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

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; 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; 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).

127 Interestingly, many of the pathways that harbor clinal loci are known from
128 functional genetic studies to be implicated in the physiological regulation of life
129 history in organisms such as *C. elegans*, *Drosophila* and the mouse (Tatar et al.
130 2003; Fielenbach and Antebi 2008; Flatt and Heyland 2011; Flatt et al. 2013). In
131 particular, we found strongly clinal SNPs in multiple components of the IIS/TOR
132 pathway, including SNPs in *Drosophila insulin-like peptide* genes *dilp3* and *dilp5*,
133 *insulin-like receptor* (*InR*), *phosphatidyl-inositol-4,5-bis-phosphate 3-kinase* (*Pi3K*),
134 forkhead box-O transcription factor *foxo*, the *foxo* regulator *14-3-3ε*, *target of brain*
135 *insulin* (*tobi*), *tuberous sclerosis complex 1* (*Tsc1*), and *target of rapamycin* (*Tor*) (Fig.
136 1; Fabian et al. 2012; Kapun et al. 2016b). This pattern is compelling since loss-of-
137 function mutations in the IIS/TOR pathway have major, evolutionarily conserved
138 effects on growth, size, reproduction, lifespan and stress resistance in *Drosophila*, *C.*
139 *elegans*, and the mouse (Kenyon et al. 1993; Gems et al. 1998; Böhni et al. 1999;
140 Brogiolo et al. 2001; Tatar and Yin 2001; Clancy et al. 2001; Kenyon 2001; Oldham
141 et al. 2002; Oldham and Hafen 2003; Holzenberger et al. 2003; Tatar et al. 2001;
142 Partridge et al. 2005).
143 Since many fitness-related traits affected by IIS/TOR also exhibit phenotypic
144 clines, it is tempting to hypothesize that natural variation in this pathway contributes
145 to life-history clines, especially with regard to body size (de Jong and Bochdanovits
146 2003); yet, the evolutionary significance of natural variants in this pathway is poorly
147 understood. An exception is an indel polymorphism in the *D. melanogaster InR* gene,
148 which varies clinally along both the North American and Australian east coasts and
149 which has multifarious life-history effects (Paaby et al. 2010, 2014). Consistent with
150 the idea that IIS polymorphisms affect adaptation, natural variation in adult
151 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 (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 (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 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 (this paper; also see analyses in our companion study, Betancourt et al. 2018). Here we characterize the effects of

177 these clinal *foxo* genotypes on several fitness-related traits (egg-to-adult survival,
178 proxies of size, starvation resistance, fat content) by measuring phenotypes on
179 replicate populations of the two alternative alleles under different environmental
180 assay conditions in the laboratory. Since temperature gradients are thought to
181 underpin – at least partly – latitudinal clines (e.g., de Jong and Bochdanovits 2003;
182 Kapun et al. 2016b; and references therein), and because both diet and temperature
183 modulate IIS (e.g., Britton et al. 2002; Kramer et al. 2003; Puig and Tijan 2005;
184 Giannakou et al. 2008; Teleman 2010; Puig and Mattila 2011; Li and Gong 2015;
185 Zhang et al. 2015), we phenotyped replicated population cage cultures of the
186 alternative alleles at two temperatures (18°C, 25°C) and on two commonly used diets
187 that differ mainly in their sugar source (sucrose vs. molasses) and content.
188 Measuring reaction norms to assess phenotypic plasticity and genotype-by-
189 environment interactions (G × E) for this variant is of interest since still little is known
190 about whether and how clinality and plasticity interact (van Heerwaarden and Sgrò
191 2017), and with previous work having mainly focused on gene expression, not whole-
192 organism traits (e.g., see de Jong and Bochdanovits 2003; Hoffmann et al. 2005;
193 Levine et al. 2011; Overgaard et al. 2011; Chen et al. 2012; Cooper et al. 2012; Zhao
194 et al. 2015; Clemson et al. 2016; Mathur and Schmidt 2017; and references therein).
195 For example, *D. melanogaster* feeds and breeds on various kinds of rotting fruit, with
196 the protein:carbohydrate (P:C) ratios exhibiting spatiotemporal variation (Lachaise et
197 al. 1988; Hoffmann and McKechnie 1991; Markow et al. 1999; Keller 2007), but how
198 dietary plasticity affects traits in a clinal context is not well understood. Similarly, the
199 interplay between thermal plasticity and thermal adaptation is incompletely
200 understood (e.g., de Jong and Bochdanovits 2003; Overgaard et al. 2011; Mathur
201 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 below. In brief, our results show that the *foxo* polymorphism affects multiple components of fitness according to these predictions, in particular the clinality of size-related traits; we also observe that two alternative *foxo* alleles respond plastically to changes in temperature and diet but overall we find little evidence for G x E interactions.

In a companion study (Betancourt et al. 2018) we analyze all polymorphic SNPs at the *foxo* locus in a genomic dataset from 10 populations along the North American cline; use these data to show that our candidate *foxo* polymorphism represents an extreme outlier in terms of clinality and is likely maintained non-neutrally; report life-history effects of this polymorphism from independent assays performed under constant environmental conditions in a different laboratory; and directly compare the effects of this variant to new phenotypic clinal data collected from six populations along the North American east coast.

Together, our complementary studies demonstrate that this *foxo* polymorphism is an important target of spatially varying selection along the North American cline and that it makes a major contribution to life-history adaptation.

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; cf. Fabian et al.

2012 for details of outlier detection; also see 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; see Fabian et al. 2012 for details), both SNPs are also strongly clinal in a more comprehensive dataset based on 10 populations along the cline, analyzed in the companion study by Betancourt et al. (2018) and 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. Importantly, our population genomic analyses in Betancourt et al. (2018) suggest that this polymorphism exhibits much stronger clinality than the majority of other *foxo* SNPs and that it is likely maintained non-neutrally by spatially varying selection when compared to a genome-wide panel of 20,000 SNPs in short introns that presumably evolve neutrally.

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 recent 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 at the University of Pennsylvania in Philadelphia (Betancourt et al. 2018); F2 flies from these cages were transferred to the University of Lausanne for establishing population cages at our laboratory (see below).

By analyzing the genomes of the DGRP lines used to set up the experimental populations we confirmed in Betancourt et al. (2018) 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 systematic genomic differentiation (F_{ST}) in the genome-wide background of the two focal SNPs: even though there exist other SNPs that are strongly differentiated ($F_{ST} > 0.5$) between the HL and LL populations, the majority of these SNPs are different between the independently replicated sets (blocks) of DGRP lines that were used to make the HL vs. LL contrast. Thus, strongly differentiated SNPs that are specific ('private') 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 in our laboratory in Lausanne 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 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: ~22ºC, ~10:14 h light:dark, ~50% humidity). Thus, phenotypes were measured after a total of 12 generations of recombination.

PHENOTYPE ASSAYS

All assays reported here were performed in our previous laboratory in Lausanne; in our companion study we report independent assays performed under constant

environmental conditions in Philadelphia (Betancourt et al. 2018), allowing us to assess the reproducibility of the allelic effects and to account for potential variation in life-history traits due to potential differences in local laboratory assay conditions (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 $\times$ 8 cages $\times$ 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 $\times$ 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 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 (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 vials $\times$ 10 flies per vial $\times$ 2 sexes $\times$ 8 cages $\times$ 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).

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, repeatibility 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 clinal *foxo* polymorphism to the overall phenotypic cline for wing area, a trait which we have recently measured across 6 populations along the North American east coast (Betancourt et al. 2018). This analysis was performed for flies raised on a molasses diet at 25°C, i.e. using similar assay conditions as those used by Betancourt et al. (2018) for clinal wing area measurements. We calculated the proportional contribution of the *foxo* polymorphism to the overall cline as follows: $\Delta_{foxo} \times \Delta_{\text{frequency}} / \Delta_{\text{cline}}$, where Δ_{foxo} is the difference in mean wing area between the HL and LL allelic states, $\Delta_{\text{frequency}}$ is the allele frequency gradient for the *foxo* polymorphism between the cline ends (Maine vs. Florida, ~60%)

