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

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Running head: Adaptive Clinal Polymorphism in *Drosophila*

Key words: cline, life history, adaptation, insulin signaling, pleiotropy, plasticity

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

54 *Introduction*

55 Much has been learned about the genetics of fitness traits (e.g., size, lifespan),
56 mainly from studies of large-effect mutants and transgenes in yeast, *C. elegans*,
57 *Drosophila* and the mouse (Finch and Rose 1995; Oldham and Hafen 2003; Tatar et
58 al. 2003; Fielenbach and Antebi 2008; Kenyon 2010; Flatt and Partridge 2018), but
59 loci identified in such laboratory analyses do not necessarily harbor segregating
60 alleles that would contribute to genetic variance for traits in natural populations (Flatt
61 2004; Flatt and Schmidt 2009; Vonesch et al. 2016; Birney 2016; Fabian et al. 2018).
62 In particular, the identity and presumably subtle effects of naturally occurring life-
63 history polymorphisms are poorly known (Flatt and Schmidt 2009; Paaby and
64 Schmidt 2009; Flatt and Heyland 2011). While adaptation genomics can in principle
65 quite readily identify such candidate polymorphisms, a major – but rarely
66 accomplished – objective is to experimentally validate these candidates as genic
67 targets of selection (Barrett and Hoekstra 2011; Turner 2014; Flatt 2016; Siddiq et al.
68 2017). Thus, with a few exceptions, examples of causative life-history variants
69 remain rare (Schmidt et al. 2008; McKechnie et al. 2010; Paaby et al. 2010; Jones et
70 al. 2012; Johnston et al. 2013; Méndez-Vigo et al. 2013; Paaby et al. 2014; Barson et
71 al. 2015; Catalán et al. 2016; reviewed in Mackay et al. 2009; Barrett and Hoekstra
72 2011).

73 Despite conceptual and methodological limitations of the so-called quantitative trait
74 nucleotide (QTN) program (Rockman 2012), the identification of life-history
75 polymorphisms allows addressing fundamental questions about the genetic basis of
76 adaptation, including: (1) Which pathways and molecular functions underpin variation
77 in fitness-related traits? (2) Are these mechanisms evolutionarily conserved? (3)
78 What are the phenotypic effects of naturally segregating life-history variants? (4)

79 What is the molecular nature of life-history epistasis, pleiotropy and trade-offs? (5)
80 Do life-history polymorphisms mediate plasticity and how? (6) Is the genetic basis of
81 evolutionary changes in life history 'predictable', i.e. relying on variation in the same
82 pathways or genes? Or do life-history traits evolve unpredictably, i.e. via different
83 pathways or loci, in different contexts?
84 A powerful model for dissecting the genetics of life-history adaptation is the
85 vinegar fly *Drosophila melanogaster*, a species of sub-Saharan African origin, which
86 has migrated out of Africa ~15,000 years ago and subsequently colonized the rest of
87 the world (David and Bocquet 1975; David and Capy 1988; de Jong and
88 Bochdanovits 2003; Hoffmann and Weeks 2007; Adrion et al. 2015). During the
89 colonization of new climate zones, this ancestrally tropical insect has undergone a
90 series of life-history adaptations to temperate, seasonal habitats (David and Capy
91 1988; de Jong and Bochdanovits 2003; Paaby and Schmidt 2009). This is particularly
92 evident in the case of clines, i.e. directional patterns of phenotypic or genetic change
93 across environmental gradients. Many studies have documented patterns of
94 latitudinal differentiation among *D. melanogaster* populations that are presumably
95 driven by spatially varying selection, for example along the North American and
96 Australian east coasts, with the corresponding clines spanning subtropical/tropical
97 and temperate habitats (de Jong and Bochdanovits 2003; Schmidt et al. 2005a,
98 2005b; Hoffmann and Weeks 2007; Schmidt and Paaby 2008; Kolaczkowski et al.
99 2011; Fabian et al. 2012; Adrion et al. 2015; Cogni et al. 2017). Clinal trait
100 differentiation has been found, for instance, for body size, fecundity, reproductive
101 dormancy, stress resistance and lifespan, typically in a parallel fashion on multiple
102 continents, suggesting that these patterns are likely adaptive (Coyne and Beecham
103 1987; Schmidt et al. 2000; Weeks et al. 2002; de Jong and Bochdanovits 2003;

104 Schmidt et al. 2005a, 2005b; Hoffmann and Weeks 2007; Schmidt and Paaby 2008;
105 Adrion et al. 2015; Fabian et al. 2015; Kapun et al. 2016a).
106 To begin to identify the genetic basis of life-history clines in *D. melanogaster*, we
107 have previously performed genome-wide analyses of latitudinal differentiation along
108 the North American cline (Fabian et al. 2012; Kapun et al. 2016b) (also see Turner et
109 al. 2008; Bergland et al. 2014; Reinhardt et al. 2014; Machado et al. 2018). Our
110 analysis based on SNP F_{ST} outliers uncovered pervasive genome-wide patterns of
111 clinality, with hundreds of clinal SNPs mapping to loci involved in the insulin/insulin-
112 like growth factor signaling (IIS)/target of rapamycin (TOR), ecdysone, torso, EGFR,
113 TGFβ/BMP, JAK/STAT, lipid metabolism, immunity and circadian rhythm pathways
114 (Fabian et al. 2012). Many of the identified variants also exhibit parallel differentiation
115 in Australia (Fabian et al. 2012; Kapun et al. 2016b; also cf. Kolaczkowski et al. 2011;
116 Reinhardt et al. 2014; Machado et al. 2016), thereby strengthening the case for clinal
117 adaptation. However, while many clinal variants might be shaped by selection, some
118 of the observed differentiation might be due to non-adaptive factors, including
119 population structure, demography, admixture or hitchhiking with causative sites
120 (Endler 1977; Duchen et al. 2013; Kao et al. 2015; Bergland et al. 2016). Identifying
121 adaptive clinal variants as targets of selection thus requires comparing clinal patterns
122 against neutral expectations and – optimally – functional genetic testing (Barrett and
123 Hoekstra 2011; Kapun et al. 2016a, 2016b; Flatt 2016). To date, however, functional
124 analyses of clinal polymorphisms that are potentially subject to spatially varying
125 selection remain scarce (for some exceptions see e.g. Schmidt et al. 2008;
126 McKechnie et al. 2010; Paaby et al. 2010; Lee et al. 2013; Paaby et al. 2014; Kapun
127 et al. 2016a; Durmaz et al. 2018; Svetec et al. 2018).

