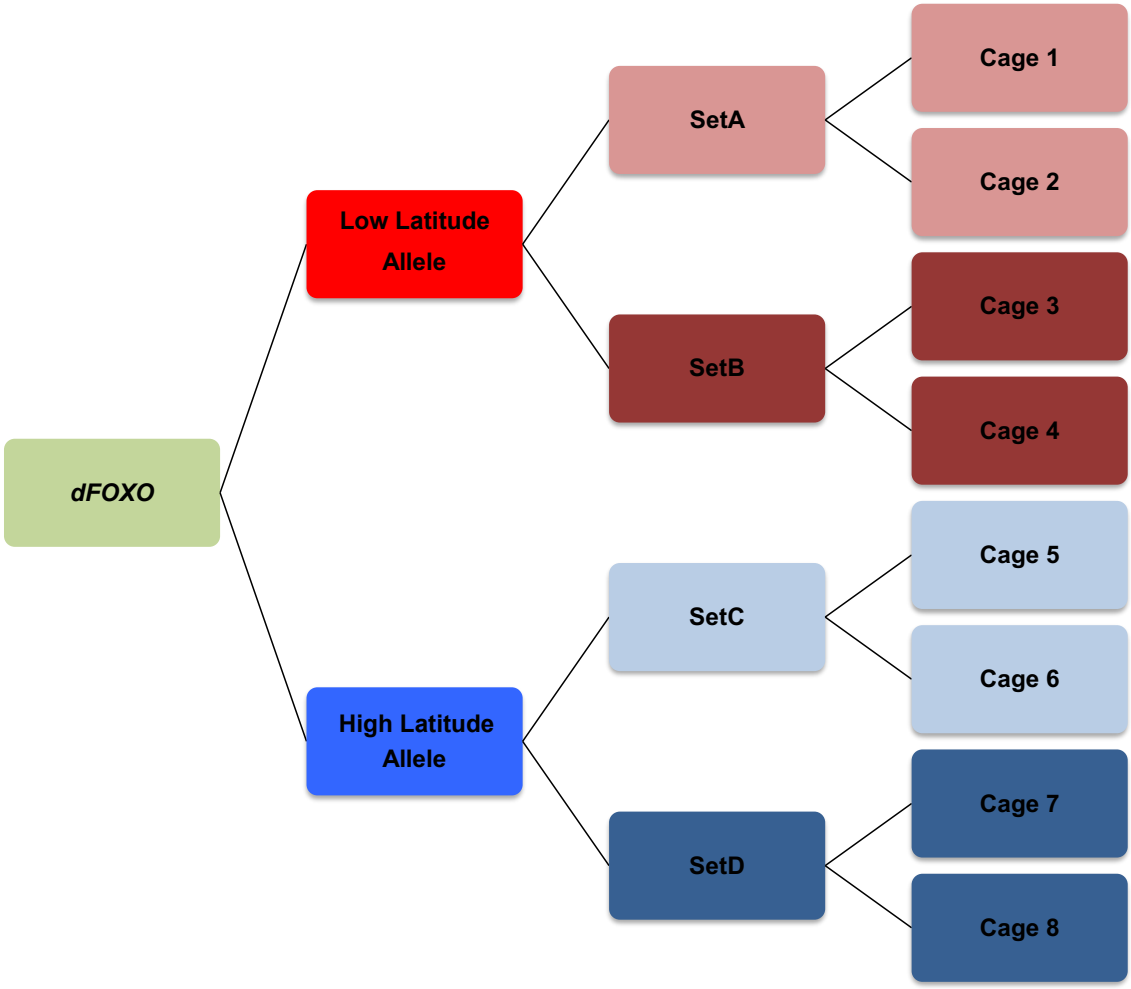

dFOXO
Low Latitude Allele
High Latitude Allele
SetA
SetB
SetC
SetD
Cage 1
Cage 2
Cage 3
Cage 4
Cage 5
Cage 6
Cage 7
Cage 8