425 and Δ_{cline} is the difference in mean wing area between the cline ends as estimated
426 from the data in Betancourt et al. (2018).
427
428 **PREDICTIONS**
429 Here we make some qualitative predictions for the expected behavior of the *foxo*
430 polymorphism with regard to (1) clinal phenotypic effects, (2) patterns of trait
431 covariation determined by IIS, and (3) plasticity, G × E, and local adaptation. We
432 compare our results to these predictions in the Results section below.
433 (1) Latitudinal clinality. Traits which have been found to covary positively with
434 latitude include, for example, faster development, lower egg-to-adult survival
435 (viability), increased body size, reduced wing loading, reduced fecundity, prolonged
436 lifespan, and increased resistance to starvation, cold and heat stress (e.g., Coyne
437 and Beecham 1987; Azevedo et al. 1998; Bochdanovits and de Jong 2003a; de Jong
438 and Bochdanovits 2003; Schmidt et al. 2005a, 2005b; Folguera et al. 2008; Schmidt
439 and Paaby 2008; Bhan et al. 2014; Mathur and Schmidt 2017; Durmaz et al. 2018).
440 For some traits clinal patterns have been observed in a parallel fashion on multiple
441 continents, but there can also be major differences among continents (e.g., see
442 discussion in Fabian et al. 2015); for example, contrasting predictions have been
443 made for viability (van 't Land et al. 1999), starvation resistance (Karan et al. 1998,
444 Robinson et al. 2002; Hoffmann et al. 2005; Goenaga et al. 2013) and heat tolerance
445 (Hoffmann et al. 2002; Sgrò et al. 2010).
446 In general, we would expect that the effects of the high- and low-latitude *foxo*
447 alleles agree with the overall phenotypic patterns across latitude, especially for those
448 traits that have previously been examined along the North American cline (e.g.,

449 Coyne and Beecham 1987; Schmidt and Paaby 2008; Paaby et al. 2014; Kapun et
450 al. 2016a; Mathur and Schmidt 2017; Durmaz et al. 2018).
451 (2) IIS. Traits that are associated with reduced IIS include reduced body size,
452 increased lifespan, resistance to starvation and cold, increased fat content, reduced
453 fecundity, and activation of FOXO (Tatar et al. 2001, 2003; Oldham and Hafen 2003;
454 Broughton et al. 2005; Teleman 2010). For example, loss-of-function (LOF) mutants
455 of *foxo* exhibit (depending on the allele) prolonged development, reduced weight,
456 smaller wing size, reduced fecundity, shortened lifespan, and reduced survival upon
457 oxidative and starvation stress (Jünger et al. 2003; Kramer et al. 2003, 2008;
458 Hwangbo et al. 2004; Giannakou et al. 2004; Giannakou et al. 2008; Slack et al.
459 2011); the effects of IIS (or of *foxo*) on viability are, however, not well understood.
460 Conversely, overexpression of *foxo* has opposite effects on most of these traits (e.g.,
461 increased lifespan), yet – like LOF alleles – causes decreased size (Kramer et al.
462 2003; Puig et al. 2003; Hwangbo et al. 2004; Kramer et al. 2008; Tang et al. 2011).
463 We predict that the naturally occurring *foxo* alleles tested here differ consistently
464 along this IIS/*foxo* axis of trait covariation. Notably, traits observed in flies from high-
465 versus low-latitude populations in North America resemble those of flies with low
466 versus high IIS, respectively (e.g., de Jong and Bochdanovits 2003; Flatt et al. 2013;
467 Paaby et al. 2014): lower fecundity, improved stress resistance, and longer lifespan
468 observed in high-latitude flies are traits that tend to be co-expressed in IIS mutants;
469 however, flies from high-latitude populations are larger than low-latitude flies, yet
470 reduced IIS causes smaller size.
471 (3) Plasticity, G × E, and local adaptation. With regard to thermal effects, we would
472 expect flies raised at lower temperature to exhibit prolonged development, reduced
473 viability, larger size, reduced wing loading, lower fecundity, increased lifespan, and

474 improved starvation resistance (David et al. 1994; Partridge et al. 1994a, 1994b;
475 James and Partridge 1995; Bochdanovits and de Jong 2003b; Trotta et al. 2006;
476 Folguera et al. 2008; Klepsatel et al. 2013, 2014; Mathur and Schmidt 2017; cf.
477 Hoffmann et al. 2005 for a contrasting prediction for starvation survival).

478 With respect to dietary effects, higher P:C ratios, for instance, might be expected
479 to cause increased viability, larger size but reduced starvation resistance (Lee and
480 Jang 2014; Lihoreau et al. 2016; Reis 2016). In terms of G × E, genotypes from
481 temperate, seasonal high-latitude habitats might be more plastic than those from low-
482 latitude habitats (Overgaard et al. 2011; Klepsatel et al. 2013); if so, patterns of
483 differential plasticity between high- and low-latitude alleles might be consistent with
484 patterns of local adaptation (Mathur and Schmidt 2017).

485

486 *Results*

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

494

495 **ALLELIC VARIATION AT *FOXO* AFFECTS VIABILITY**

496 The *foxo* polymorphism significantly affected viability, with the LL allele exhibiting
497 higher egg-to-adult survival than the HL allele (Fig. 2; Table 1), consistent with
498 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 × 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; Betancourt et al. 2018) 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 as measured in our experiments makes a proportional contribution of ~14% to the total cline for wing area as measured by Betancourt et al. (2018) (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 (see 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 × 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 (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 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

549 Information), potentially in support of the finding that lower P:C ratios favor higher
550 resistance (Chippindale et al. 1993; Lee and Jang 2014). We also found evidence for
551 an allele by diet interaction: allelic differences in resistance were more pronounced
552 on molasses than sucrose diet (Fig. 5; Table 1; Fig. S7; Table S4, Supporting
553 Information).

554 To further examine the physiological basis of starvation resistance we quantified
555 how much fat female flies mobilize upon starvation (Fig 6; Table 2; males were not
556 examined since they did not show allelic differences in resistance). Paralleling our
557 result that LL females are more resistant than HL females, the amount of fat
558 catabolized under starvation was greater in LL than in HL females, under almost all
559 conditions (except for females raised on sucrose diet at 25°C; see Fig. 6 and Table 2:
560 significant allele by diet by starvation treatment interaction at 25°C but not at 18°C).
561 Fat loss upon starvation was greater for flies raised on molasses than on sucrose
562 diet (Fig 6; Table 2), again matching the results for starvation resistance itself.
563

564 **_FOXO_ ALLELES DIFFER IN TRANSCRIPTIONAL FEEDBACK CONTROL OF _InR_**

565 From the above patterns we predicted that the LL allele would exhibit decreased IIS
566 and increased FOXO activity: the LL allele has smaller size but higher starvation
567 resistance, i.e. traits that co-occur in IIS mutants or flies with increased FOXO
568 activity. To test this hypothesis we performed qRT-PCR analysis of a major
569 transcriptional target of FOXO, *InR*: when IIS is low, FOXO becomes active and
570 upregulates *InR* transcription, while under high IIS FOXO is inactive and represses
571 *InR* (Puig et al. 2003; Puig and Tjian 2005). In support of this hypothesis we found
572 that the LL allele had a ~12% higher level of *InR* transcript than the HL allele (Fig.
573 S8; Table S6, Supporting Information). Dietary conditions also affected *InR* levels,

574 with flies raised on molasses producing more *InR* than flies raised on sucrose diet
575 (Fig. S8; Table S6, Supporting Information).
576
577

578 *Discussion*

579 CONNECTING ADAPTIVE CLINAL PHENOTYPES TO GENOTYPES

580 Here we have studied the life-history effects of a strongly clinally varying, presumably
581 adaptive polymorphism in the IIS gene *foxo*, a naturally segregating variant identified
582 from our genomic analysis of the North American latitudinal cline (Fabian et al. 2012;
583 Betancourt et al. 2018).

584 As hypothesized by de Jong and Bochdanovits (2003), genes of the IIS/TOR
585 pathway might represent particularly promising candidates underlying clinal life-
586 history adaptation in *D. melanogaster*: (1) laboratory mutants in this pathway often
587 mirror life-history traits and trade-offs observed in natural populations (de Jong and
588 Bochdanovits 2003; Clancy et al. 2001; Tatar et al. 2001; Tatar and Yin 2001; Tatar
589 et al. 2003; Paaby et al. 2010; Flatt et al. 2013; Paaby et al. 2014; Flatt and Partridge
590 2018); (2) reproductive dormancy in response to cool temperature and short
591 photoperiod, a genetically variable and clinal trait (Williams and Sokolowski 1993;
592 Schmidt et al. 2005a; Schmidt and Conde 2006; Schmidt et al. 2005b; Schmidt and
593 Paaby 2008), is physiologically regulated by IIS (Williams et al. 2006; Flatt et al.
594 2013; Kubrak et al. 2014; Schiesari et al. 2016; Zhao et al. 2016; Andreatta et al.
595 2018); (3) genomic analyses of clinal differentiation has identified many clinal SNPs
596 in the IIS/TOR pathway presumably shaped by spatially varying selection (Fig. 1;
597 Kolaczkowksi 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

623 known to be affected by the *foxo* locus. With regard to growth and size, our findings
624 from natural variants agree well with functional genetic studies showing that genetic
625 manipulations of the *foxo* locus affect body size and wing area (Jünger et al. 2003;
626 Slack et al. 2011; Tang et al. 2011). Similarly, our observation that variation at *foxo*
627 affects survival and fat content upon starvation is consistent with the fact that *foxo*
628 mutants display reduced starvation resistance (Jünger et al. 2003; Kramer et al.
629 2003, 2008). In contrast, although *foxo* null mutants produce viable adults (Jünger et
630 al. 2003; Slack et al. 2011), whether distinct *foxo* alleles vary in viability has not yet
631 been examined; here we find that the two natural alleles differ in egg-to-adult
632 survival. We also asked whether the alleles differentially affect mRNA abundance of
633 *InR*, a transcriptional target of FOXO (Puig et al. 2003; Puig and Tjian 2005). Indeed,
634 the LL allele had higher *InR* mRNA levels, consistent with the LL genotype exhibiting
635 reduced IIS and higher FOXO activity.