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

153 (Williams et al. 2006), and work in *Caenorhabditis remanei* has identified a global
154 selective sweep in the *Caenorhabditis* homolog of *Pi3K*, *age-1* (Jovelin et al. 2014).
155 Multiple lines of evidence also indicate that insulin-like growth factor-1 (IGF-1)
156 signaling mediates physiological life-history variation in vertebrate populations
157 (Dantzer and Swanson 2011; Swanson and Dantzer 2014). Together, these findings
158 suggest that allelic variation in IIS/TOR might profoundly affect life-history adaptation,
159 but experimental evidence remains scarce (Flatt et al. 2013; Flatt and Partridge
160 2018).

161 Here we provide a comprehensive examination of the life-history effects of a
162 clinally varying polymorphism in the forkhead box-O transcription factor gene *foxo* of
163 *D. melanogaster* (Fig. 1), a major regulator of IIS that is homologous to *C. elegans*
164 *daf-16* and mammalian *FOXO3A*. Molecular studies – mainly in the fly and nematode
165 – have shown that FOXO plays a key role in regulating growth, lifespan and
166 resistance to starvation and oxidative stress (Jünger et al. 2003; Puig et al. 2003;
167 Libina et al. 2003; Murphy et al. 2003; Kramer et al. 2003; Kramer et al. 2008;
168 Hwangbo et al. 2004; Puig and Tijan 2005; Fielenbach and Antebi 2008; Mattila et al.
169 2009; Slack et al. 2011). Moreover, genetic association studies in humans have
170 linked polymorphisms in *FOXO3A* to longevity in centenarians (Flachsbart et al.
171 2009; Willcox et al. 2008). Natural *foxo* variants thus represent promising candidates
172 for mediating life-history variation in natural populations.

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

178 survival, proxies of size, starvation resistance, fat content) by measuring phenotypes
179 on 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 interesting because still relatively
190 little is known about whether and how clinality and plasticity interact (James et al.
191 1997; de Jong and Bochdanovits 2003; Hoffmann et al. 2005; Levine et al. 2011;
192 Overgaard et al. 2011; Chen et al. 2012; Cooper et al. 2012; Zhao et al. 2015;
193 Clemson et al. 2016; Mathur and Schmidt 2017; van Heerwaarden and Sgrò 2017).
194 For example, *D. melanogaster* feeds and breeds on various kinds of rotting fruit, with
195 the protein:carbohydrate (P:C) ratios exhibiting spatiotemporal variation (Lachaise et
196 al. 1988; Hoffmann and McKechnie 1991; Markow et al. 1999; Keller 2007), but how
197 dietary plasticity affects traits in a clinal context remains largely unclear. Similarly, the
198 interplay between thermal plasticity and adaptation is incompletely understood (e.g.,
199 de Jong and Bochdanovits 2003; Overgaard et al. 2011; Mathur and Schmidt 2017;
200 van Heerwaarden and Sgrò 2017).
201 We give predictions for the expected phenotypic effects of the *foxo* variant in terms
202 of clinality, plasticity and the physiology of IIS in the Methods section. Our results

203 show that the *foxo* polymorphism affects multiple fitness components according to
204 these predictions and suggest that it contributes to clinal life-history adaptation; we
205 also find that the alternative alleles respond plastically to temperature and diet but
206 with little evidence for G x E.
207

208 ## *Methods*

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

210 We identified two strongly clinal SNPs in *foxo* in the genomic data of Fabian et al.
211 (2012) by using an F_{ST} outlier approach: an A/G polymorphism at position *3R*:
212 9892517 (position in the *D. melanogaster* reference genome v.5.0; F_{ST} = 0.48
213 between Florida and Maine) and a T/G polymorphism at position *3R*: 9894559 (F_{ST} =
214 0.42 between Florida and Maine) (Fig. S1A, Supporting Information; see Fabian et al.
215 2012 for details of outlier detection). The A/G polymorphism is a synonymous coding
216 SNP, predicted to be located in the PEST region of the FOXO protein, which serves
217 as a protein degradation signal (analysis with ExPASy [Artimo et al. 2012]; Fig. S2,
218 Supporting Information). The T/G SNP is located in the first intron of *foxo*, with no
219 biological function attributed to this position (Attrill et al. 2016).