Figure S4

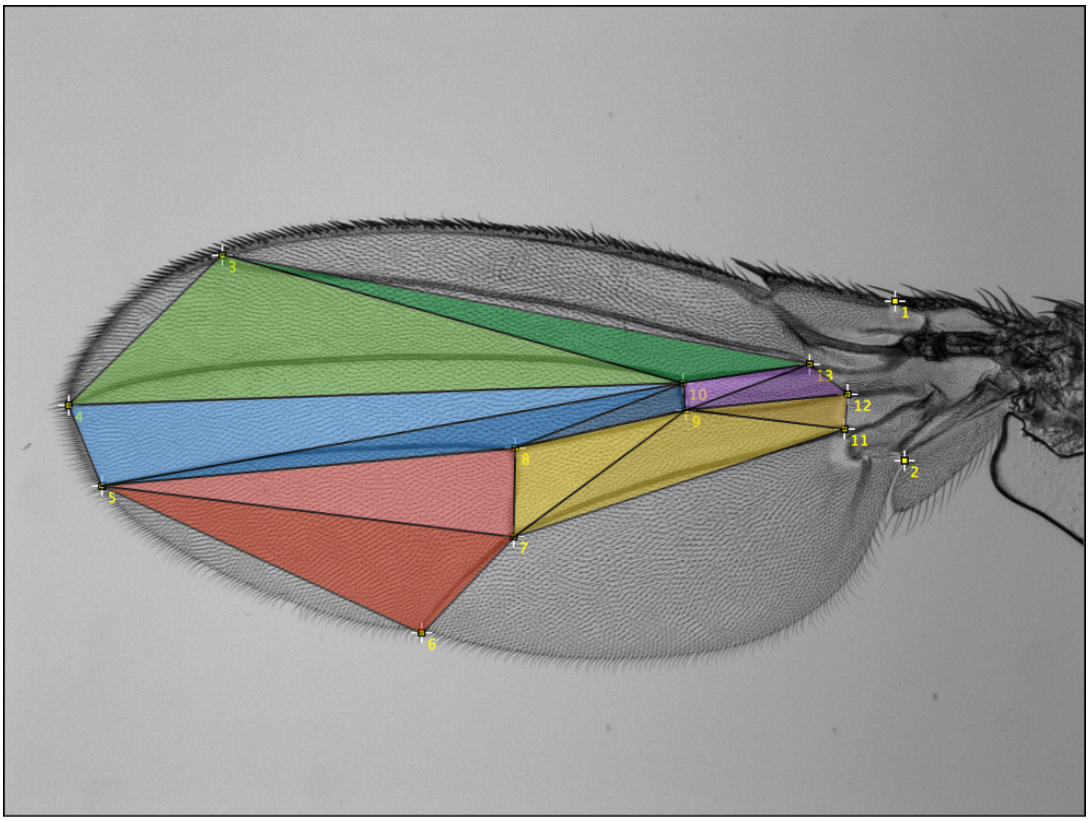


Figure S5

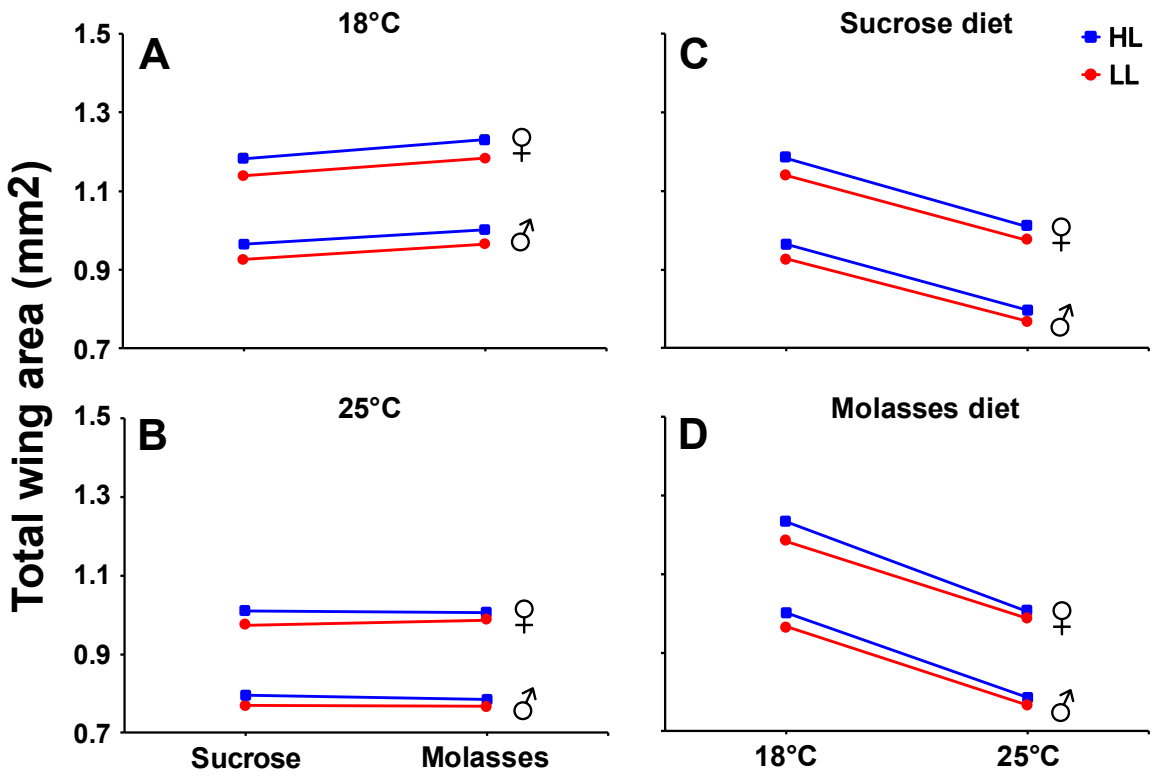

Total wing area (mm2)
1.5
1.3
1.1
0.9
0.7
A
18°C
B
25°C
C
Sucrose diet
D
Molasses diet
HL
LL
♀
♂
Sucrose
Molasses
18°C
25°C