636 For most traits measured, both alleles reacted plastically to changes in diet and
637 temperature in the direction predicted from previous work (Partridge et al. 1994a,
638 1994b; Lee and Jang 2014; Lihoreau et al. 2016; Mathur and Schmidt 2017), yet we
639 found very little evidence for allele by environment (G × E) interactions.

640 While our experimental design does not allow us to disentangle the contribution of
641 the 2 individual SNPs to the total effects seen for the *foxo* polymorphism, our results
642 suggest that the naturally occurring alternative alleles at *foxo* we have examined here
643 – and which are defined by only two linked SNP positions –can apparently have quite
644 strong pleiotropic (or, via LD, correlational) effects upon multiple complex life-history
645 traits, including on viability, several proxies of size and on starvation resistance (for
646 estimates of allelic effect sizes see Table S4, Supporting Information). This is
647 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 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

673 cold temperature (Stalker 1980; David et al. 1994; Azevedo et al. 1998; Frazier et al.
674 2008; Bhan et al. 2014); and reduced viability (Folguera et al. 2008). Conversely, the
675 LL allele exhibits smaller size, increased wing loading, and higher viability. Thus, the
676 *foxo* variant contributes to the observed phenotypic cline in the predicted direction
677 (gradient or cogradient variation) and appears to be maintained by spatially varying
678 selection (for a remarkable example where size is subject to countergradient – not
679 cogradient – variation along an altitudinal gradient in Puerto Rican *D. melanogaster*
680 see Levins, 1968, 1969). Importantly, our results for the allelic effects of this
681 polymorphism on size-related traits are fully consistent with the independent assays
682 performed by Betancourt et al. (2018) under constant laboratory conditions and
683 suggest a major contribution of the *foxo* polymorphism to clinal size variation (the
684 polymorphism seems to account for ~14% of the total latitudinal cline in wing area;
685 see Results section).
686 For starvation resistance, we found – contrary to clinal predictions – that the HL
687 allele is less resistant than the LL allele, consistent with countergradient variation (but
688 see Betancourt et al. 2018; discussion below). Interestingly, a similar countergradient
689 effect (on body size) was found for the *InR* polymorphism mentioned above: the high-
690 latitude InR^{short} allele confers smaller size, even though flies from high-latitude
691 populations are normally larger (Paaby et al. 2014). Likewise, for a clinal variant of
692 *neurofibromin 1* (*Nf1*) the high-latitude haplotype has smaller wing size, an effect that
693 runs counter to the cline (Lee et al. 2013). However, as mentioned in the methods,
694 we can of course not completely rule out potentially confounding LD effects that
695 might account for this unexpected result with regard to starvation resistance.
696 In terms of the physiological effects of IIS, temperate fly populations might be
697 characterized by 'thrifty' genotypes with high IIS, whereas tropical populations might

698 have a higher frequency of 'spendthrift' genotypes with low IIS (de Jong and
699 Bochdanovits 2003). Our finding that the low-latitude *foxo* allele likely exhibits
700 increased FOXO activity and lower IIS seems to support this, yet Paaby et al. (2014)
701 found that IIS was lower for the high-latitude *InR* allele. The directionality of IIS
702 effects along the cline thus remains difficult to predict.

703 As noted by Lee et al. (2013) and Paaby et al. (2014), clinal variants subject to
704 countergradient effects might interact epistatically with other loci affecting the trait, or
705 they might be affected by antagonistic selection pressures (Schluter et al. 1991).
706 Conflicting selection pressures on clinal variants might be particularly acute when
707 they exhibit pleiotropic effects on multiple traits, as is the case for the polymorphisms
708 at *Nf1, InR*, and *foxo*. These examples illustrate the complexity of dissecting clinal
709 selection and the genotype-phenotype map underlying clinal adaptation (Lee et al.
710 2013; Paaby et al. 2014; Flatt 2016).

711 With regard to starvation resistance, an important caveat is that the results for this
712 trait were opposite between our laboratories: in our assays in Lausanne the low-
713 latitude *foxo* allele was more starvation resistant, while in Philadelphia the low-
714 latitude conferred increased resistance (Betancourt et al. 2018). This discrepancy
715 might be due to differences in the assay protocols used for measuring starvation
716 resistance in our laboratories: in contrast to our protocol using agar (see above), the
717 assay used in Betancourt et al. (2018) might additionally impose some degree of
718 desiccation stress. Interestingly, desiccation resistance is known to vary latitudinally
719 along the North America east coast (Rajpurohit et al. 2018), but whether the *foxo*
720 polymorphism examined here affects survival upon desiccation remains unknown
721 and awaits future study. Overall, however, our independent life-history assays across
722 two laboratories suggest that our phenotypic results are qualitatively robust and

723 repeatable (for a discussion of the effects of local laboratory assay conditions see
724 Ackermann et al. 2001).
725
726 **GROWING EVIDENCE FOR A ROLE OF IIS IN LIFE-HISTORY ADAPTATION**
727 The IIS pathway provides an excellent example of how mechanistic and evolutionary
728 insights might be combined to gain a more complete understanding of the ultimate
729 and proximate determinants of life-history adaptation (Finch and Rose 1995; Houle
730 2001; Flatt and Heyland 2011). Since the 1990s, a great deal has been learned
731 about the genetic, developmental and physiological effects of this pathway in model
732 organisms. This work has shown that IIS mutants affect major fitness-related traits,
733 and this in turn has illuminated our understanding of the molecular underpinnings of
734 growth, size, lifespan and trade-offs (Partridge and Gems 2002; Tatar et al. 2003;
735 Flatt et al. 2005; Flatt and Heyland 2011; Flatt et al. 2013). In particular, these
736 studies have revealed that the IIS pathway plays an evolutionarily conserved role in
737 the physiological regulation of longevity (Partridge and Gems 2002; Tatar et al.
738 2003); they have also given us some of the clearest examples of alleles exhibiting
739 antagonistic pleiotropy (Williams 1957; Flatt and Promislow 2007; and references
740 above).
741 The functional characterization of this pathway therefore promised an opportunity
742 for evolutionary geneticists to identify natural variants involved in life-history evolution
743 (de Jong and Bochdanovits 2003). Yet, 'life history loci' identified via functional
744 genetic analysis need not necessarily contribute to standing variation for these traits
745 in the wild (Flatt 2004; Flatt and Schmidt 2009; Fabian et al. 2018). For some time, it
746 thus remained unclear whether natural variation in this pathway impacts variation in
747 fitness-related traits in natural populations (see Reznick 2005; Fabian et al. 2018).

748 Today, we have growing evidence that variation in IIS indeed can make an
749 important contribution to life-history variation in flies and other insects, worms, fish,
750 reptiles and mammals, including effects on longevity in humans (e.g., de Jong and
751 Bochdanovits 2003; Williams et al. 2006; Flachsbart et al. 2008; Suh et al. 2008;
752 Willcox et al. 2008; Alvarez-Ponce et al. 2009; Sparkman et al. 2009, 2010; Paaby et
753 al. 2010; Stuart and Page 2010; Dantzer and Swanson 2012; Jovelin et al. 2014;
754 Paaby et al. 2014; Swanson and Dantzer 2014; McGaugh et al. 2015; Schwartz and
755 Bronikowski 2016; Zhao et al. 2016; and references therein). On the other hand,
756 'evolve and resequence' studies of *Drosophila* longevity have failed to find a major
757 contribution of standing variation in IIS to evolved changes in life history and lifespan,
758 perhaps suggesting that the IIS pathway might be selectively constrained, at least
759 with regard to the evolution of certain traits (e.g., Remolina et al. 2012; Fabian et al.
760 2018; Flatt and Partridge 2018). In sum, this body of work illustrates how one might
761 be able to connect genotypes to molecular mechanisms to components of fitness by
762 studying a fundamentally important physiological pathway from multiple angles
763 (Finch and Rose 1995; Houle 2001; Flatt and Heyland 2011; Flatt et al. 2013).
764