220 While our initial identification of these SNPs was based on only three populations
221 (Florida, Pennsylvania, Maine; Fabian et al. 2012), both SNPs are also strongly clinal
222 in a more comprehensive dataset from 10 populations along the cline (Betancourt et
223 al. 2018), collected by the *Drosophila* Real Time Evolution Consortium (DrosRTEC;
224 Bergland et al. 2014; Kapun et al. 2016b; Machado et al. 2018). The frequency of the
225 high-latitude [HL] allele (A, T) for this 2-SNP variant ranges from ~10% in Florida to
226 ~70% in Maine; conversely, the alternative low-latitude [LL] allele (G,G) is prevalent
227 in Florida but at low frequency in Maine (Fig. S1A, Supporting Information). Because

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

238 To isolate the two alternative *foxo* alleles for experiments we used whole-genome
239 sequenced inbred lines from the *Drosophila* Genetic Reference Panel (DGRP;
240 Mackay et al. 2012) to reconstitute outbred populations either fixed for the LL (G,G)
241 and the HL (A,T) alleles. This 'reconstituted or recombinant outbred population'
242 (ROP) or 'Mendelian randomization' approach produces populations that are
243 consistently and completely fixed for the two alternative allelic states to be compared,
244 with the rest of the genetic background being randomized (see Behrman et al. 2018
245 and Lafuente et al. 2018 for examples using this method). For each allele we used
246 two independent sets of DGRP lines (sets A and B for HL; sets C and D for LL; each
247 set consisting of 20 distinct lines) and two replicate population cages per set, giving a
248 total of 8 population cages (Fig. S3, Table S1, Supporting Information). ROP cages
249 were established from the DGRP lines by Betancourt et al. (2018); F2 flies were
250 transferred to Lausanne for establishing cages at our laboratory.

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

253 alleles, respectively; this also showed that, although there exist other SNPs that are
254 strongly differentiated (F_{ST} >0.5) between the HL and LL populations, the majority of
255 them are different between the independently replicated sets (blocks) of DGRP lines
256 used to make the HL vs. LL contrast (Betancourt et al. 2018). Such SNPs, which are
257 specific to a given set of lines, do not make a consistent contribution to the overall HL
258 vs. LL contrast.
259 The most parsimonious interpretation of our results is therefore that the effects
260 reported below are caused by the two *foxo* SNPs which we have studied. However,
261 we cannot completely rule out that other (causative) sites are potentially in long-
262 range LD with our focal SNPs (see Fig. S1B, Supporting Information). A conservative
263 interpretation of our results is thus to view the two focal *foxo* SNPs as representing
264 'tags' or markers for functionally significant variants segregating at the *foxo* locus that
265 are in LD with the causative site(s), similar to those used in genome-wide association
266 studies (GWAS; e.g., Wang et al. 2010).
267
268 **POPULATION CAGES**
269 Population cages were maintained at 25ºC, 12:12 h light:dark, 60% relative air
270 humidity and controlled larval density. Larval density was kept constant via egg
271 collections (200-300 eggs per bottle [6 oz. = 177 mL]; 10 bottles per cage), with
272 eclosing adults being released into cages (17.5 x 17.5 x 17.5 cm; BugDorm®) at a
273 density of ~2000-2500 adults per cage. Prior to the phenotypic assays population
274 cages were kept for 10 generations to allow for free recombination among lines
275 within each cage and allelic state and to homogenize (randomize) differences in
276 genomic background between the two allelic states to be compared. Before setting
277 up assays, we kept cages for 2 generations under common garden conditions (room

278 temperature: ~22ºC, ~10:14 h light:dark, ~50% humidity). Thus, phenotypes were
279 measured after a total of 12 generations of recombination.
280
281 **PHENOTYPE ASSAYS**
282 Assays reported here were performed in our laboratory in Lausanne; independent
283 assays were performed under constant environmental conditions by Betancourt et al.
284 (2018), thus allowing us to assess the reproducibility of our results and to identify
285 potential differences in assay conditions between laboratories (cf. Ackermann et al.
286 2001).
287 In generation 13 (see above) we assayed flies for viability, size, starvation
288 resistance and lipid content. Phenotypes were assayed under four environmental
289 conditions, using a fully factorial 2-way design: 2 rearing temperatures (18°C, 25°C)
290 by 2 commonly used diets that differ mainly in their sugar source (sucrose [cornmeal-
291 agar-yeast-sucrose] vs. molasses [cornmeal-agar-yeast-molasses] diet and their
292 protein:carbohydrate ratio (P:C ~1:3.6 vs. ~1:12.3, respectively; see Table S2,
293 Supporting Information, for details of nutrient content and media recipes). To initiate
294 assays we collected ~6400 eggs from each cage, distributed them across 32 bottles
295 (each with 200 eggs; 25 mL medium), and allocated 8 bottles to each of the 4
296 conditions (8 bottles × 8 cages × 4 conditions = 256 bottles). For all assays (except
297 viability; see below), we collected eclosed adults in 48-h cohorts, allowed them to
298 mate for 4 days under their respective thermal and dietary conditions, sexed them
299 under light CO_2 anesthesia 4-6 days post-eclosion, and transferred them to fresh
300 vials 24 h prior to assays. Flies used for size assays were stored at -20°C until
301 measurement.

302 Viability (egg-to-adult survival) was calculated as the proportion of adult flies
303 successfully developing from eggs by collecting 600 eggs per cage and placing them
304 into vials containing 8 mL of medium, with 30 eggs per vial (5 vials × 8 cages × 4
305 conditions = 160 vials).
306 Body size was examined by measuring three proxies: wing area, thorax length and
307 femur length (*N* = 26-30 wings, 9-15 thoraces, and 19-21 femurs per cage, treatment,
308 and sex). Right wings and femurs were mounted on slides with CC/Mount™ tissue
309 mounting medium (Sigma Aldrich) and slides sealed with cover slips. Thorax length
310 was defined as the lateral distance between the upper tip of the thorax and the end of
311 the scutellar plate (*N* = 10-15 individuals per cage, treatment, and sex). Images for
312 morphometric measurements were taken with a digital camera (Leica DFC 290)
313 attached to a stereo dissecting microscope (Leica MZ 125; Leica Microsystems
314 GmbH, Wetzlar, Germany). We used ImageJ software (v.1.47) to measure femur and
315 thorax length (mm) and to define landmarks for calculating wing area (mm^2). To
316 measure wing area we defined 12 landmarks located at various vein intersections
317 along the wing; the total area encompassed by these landmarks was estimated using
318 a custom-made Python script (available upon request). In brief, we split the polygon
319 defined by the landmarks up into triangles and summed across their areas (Fig. S4,
320 Supporting Information). Thorax and femur (but not wing area) measurements were
321 repeated three times per individual (see below for estimates of 'repeatability'). From
322 these data, we calculated the ratio of wing area:thorax length, which is inversely
323 related to ‘wing loading’ (Azevedo et al. 1998; Gilchrist et al. 2000); reduced wing
324 loading (i.e., increased wing dimensions relative to body size) can improve flight
325 performance at low temperature (Frazier et al. 2008).