Figure S6

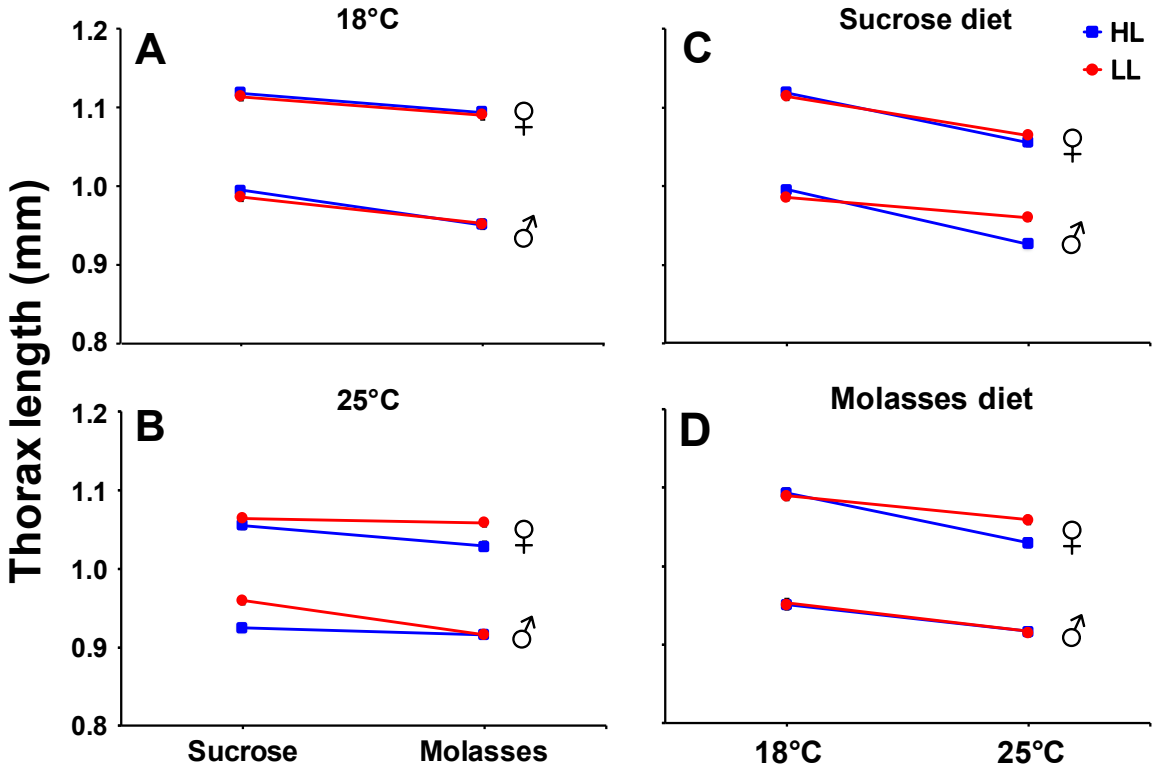

Thorax length (mm)
1.2
1.1
1.0
0.9
0.8
A
18°C
B
25°C
C
Sucrose diet
D
Molasses diet
HL
LL
♀
♂
Sucrose
Molasses
18°C
25°C