765 ## *Conclusions*

766 Here we have found that a strongly clinal polymorphism (which might be viewed as a
767 marker for alleles of functional significance) at the *foxo* locus has pleiotropic (or
768 correlational) effects upon several fitness-related traits known to vary clinally across
769 latitude, including egg-to-adult survival, several size-related traits, starvation
770 resistance and fat content. Depending on the thermal and dietary assay conditions,
771 the polymorphism had moderate to large allelic effects on these traits, but we found
772 little evidence for G × E interactions. The directionality of most of the observed allelic

773 effects matches previously observed phenotypic clines, especially with regard to
774 size-related traits (e.g., Schmidt et al. 2005a, 2005b; Schmidt and Paaby 2008;
775 Durmaz et al. 2018; Betancourt et al. 2018). In particular in terms of wing area, the
776 *foxo* polymorphism seems to make a substantial contribution to the total phenotypic
777 cline. These results – except for stress resistance – are corroborated by independent
778 assays reported in Betancourt et al. (2018). Our observations on a naturally
779 segregating polymorphism are also in good qualitative agreement with functional
780 genetic studies of the *foxo* locus using mutants and transgenes (Jünger et al. 2003;
781 Kramer et al. 2008; Slack et al. 2011). Together with the study of Betancourt et al.
782 (2018), whose genomic analyses indicate that this polymorphism likely evolves non-
783 neutrally, our results suggest that standing genetic variation in the IIS pathway
784 makes an important and – at least partly – predictable contribution to clinal life-history
785 adaptation in *Drosophila*.
786
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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.

1356 **Table 1.** Summary of ANOVA results for viability; femur length; wing area:thorax
1357 length ratio; female starvation resistance (also cf. Table S5). White and grey cells
1358 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*	

1359

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

1390 inhibits the negative regulator 4E-BP. The phenotypic effects of naturally occurring
1391 alleles of the genes in the IIS/TOR pathway remain poorly understood, but clinal
1392 polymorphisms in *InR* (Paaby et al. 2010; Paaby et al. 2014) and *foxo* (this study)
1393 have pleiotropic effects on life history in *Drosophila*.

1394

1395 **Figure 2.** Viability (egg-to-adult survival). Effects of the clinal *foxo* variant on the
1396 proportion viability (egg-to-adult survival). (A) Dietary reaction norms at 18°C. (B)
1397 Dietary reaction norms at 25°C. (C) Thermal reaction norms measured on sucrose
1398 diet. (D) Thermal reaction norms measured on molasses diet. Data in (A, B) are the
1399 same as those shown in (C, D). Shown are means and standard errors. Red lines:
1400 low-latitude (LL) allele, blue lines: high-latitude (HL) allele.

1401

1402 **Figure 3.** Femur length. Effects of the *foxo* polymorphism on femur length (mm) in
1403 females and males. (A) Dietary reaction norms at 18°C. (B) Dietary reaction norms at
1404 25°C. (C) Thermal reaction norms measured on sucrose diet. (D) Thermal reaction
1405 norms measured on molasses diet. Data in (A, B) are the same as those shown in
1406 (C, D). Shown are means and standard errors. Red lines: low-latitude (LL) allele, blue
1407 lines: high-latitude (HL) allele.

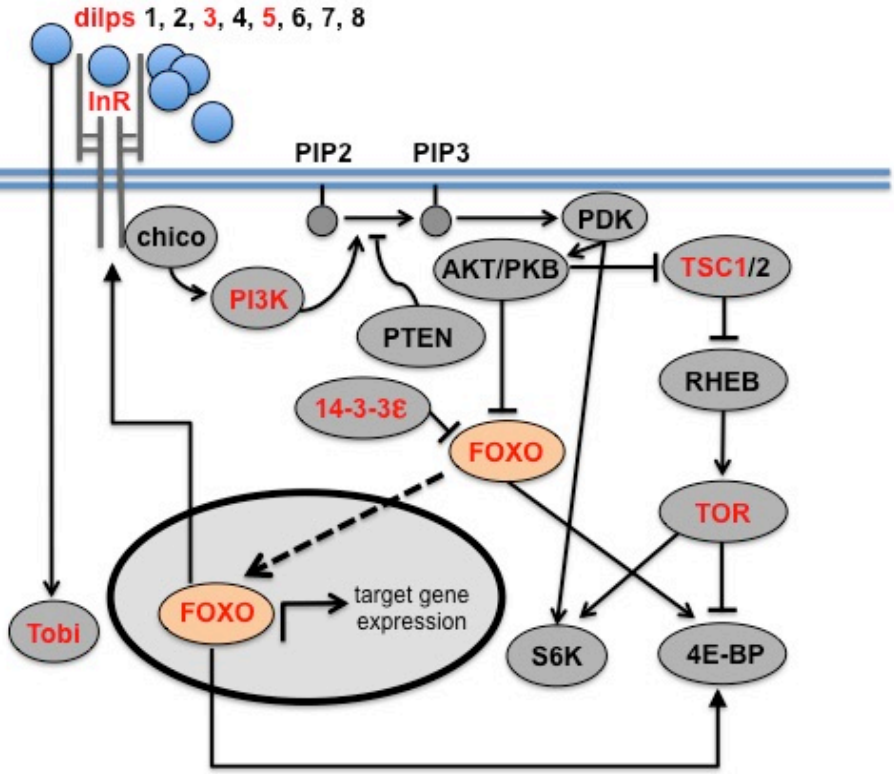
1408

1409 **Figure 4.** Wing:thorax ratio. Effects of the *foxo* variant on the ratio of wing
1410 area:thorax length (mm) in females and males. (A) Dietary reaction norms at 18°C.
1411 (B) Dietary reaction norms at 25°C. (C) Thermal reaction norms measured on
1412 sucrose diet. (D) Thermal reaction norms measured on molasses diet. Data in (A, B)
1413 are the same as those shown in (C, D). Shown are means and (propagated)
1414 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.

1430 **Figure 1**



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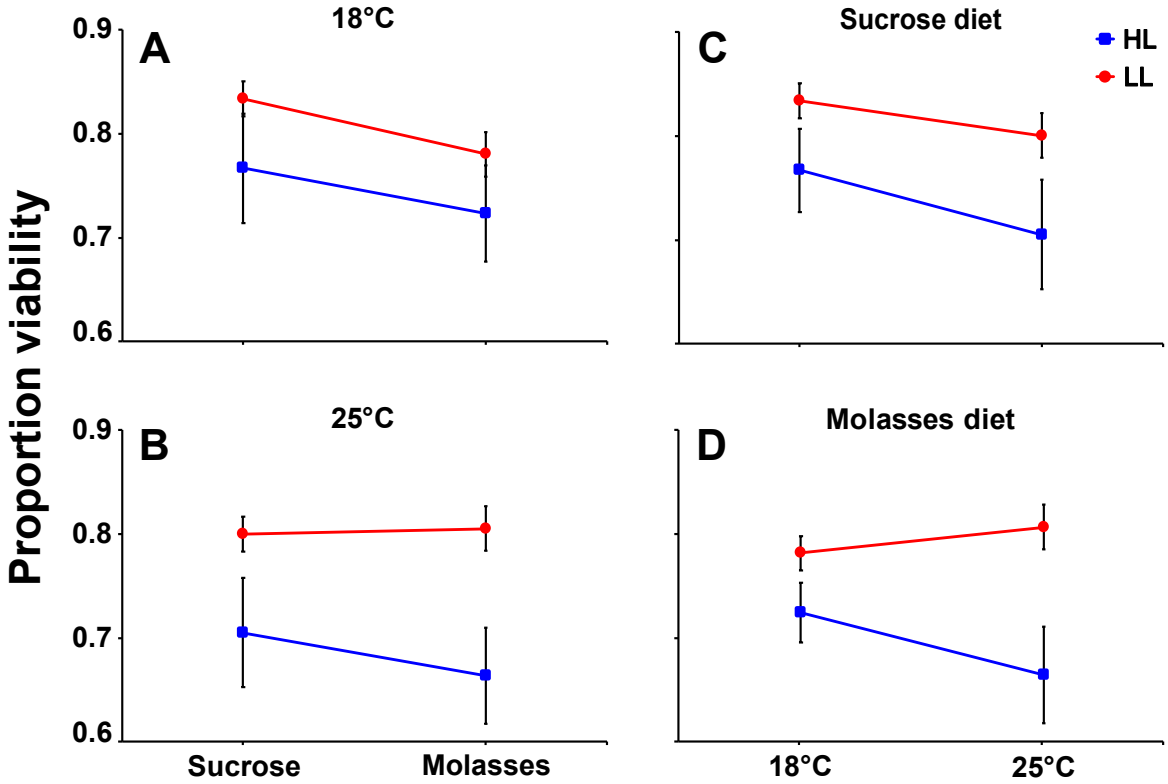
1433 **Figure 2**