326 To measure starvation resistance (i.e., survival upon starvation) we placed flies
327 into vials containing 0.5% agar/water medium and scored the duration of survival (h)
328 upon starvation every 6 h until all flies had died (N = 5 vials × 10 flies per vial × 2
329 sexes × 8 cages × 4 conditions = 320 vials or 3200 flies).
330 Since there is typically a positive correlation between starvation resistance and
331 lipid content (Hoffmann and Harshman 1999), we also determined whole-body
332 triacylglyceride (TAG) content (in μg per fly) using a serum triglyceride determination
333 kit (Sigma Aldrich; Tennessen et al. 2014). For each cage and treatment, triglyceride
334 content was estimated from 5-7-day-old females, either kept under fed or starved (24
335 h) conditions, by preparing 10 replicate homogenates, each made from 2 flies (8
336 cages × 4 conditions × 2 treatments × 10 replicates = 640 homogenates). To
337 estimate fat loss upon starvation we calculated the difference between fat content
338 under fed versus starved conditions, using treatment (fed vs. starved) means from
339 each population cage (mean fat loss per fly, in μg).
340
341 **QRT-PCR ANALYSIS OF INSULIN SIGNALING STATE**
342 A well-established transcriptional read-out of FOXO signaling is the insulin-like
343 receptor InR: under conditions of high insulin (e.g., after a meal), InR synthesis is
344 repressed by a feedback mechanism controlled by FOXO; conversely, under
345 conditions of low insulin, activation of FOXO leads to upregulation of *InR* mRNA
346 (Puig et al. 2003; Puig and Tjian 2005). To test whether the *foxo* alleles differ in IIS
347 state we performed qRT-PCR, measuring *InR* mRNA abundance. For each cage and
348 treatment, we extracted total RNA from 5-7-day-old snap-frozen females in triplicate,
349 with each replicate prepared from 5 flies. RNA was extracted with the RNeasy kit
350 (Qiagen) and reverse transcribed with the GoScript Reverse Transcription System

351 (Promega). From each triplicate biological sample we prepared 3 technical replicates
352 (8 cages × 4 conditions × 3 biological replicates × 3 technical replicates = 288
353 samples). Relative transcript abundance was normalized by using *Actin5C* as an
354 endogenous control (Ponton et al. 2011). qRT-PCR was carried out using a
355 QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) and SYBR Green
356 GoTaq qPCR Master Mix (Promega). Thermal cycling was conducted at 95°C for 2
357 min, followed by 42 cycles of amplification at 95°C for 15 s and 60°C for 1 min, and
358 using the following melting curve: 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s.
359 Quantification of relative abundance for each sample was based on the Δ*CT* method.
360 We used the following primer sequences (Casas-Tinto et al. 2007; Ponton et al.
361 2011): *Actin5C forward*, 5'-GCGTCGGTCAATTCAATCTT-3'; *Actin5C reverse*, 5'-
362 AAGCTGCAACCTCTTCGTCA-3'; *InR forward*, 5'-CACAAGCTGGAAAGAAAGTGC-
363 3'; *InR reverse*, 5'- CAAACACGTTTCGATAATATTTTTCT-3'.
364
365 **STATISTICAL ANALYSIS**
366 Analyses were performed with JMP (SAS, Raleigh, NC, USA; v.11.1.1). Data were
367 analyzed with analysis of variance (ANOVA), testing the fixed effects of allele (*A*; HL
368 vs. LL), temperature (*T*; 18°C vs. 25°C), diet (*D*; sucrose vs. molasses), set (*S*;
369 independent blocks of DGRP lines) nested within *A*, replicate cage (*C*) nested within
370 the combination of *A* and *S*, and all 2- and 3-way interactions: $y = A + T + D + A \times T$
371 $+ A \times D + T \times D + A \times T \times D + S(A) + C(A,S)$, where *y* denotes the response variable
372 (trait). For simplicity, the sexes were analyzed separately (i.e., to reduce the number
373 of higher-order interactions).
374 For starvation resistance we measured age at death from multiple individuals per
375 replicate vial; we thus estimated and accounted for the random effect of vial (*V*),

376 nested within the combination of *A*, *S* and *C*, using restricted maximum likelihood
377 (REML) (see Supporting Information for these estimates).
378 Viability (proportion) data were arcsine square-root transformed prior to analysis.
379 ANOVA on thorax and femur length data was performed using means across 3
380 measures per individual. From the repeat measurements of these traits on the same
381 individuals, we estimated the 'repeatability' of our measurements (i.e., the intraclass
382 correlation; see Whitlock and Schluter 2009) by performing random-effects ANOVAs
383 with REML. Overall, repeatibility was very high for femur length (~91.9% for females;
384 94.4% for males) but less so for thorax length (~29.9% for females; 36.6% for males)
385 (details not shown). Because wings and thoraces were measured on separate
386 individuals, analysis of wing:thorax ratio was performed on population (cage) means.
387 For fat content, we included the fixed effect of starvation treatment (*Tr*; fed vs.
388 starved); interactions involving *A* and *Tr* (i.e., $A \times Tr$; $A \times D \times Tr$) test for allelic
389 differences in fat loss upon starvation. For simplicity, this analysis was performed
390 separately for the two rearing temperatures.
391 To estimate the magnitude of the allelic effects of the *foxo* polymorphism upon the
392 assayed fitness components we calculated Cohen's *d* (Table S3, Supporting
393 Information), a standardized measure of effect size (i.e., a signal to noise ratio,
394 defined as the difference between two means divided by their pooled standard
395 deviation) (Cohen 1988; Sawilowsky 2009). Low values of Cohen's *d* (e.g., 0.01) are
396 commonly interpreted as representing very small effect sizes, whereas effect sizes
397 >0.8 are interpreted as being qualitatively large to very large (Sawilowsky 2009).
398 We also estimated the relative contribution of the *foxo* polymorphism assayed in
399 our laboratory to the overall phenotypic cline for wing area, recently measured on
400 flies from 6 populations along the North American east coast (Betancourt et al. 2018;