Figure S7

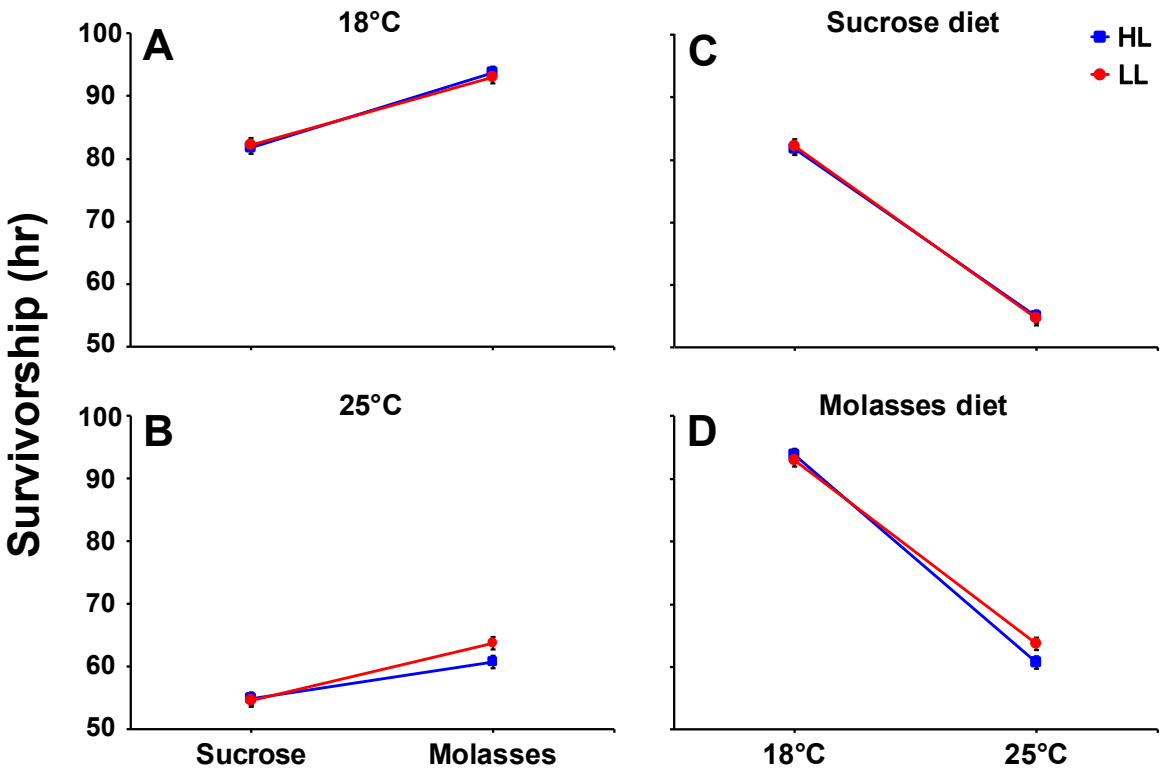

Survivorship (hr)
A
18°C
B
25°C
C
Sucrose diet
D
Molasses diet
HL
LL
100
90
80
70
60
50
Sucrose
Molasses
18°C
25°C

Figure S8

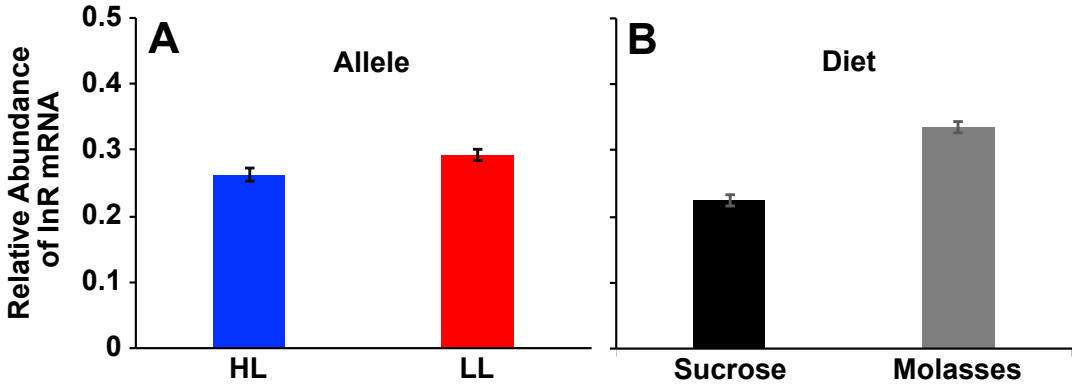

A
Allele
B
Diet
Relative Abundance of InR mRNA
0.5
0.4
0.3
0.2
0.1
0
HL
LL
Sucrose
Molasses

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

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

Table S2. Nutritional value and composition of sucrose and molasses diets. Table S2a: nutritional values of fly food ingredients per 100 g; Table S2b: recipe for sucrose and molasses diets; Table S2c: comparison of nutritional values of sucrose and molasses diets. See Materials and Methods section for details. The sucrose diet is the standard medium used in our laboratory in Lausanne; the recipe for the molasses diet follows that recipe of the Bloomington *Drosophila* Stock Center (BDSC) but uses different products for the food ingredients. 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) (*Polenta, Migros*)	50	61.3
Yeast (g/L) (*Actilife, Migros*)	50	12.4
Sugar (g/L) (*Cristal, Migros*)	50	0
Molasses (g/L) (*Zuckerrohrmelasse, EM Schweiz*)	0	109.6
Agar (g/L) (*Drosophila Agar Type II, Genesee*)	7	6
Nipagin 10% (ml/L) (*Sigma Aldrich*)	10	14.3
Propionic acid (ml/L) (*Sigma Aldrich*)	6	6

S2c. Nutritional values of sucrose and molasses diets

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

Table S3. Summary of ANOVA results for wing area, thorax length, and male starvation resistance. White and grey cells show the results for females and males, respectively; 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 S4. Summary of ANOVA results for relative abundance of *insulin-like receptor* (*InR*) transcript levels. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Factor in ANOVA	Relative Abundance of *InR*
Allele	$F_{1,80}=4.54^{*}$
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)	$F_{4,80}=5.73^{***}$