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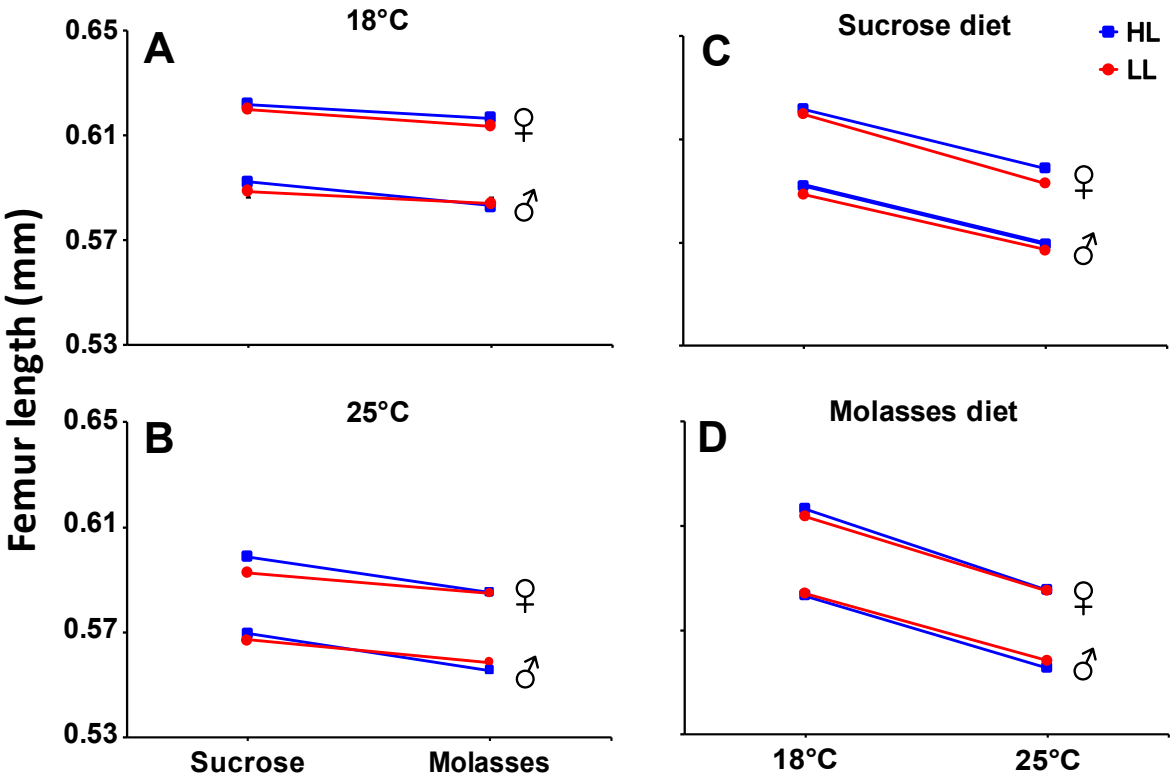
1435 **Figure 3**



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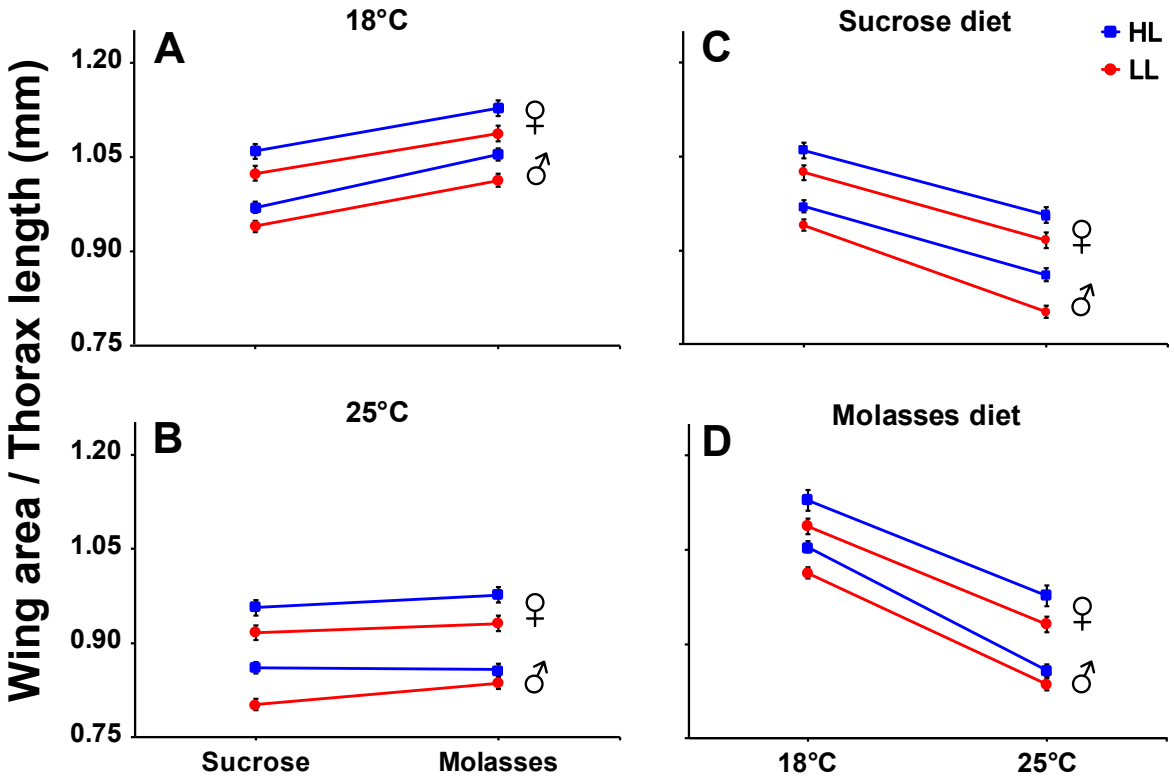
1438 **Figure 4**



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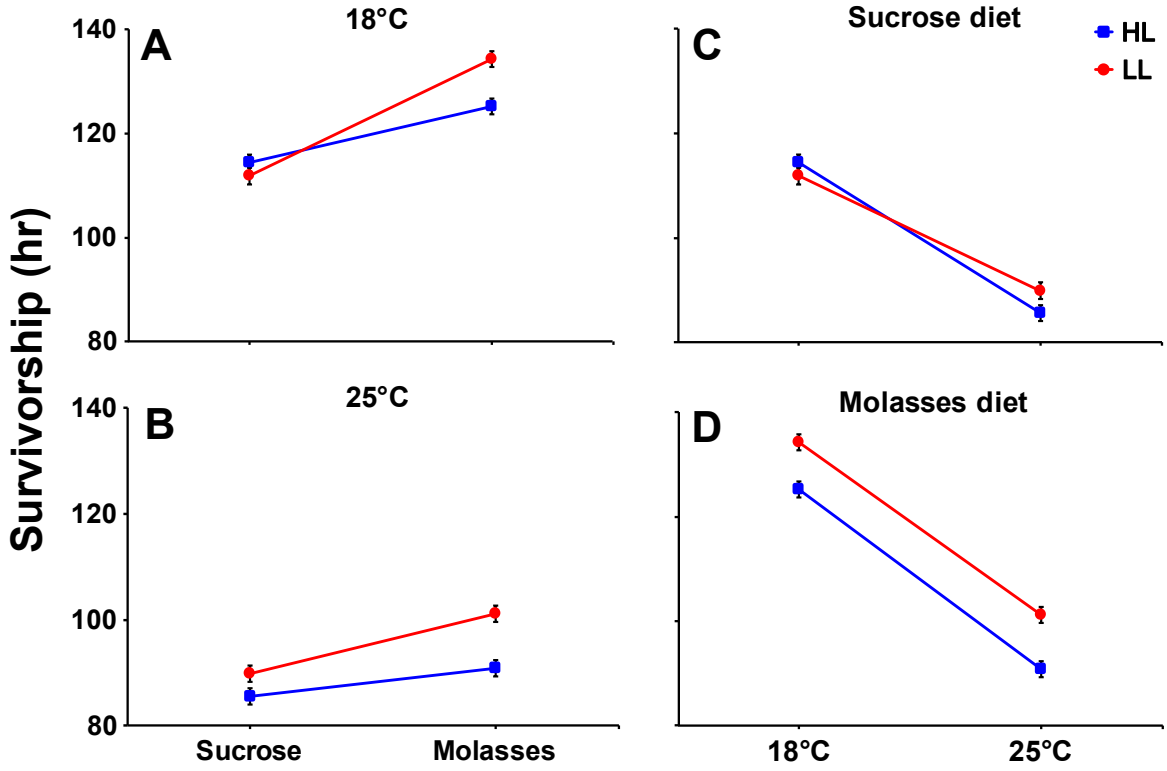
1441 **Figure 5**