401 using flies assayed on molasses diet at 25°C). We calculated the proportional
402 contribution of the polymorphism to the overall cline as follows: $\Delta_{foxo} \times \Delta_{\text{frequency}} / \Delta_{\text{cline}}$,
403 where Δ_{foxo} is the difference in mean wing area between the HL and LL alleles,
404 $\Delta_{\text{frequency}}$ is the allele frequency gradient for the polymorphism between cline ends
405 (Maine vs. Florida, ~60%) and Δ_{cline} is the difference in mean wing area between
406 cline ends.
407
408 **PREDICTIONS**
409 Here we make some qualitative predictions for the expected behavior of the *foxo*
410 polymorphism with regard to (1) clinal phenotypic effects, (2) patterns of trait
411 covariation determined by IIS, and (3) plasticity, G × E, and local adaptation. We
412 compare our results to these predictions in the Results section below.
413 (1) Latitudinal clinality. Traits which have been found to covary positively with
414 latitude include, for example, faster development, lower egg-to-adult survival
415 (viability), increased body size, reduced wing loading, reduced fecundity, prolonged
416 lifespan, and increased resistance to starvation, cold and heat stress (e.g., Coyne
417 and Beecham 1987; Azevedo et al. 1998; Bochdanovits and de Jong 2003a; de Jong
418 and Bochdanovits 2003; Schmidt et al. 2005a, 2005b; Folguera et al. 2008; Schmidt
419 and Paaby 2008; Bhan et al. 2014; Mathur and Schmidt 2017; Durmaz et al. 2018).
420 For some traits clinal patterns have been observed in a parallel fashion on multiple
421 continents, but there can also be major differences among continents (e.g., see
422 discussion in Fabian et al. 2015); for example, contrasting predictions have been
423 made for viability (van 't Land et al. 1999), starvation resistance (Karan et al. 1998,
424 Robinson et al. 2002; Hoffmann et al. 2005; Goenaga et al. 2013) and heat tolerance
425 (Hoffmann et al. 2002; Sgrò et al. 2010).

426 In general, we would expect that the effects of the high- and low-latitude *foxo*
427 alleles agree with the overall phenotypic patterns across latitude, especially for those
428 traits that have previously been examined along the North American cline (e.g.,
429 Coyne and Beecham 1987; Schmidt and Paaby 2008; Paaby et al. 2014; Kapun et
430 al. 2016a; Mathur and Schmidt 2017; Durmaz et al. 2018).
431 (2) IIS. Traits that are associated with reduced IIS include reduced body size,
432 increased lifespan, resistance to starvation and cold, increased fat content, reduced
433 fecundity, and activation of FOXO (Tatar et al. 2001, 2003; Oldham and Hafen 2003;
434 Broughton et al. 2005; Teleman 2010). For example, loss-of-function (LOF) mutants
435 of *foxo* exhibit (depending on the allele) prolonged development, reduced weight,
436 smaller wing size, reduced fecundity, shortened lifespan, and reduced survival upon
437 oxidative and starvation stress (Jünger et al. 2003; Kramer et al. 2003, 2008;
438 Hwangbo et al. 2004; Giannakou et al. 2004; Giannakou et al. 2008; Slack et al.
439 2011); the effects of IIS (or of *foxo*) on viability are, however, not well understood.
440 Conversely, overexpression of *foxo* has opposite effects on most of these traits (e.g.,
441 increased lifespan), yet – like LOF alleles – causes decreased size (Kramer et al.
442 2003; Puig et al. 2003; Hwangbo et al. 2004; Kramer et al. 2008; Tang et al. 2011).
443 We predict that the naturally occurring *foxo* alleles tested here differ consistently
444 along this IIS/*foxo* axis of trait covariation. Notably, traits observed in flies from high-
445 versus low-latitude populations in North America resemble those of flies with low
446 versus high IIS, respectively (e.g., de Jong and Bochdanovits 2003; Flatt et al. 2013;
447 Paaby et al. 2014): lower fecundity, improved stress resistance, and longer lifespan
448 observed in high-latitude flies are traits that tend to be co-expressed in IIS mutants;
449 however, flies from high-latitude populations are larger than low-latitude flies, yet
450 reduced IIS causes smaller size.

451 (3) Plasticity, G × E, and local adaptation. With regard to thermal effects, we would
452 expect flies raised at lower temperature to exhibit prolonged development, reduced
453 viability, larger size, reduced wing loading, lower fecundity, increased lifespan, and
454 improved starvation resistance (David et al. 1994; Partridge et al. 1994a, 1994b;
455 James and Partridge 1995; Bochdanovits and de Jong 2003b; Trotta et al. 2006;
456 Folguera et al. 2008; Klepsatel et al. 2013, 2014; Mathur and Schmidt 2017; cf.
457 Hoffmann et al. 2005 for a contrasting prediction for starvation survival).
458 With respect to dietary effects, higher P:C ratios, for instance, might be expected
459 to cause increased viability, larger size but reduced starvation resistance (Lee and
460 Jang 2014; Lihoreau et al. 2016; Reis 2016). In terms of G × E, genotypes from
461 temperate, seasonal high-latitude habitats might be more plastic than those from low-
462 latitude habitats (Overgaard et al. 2011; Klepsatel et al. 2013); if so, patterns of
463 differential plasticity between high- and low-latitude alleles might be consistent with
464 patterns of local adaptation (Mathur and Schmidt 2017).
465