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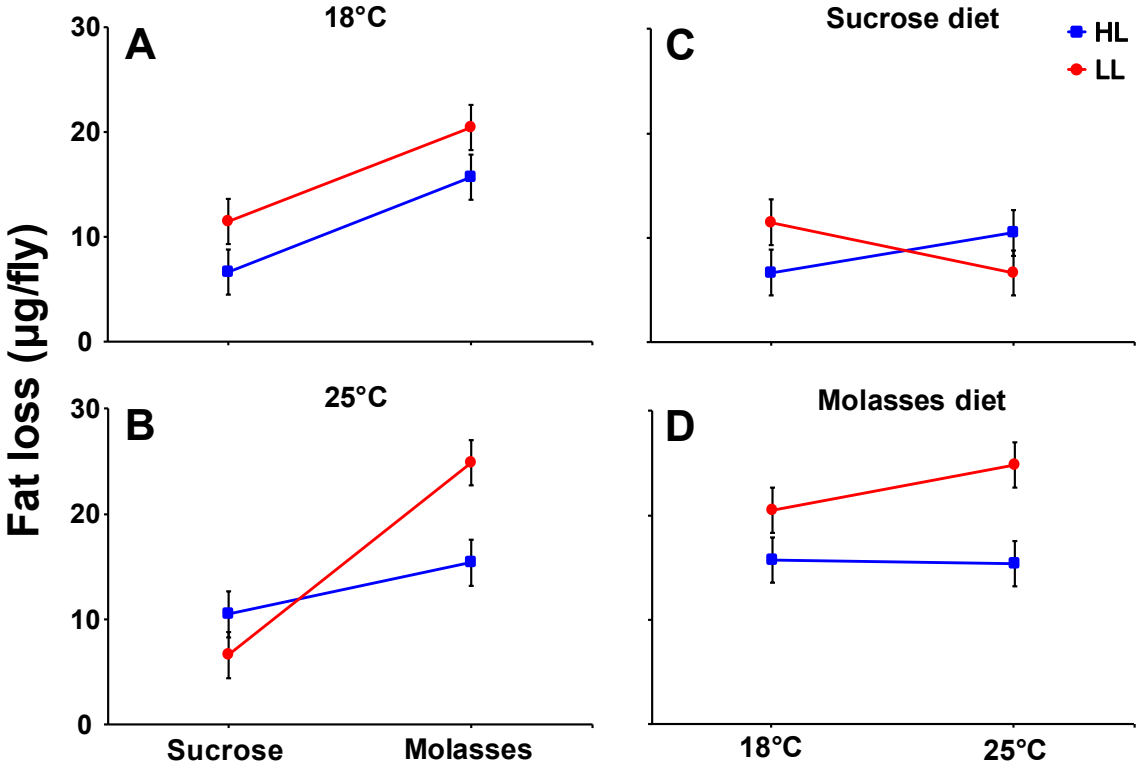
1444 **Figure 6**



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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

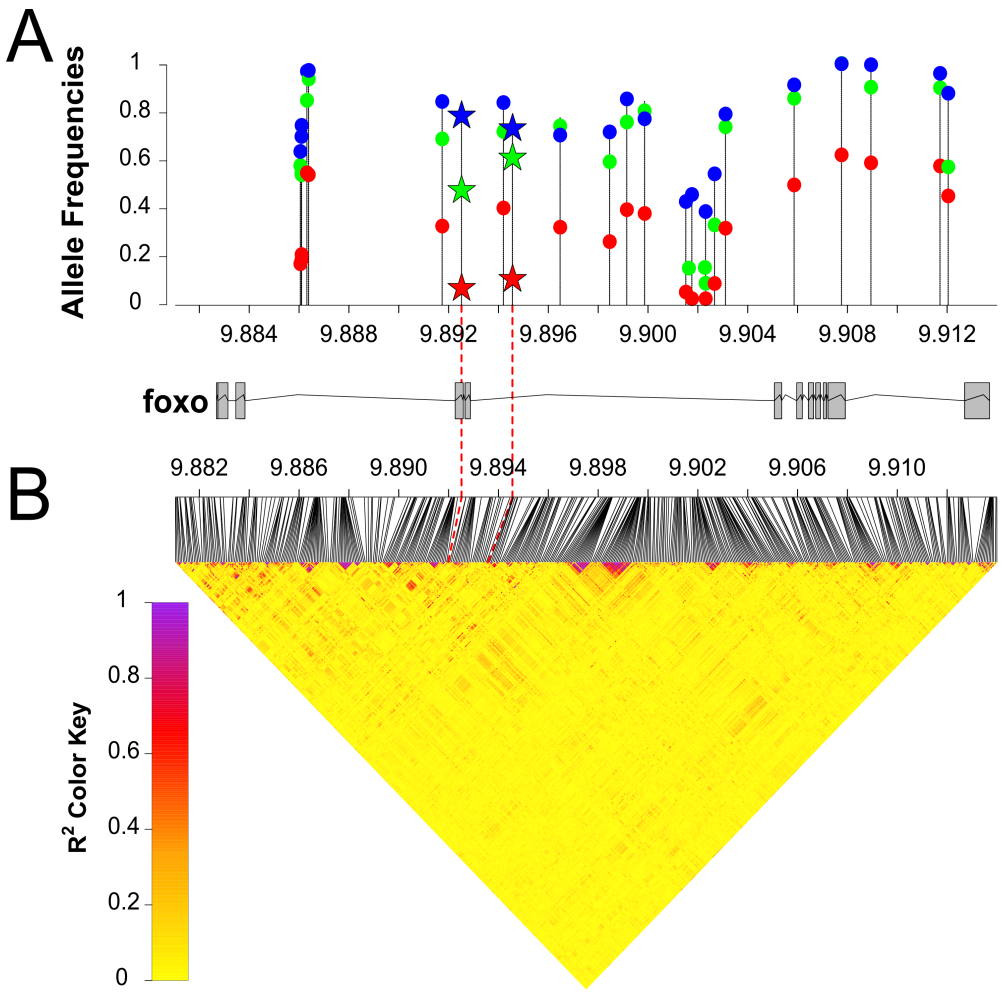


Figure S2

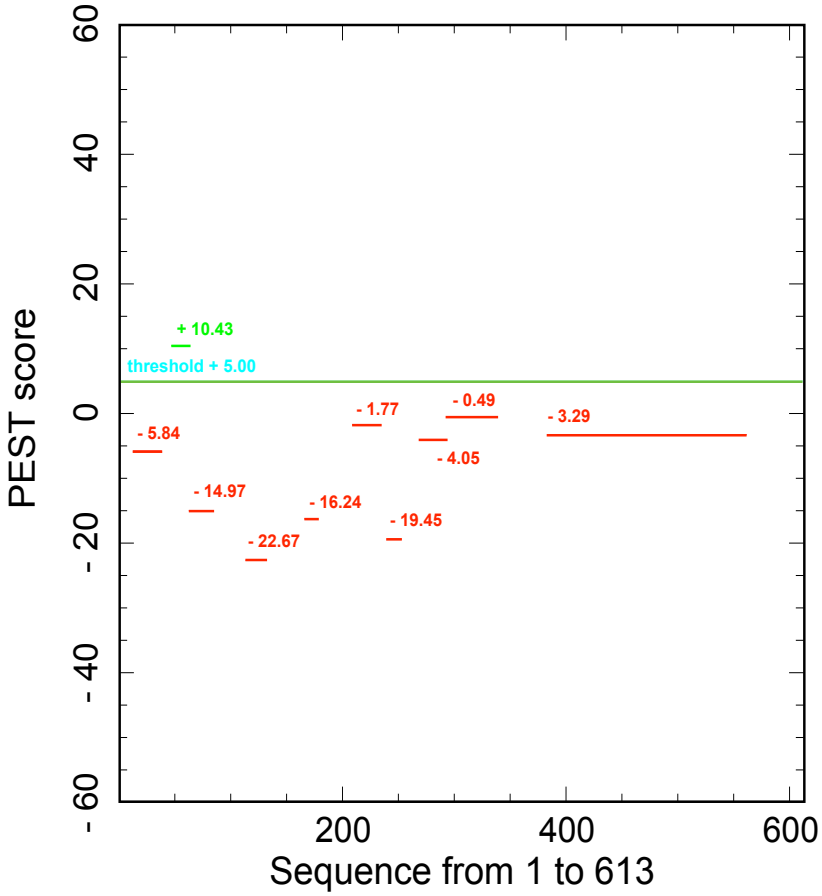


Figure S3

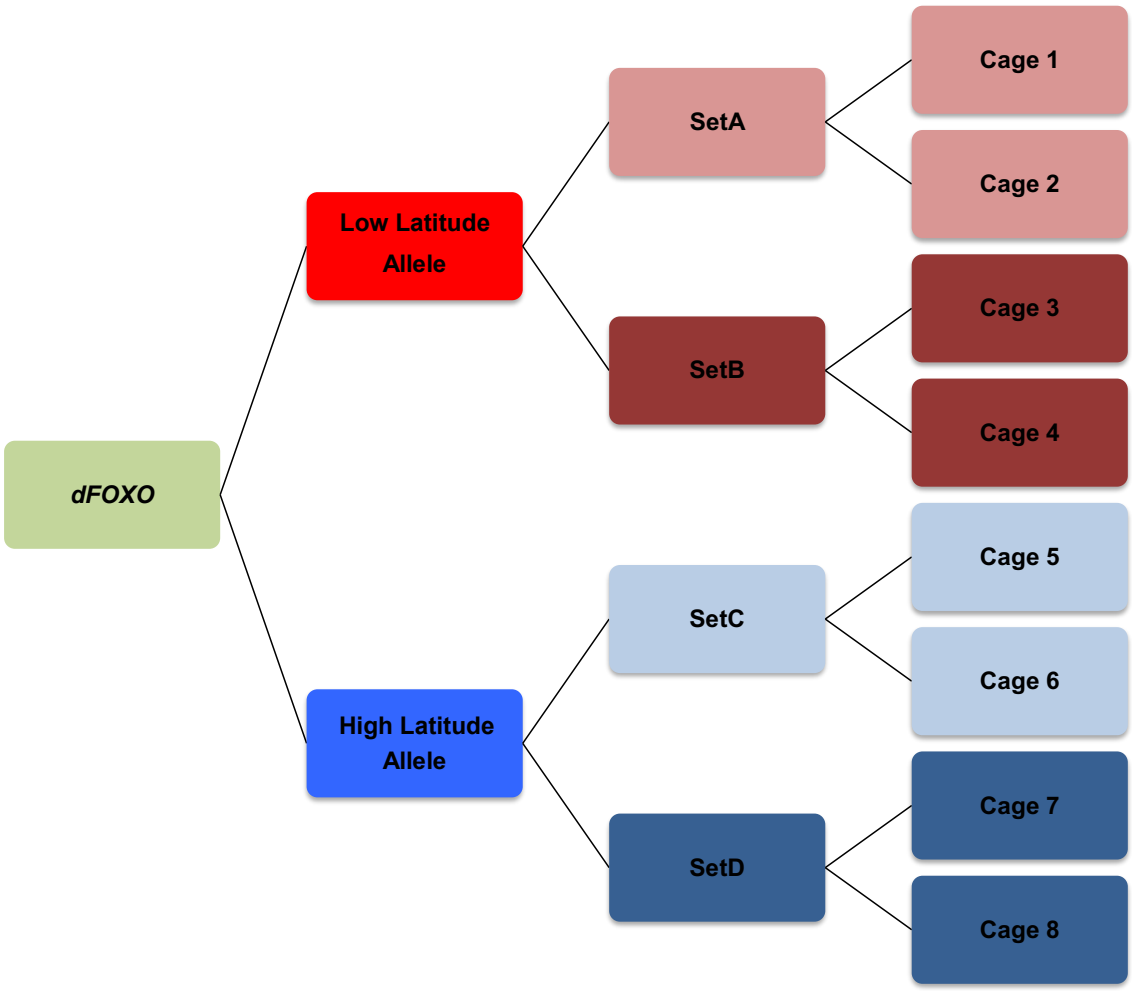


Figure S4

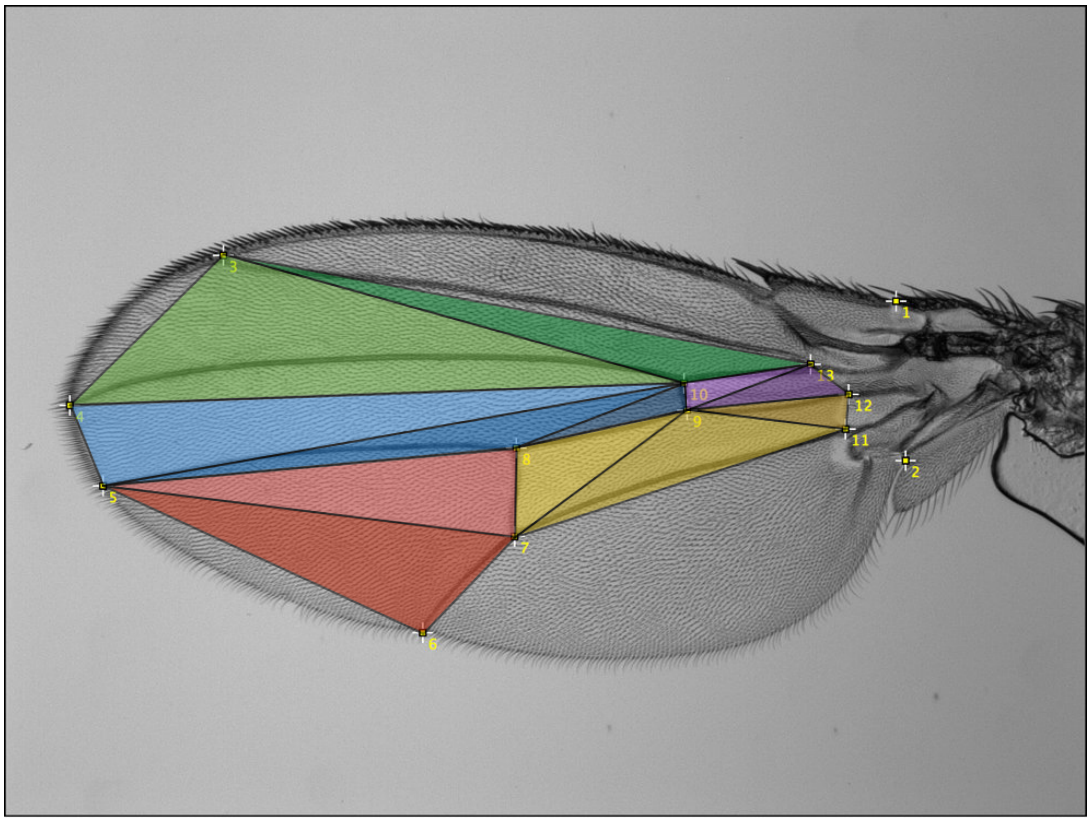
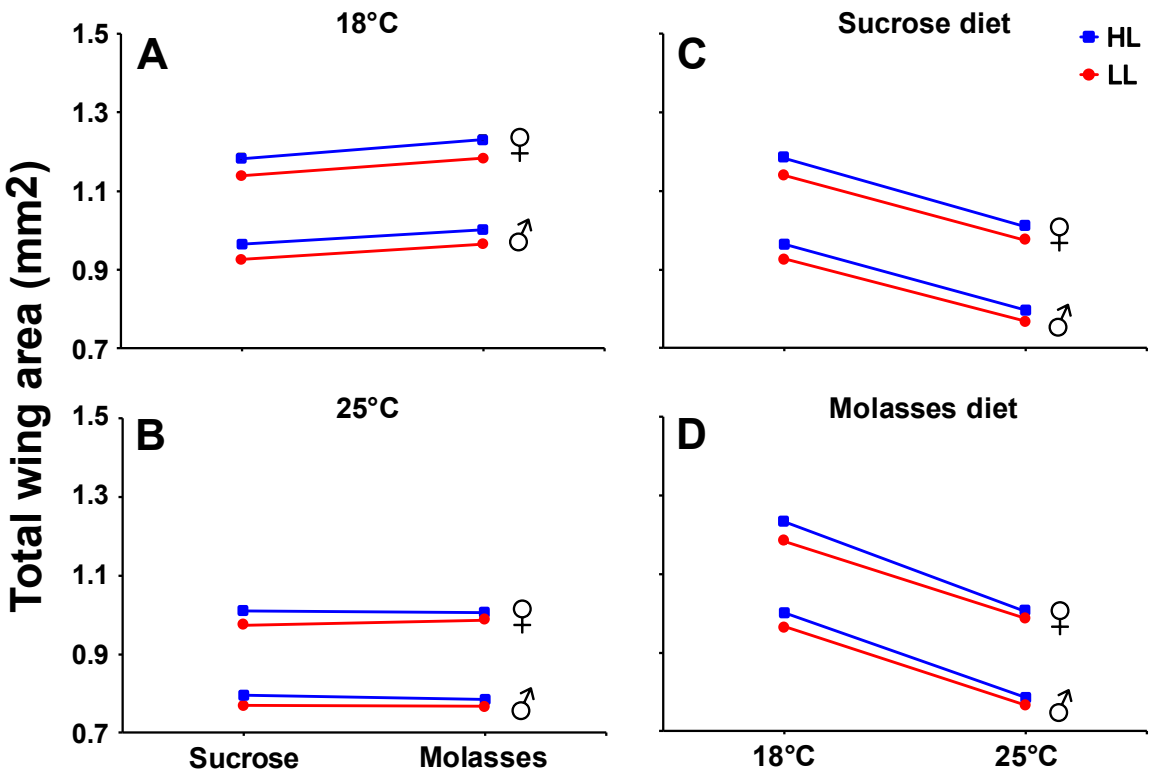