466 *Results*

467 The clinal *foxo* polymorphism examined here (or causative SNPs in LD with it; see
468 caveat in the Methods section) impacted all fitness components assayed (Table 1;
469 Tables S3 and S4, Supporting Information), including significant effects on egg-to-
470 adult survival (viability) (qualitatively moderate to large effects, as measured by
471 Cohen's *d*), femur length (very small to medium), wing area (medium), thorax length
472 (very small to very large), starvation resistance (very small to medium), and lipid
473 content (very small to large effects).
474

475 **ALLELIC VARIATION AT *FOXO* AFFECTS VIABILITY**

476 The *foxo* polymorphism significantly affected viability, with the LL allele exhibiting
477 higher egg-to-adult survival than the HL allele (Fig. 2; Table 1), consistent with
478 observations suggesting that viability might be higher at low latitudes (Folguera et al.
479 2008; but see van 't Land et al. 1999). Diet – but not temperature – also had an
480 effect, with viability being higher on sucrose than on molasses diet (Fig. 2; Table 1).
481 We did not find any evidence for G × E interactions affecting this trait.
482

483 **CLINAL *FOXO* ALLELES DIFFER IN BODY SIZE**

484 Since both latitude and IIS affect size (de Jong and Bochdanovits 2003), we next
485 examined three proxies of body size (wing area, thorax and femur length). The HL
486 allele conferred larger femur length (Fig. 3; Table 1; in females but not males), wing
487 area (Fig. S5; Table S4, Supporting Information), and wing:thorax ratio than the LL
488 allele (Fig. 4; Table 1; for thorax data see Fig. S6; Table S4, Supporting Information).
489 These results are consistent with the positive size cline in North America (Coyne and
490 Beecham 1987) and with reduced wing loading at high latitude (Azevedo et al. 1998;
491 Bhan et al. 2014). Remarkably, with regard to wing area, we estimate that the *foxo*
492 polymorphism makes a proportional contribution of ~14% to the total cline for wing
493 area (females: $\Delta_{foxo} \times \Delta_{\text{frequency}} / \Delta_{\text{cline}} \approx 0.017 \times 0.6 / 0.074 \approx 0.138$; males: Δ_{foxo}
494 $\times \Delta_{\text{frequency}} / \Delta_{\text{cline}} \approx 0.019 \times 0.6 / 0.083 \approx 0.137$) – this represents a major contribution
495 to the wing size cline along the North American east coast (Coyne and Beecham
496 1987; Betancourt et al. 2018).

497 For all size traits, females were larger than males (Fig. 3; Fig. 4; Table 1; Fig. S5;
498 Fig. S6; Table S4, Supporting Information), as is typically observed. With regard to
499 the plastic effects of temperature, femur length, thorax length and wing area were
500 larger at 18°C than at 25°C (Fig. 3; Fig. S5, Fig. S6, Supporting Information; Table 1;

501 Table S4, Supporting Information), as is expected based on previous work (David et
502 al. 1994; Partridge et al. 1994a). In terms of dietary plasticity, femur and thorax
503 length were larger on sucrose than on molasses diet (Fig. 3; Table 1; Fig. S6; Table
504 S4, Supporting Information), perhaps in line with the observation that more
505 carbohydrate-rich diets cause smaller size (Reis 2016); however, wing area and
506 wing:thorax ratio were larger on molasses than on sucrose diet (Fig. S5; Table S4,
507 Supporting Information; and Fig. 4; Table 1). Although we found a few G × E
508 interactions for size traits (Fig. 4; Fig. 5; Table 1; Fig. S5; Fig. S6; Table S4,
509 Supporting Information), the allelic reaction norms were overall remarkably parallel
510 across environmental conditions.
511
512 **POLYMORPHISM AT *FOXO* IMPACTS STARVATION AND FAT CATABOLISM**
513 The *foxo* alleles also differed in their effects on resistance to (survival of) starvation in
514 females (Fig. 5; Table 1), as might be expected based on the observation that *foxo*
515 mutants are more starvation sensitive than wildtype (Jünger et al. 2003; Kramer et al.
516 2003, 2008). However, contrary to clinal predictions (e.g., Schmidt and Paaby 2008;
517 Mathur and Schmidt 2017), LL females were more resistant than HL females (Fig. 5;
518 Table 1), suggesting a countergradient effect; in males, there were no allelic
519 differences in resistance (Fig. S7; Table S4, Supporting Information; for estimates of
520 the variance components of the random effect of vial see Table S5, Supporting
521 Information). Overall females were more resistant than males (Fig. 5; Table 1; Fig.
522 S7; Table S4, Supporting Information), consistent with some but not other studies
523 (Goenaga et al. 2010; but see Matzkin et al. 2009). For both females and males,
524 starvation resistance was higher at 18°C than at 25°C (Fig. 5; Table 1; Fig. S7; Table
525 S4, Supporting Information), as previously reported (Mathur and Schmidt 2017). Flies