Figure S5



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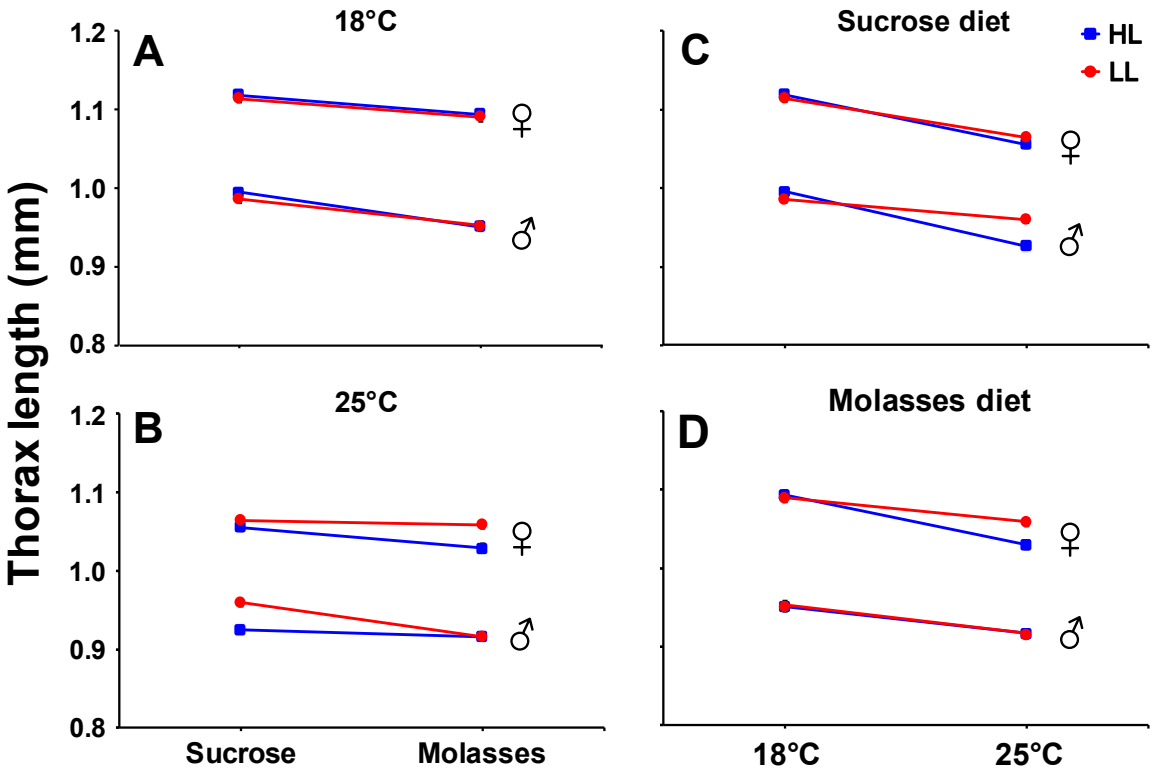

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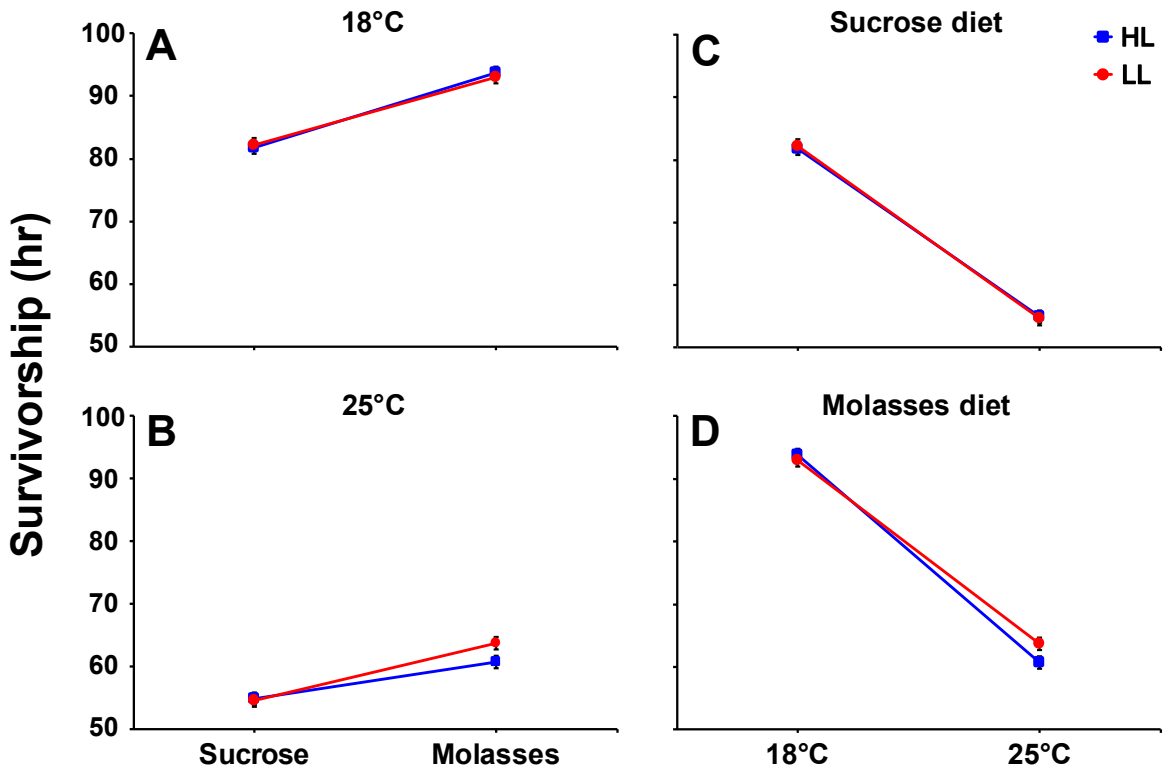

Figure S8

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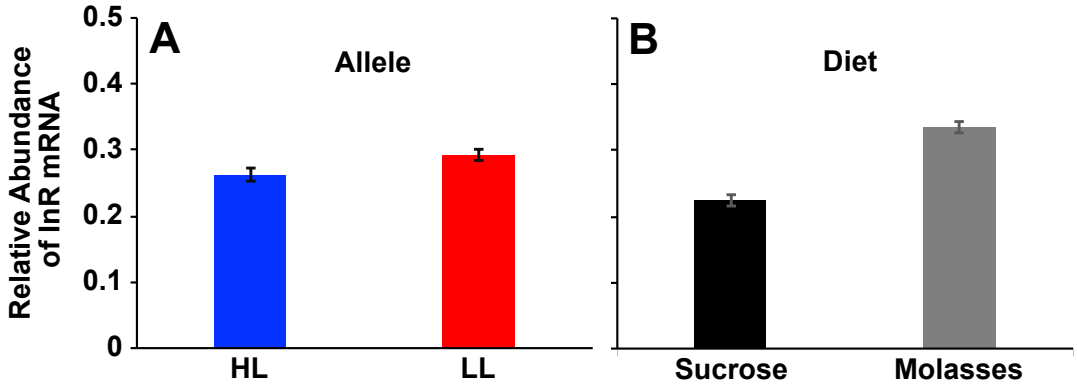

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Table S1. Details of design of reconstituted outbred population cages. HL: high-latitude *foxo* allele; LL: low-latitude *foxo* allele. See Materials and Methods section for details.

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 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

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 *p*-Value	Percentage of Total
Vial(Cage,Set,Allele)	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
Residual		474.90	17.08	443.13	510.23		100.00
		199.07	7.14	185.78	213.85		99.93
Total		474.90	17.08	443.13	510.23		100.00
		199.21	7.08	186.03	213.85		100.00

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 *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)	$F_{4,80}=5.73^{***}$