526 raised on molasses diet were more resistant than those raised on sucrose diet (Fig.
527 5; Table 1; Fig. S7; Table S4, Supporting Information), potentially in support of the
528 finding that lower P:C ratios favor higher resistance (Chippindale et al. 1993; Lee and
529 Jang 2014). We also found evidence for an allele by diet interaction: allelic
530 differences in resistance were more pronounced on molasses than sucrose diet (Fig.
531 5; Table 1; Fig. S7; Table S4, Supporting Information).
532 To further examine the physiological basis of starvation resistance we quantified
533 how much fat female flies mobilize upon starvation (Fig 6; Table 2; males were not
534 examined since they did not show allelic differences in resistance). Paralleling our
535 result that LL females are more resistant than HL females, the amount of fat
536 catabolized under starvation was greater in LL than in HL females, under almost all
537 conditions (except for females raised on sucrose diet at 25°C; see Fig. 6 and Table 2:
538 significant allele by diet by starvation treatment interaction at 25°C but not at 18°C).
539 Fat loss upon starvation was greater for flies raised on molasses than on sucrose
540 diet (Fig 6; Table 2), again matching the results for starvation resistance itself.
541
542 ***FOXO* ALLELES DIFFER IN TRANSCRIPTIONAL FEEDBACK CONTROL OF *InR***
543 From the above patterns we predicted that the LL allele would exhibit decreased IIS
544 and increased FOXO activity: the LL allele has smaller size but higher starvation
545 resistance, i.e. traits that co-occur in IIS mutants or flies with increased FOXO
546 activity. To test this hypothesis we performed qRT-PCR analysis of a major
547 transcriptional target of FOXO, *InR*: when IIS is low, FOXO becomes active and
548 upregulates *InR* transcription, while under high IIS FOXO is inactive and represses
549 *InR* (Puig et al. 2003; Puig and Tjian 2005). In support of this hypothesis we found
550 that the LL allele had a ~12% higher level of *InR* transcript than the HL allele (Fig.

551 S8; Table S6, Supporting Information). Dietary conditions also affected *InR* levels,
552 with flies raised on molasses producing more *InR* than flies raised on sucrose diet
553 (Fig. S8; Table S6, Supporting Information).
554
555

556 *Discussion*

557 **CONNECTING ADAPTIVE CLINAL PHENOTYPES TO GENOTYPES**

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

562 As hypothesized by de Jong and Bochdanovits (2003), genes of the IIS/TOR
563 pathway might represent particularly promising candidates underlying clinal life-
564 history adaptation in *D. melanogaster*: (1) laboratory mutants in this pathway often
565 mirror life-history traits and trade-offs observed in natural populations (de Jong and
566 Bochdanovits 2003; Clancy et al. 2001; Tatar et al. 2001; Tatar and Yin 2001; Tatar
567 et al. 2003; Paaby et al. 2010; Flatt et al. 2013; Paaby et al. 2014; Flatt and Partridge
568 2018); (2) reproductive dormancy in response to cool temperature and short
569 photoperiod, a genetically variable and clinal trait (Williams and Sokolowski 1993;
570 Schmidt et al. 2005a; Schmidt and Conde 2006; Schmidt et al. 2005b; Schmidt and
571 Paaby 2008), is physiologically regulated by IIS (Williams et al. 2006; Flatt et al.
572 2013; Kubrak et al. 2014; Schiesari et al. 2016; Zhao et al. 2016; Andreatta et al.
573 2018); (3) genomic analyses of clinal differentiation has identified many clinal SNPs
574 in the IIS/TOR pathway presumably shaped by spatially varying selection (Fig. 1;

575 Kolaczkowksi et al. 2011; Fabian et al. 2012; Kapun et al. 2016b); and (4) genome-
576 wide analyses of variation in size-related traits have identified novel regulators of
577 growth, several of which interact with the IIS/TOR pathway (Vonesch et al. 2016;
578 Strassburger et al. 2017). For example, in support of the idea that variation in IIS
579 contributes to clinal adaptation in *D. melanogaster,* Paaby and colleagues have
580 identified a clinal indel polymorphism in *InR* with pleiotropic effects on development,
581 body size, fecundity, lifespan, oxidative stress resistance, chill coma recovery, and
582 insulin signaling (Paaby et al. 2010, 2014). Our results on *foxo* lend further support to
583 the hypothesis of de Jong and Bochdanovits (2003).
584

585 **THE EFFECTS OF NATURAL VERSUS NULL ALLELES AT THE *FOXO* LOCUS**

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

598 Although we could not predict with certainty the directionality and/or the degree of
599 pleiotropy of the allelic effects *a priori*, we found that the *foxo* polymorphism

600 differentially affects several size-related traits and starvation resistance, phenotypes
601 known to be affected by the *foxo* locus. With regard to growth and size, our findings
602 from natural variants agree well with functional genetic studies showing that genetic
603 manipulations of the *foxo* locus affect body size and wing area (Jünger et al. 2003;
604 Slack et al. 2011; Tang et al. 2011). Similarly, our observation that variation at *foxo*
605 affects survival and fat content upon starvation is consistent with the fact that *foxo*
606 mutants display reduced starvation resistance (Jünger et al. 2003; Kramer et al.
607 2003, 2008). In contrast, although *foxo* null mutants produce viable adults (Jünger et
608 al. 2003; Slack et al. 2011), whether distinct *foxo* alleles vary in viability has not yet
609 been examined; here we find that the two natural alleles differ in egg-to-adult
610 survival. We also asked whether the alleles differentially affect mRNA abundance of
611 *InR*, a transcriptional target of FOXO (Puig et al. 2003; Puig and Tjian 2005). Indeed,
612 the LL allele had higher *InR* mRNA levels, consistent with the LL genotype exhibiting
613 reduced IIS and higher FOXO activity.
614 For most traits measured, both alleles reacted plastically to changes in diet and
615 temperature in the direction predicted from previous work (Partridge et al. 1994a,
616 1994b; Lee and Jang 2014; Lihoreau et al. 2016; Mathur and Schmidt 2017), yet we
617 found very little evidence for allele by environment (G × E) interactions.
618 While our experimental design does not allow us to disentangle the contribution of
619 the 2 individual SNPs to the total effects seen for the *foxo* polymorphism, our results
620 suggest that the naturally occurring alternative alleles at *foxo* we have examined here
621 – and which are defined by only two linked SNP positions –can apparently have quite
622 strong pleiotropic (or, via LD, correlational) effects upon multiple complex life-history
623 traits, including on viability, several proxies of size and on starvation resistance (for
624 estimates of allelic effect sizes see Table S4, Supporting Information). This is

625 consistent with the pleiotropic effects seen in *foxo* loss-of-function mutant alleles (see
626 references above) and might support the idea that the architecture of life-history
627 traits, which are connected via multiple trade-offs, is inherently pleiotropic (Williams
628 1957; Finch and Rose 1995; Flatt et al. 2005; Flatt and Promislow 2007; Flatt and
629 Schmidt 2009; Flatt et al. 2013; Paaby et al. 2014); it also provides a contrast to the
630 model from evo-devo which posits that most evolutionarily relevant mutations should
631 exhibit little or no pleiotropy (Stern 2011). In particular, the pleiotropic effects of the
632 *foxo* variant might explain why this polymorphism might be maintained, through some
633 form of balancing selection, in natural populations along the cline.
634

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

636 How does the *foxo* variant contribute to phenotypic clines observed across latitude?
637 High-latitude flies tend to be characterized, for example, by larger body size,
638 decreased fecundity, longer lifespan and improved stress resistance as compared to
639 low-latitude flies, and this differentiation is genetically based (Coyne and Beecham
640 1987; Schmidt et al. 2005a, 2005b; Schmidt and Paaby 2008; Mathur and Schmidt
641 2017; Durmaz et al. 2018). Do the allelic effects go in the same direction as the
642 latitudinal gradient, representing cogradient variation, or do certain allelic effects run
643 counter to the cline, representing countergradient variation (Levins 1968; Conover
644 and Schultz 1995)? Cogradient variation occurs when diversifying selection favors
645 different traits in different environments, as expected from selection along a cline,
646 whereas countergradient variation occurs when stabilizing selection favors similar
647 traits in different environments (Conover and Schultz 1995; Marcil et al. 2006).
648 Consistent with clinal expectation, the HL allele confers larger size (Coyne and
649 Beecham 1987; de Jong and Bochdanovits 2003); increased wing:thorax ratio, which

650 corresponds to reduced ‘wing loading’, a trait hypothesized to be adaptive for flight at
651 cold temperature (Stalker 1980; David et al. 1994; Azevedo et al. 1998; Frazier et al.
652 2008; Bhan et al. 2014); and reduced viability (Folguera et al. 2008). Conversely, the
653 LL allele exhibits smaller size, increased wing loading, and higher viability. Thus, the
654 *foxo* variant contributes to the observed phenotypic cline in the predicted direction
655 (gradient or cogradient variation) and appears to be maintained by spatially varying
656 selection (for a remarkable example where size is subject to countergradient – not
657 cogradient – variation along an altitudinal gradient in Puerto Rican *D. melanogaster*
658 see Levins, 1968, 1969). Importantly, our results for size-related traits are consistent
659 with independent assays under constant environmental conditions (Betancourt et al.
660 2018) and suggest a major contribution of the *foxo* polymorphism to the clinality of
661 body size .

662 For starvation resistance, we found – contrary to clinal predictions – that the HL
663 allele is less resistant than the LL allele, consistent with countergradient variation.
664 Interestingly, a similar countergradient effect (on body size) was found for the *InR*
665 polymorphism mentioned above: the high-latitude InR^{short} allele confers smaller size,
666 even though flies from high-latitude populations are normally larger (Paaby et al.
667 2014). Likewise, for a clinal variant of *neurofibromin 1* (*Nf1*) the high-latitude
668 haplotype has smaller wing size, an effect that runs counter to the cline (Lee et al.
669 2013). However, as mentioned in the methods, we can of course not completely rule
670 out potentially confounding LD effects that might account for this unexpected result
671 with regard to starvation resistance.

672 In terms of the physiological effects of IIS, temperate fly populations might be
673 characterized by ‘thrifty’ genotypes with high IIS, whereas tropical populations might
674 have a higher frequency of ‘spendthrift’ genotypes with low IIS (de Jong and

675 Bochdanovits 2003). Our finding that the low-latitude *foxo* allele likely exhibits
676 increased FOXO activity and lower IIS seems to support this, yet Paaby et al. (2014)
677 found that IIS was lower for the high-latitude *InR* allele. The directionality of IIS
678 effects along the cline thus remains difficult to predict.
679 As noted by Lee et al. (2013) and Paaby et al. (2014), clinal variants subject to
680 countergradient effects might interact epistatically with other loci affecting the trait, or
681 they might be affected by antagonistic selection pressures (Schluter et al. 1991).
682 Conflicting selection pressures on clinal variants might be particularly acute when
683 they exhibit pleiotropic effects on multiple traits, as is the case for the polymorphisms
684 at *Nf1, InR*, and *foxo*. These examples illustrate the complexity of dissecting clinal
685 selection and the genotype-phenotype map underlying clinal adaptation (Lee et al.
686 2013; Paaby et al. 2014; Flatt 2016).
687 With regard to starvation resistance, a caveat is that we found the LL allele to be
688 more resistant, while Betancourt et al. (2018) found the HL allele to be more
689 resistant. This discrepancy might be due to differences in assay protocols:
690 Betancourt et al. (2018) did not use agar which could impose some desiccation in
691 addition to starvation stress. Interestingly, desiccation resistance is known to vary
692 latitudinally along the North America east coast (Rajpurohit et al. 2018), but whether
693 the *foxo* polymorphism examined here affects survival upon desiccation remains
694 unknown and awaits future study.
695
696 **GROWING EVIDENCE FOR A ROLE OF IIS IN LIFE-HISTORY ADAPTATION**
697 The IIS pathway provides an excellent example of how mechanistic and evolutionary
698 insights might be combined to gain a more complete understanding of the ultimate
699 and proximate determinants of life-history adaptation (Finch and Rose 1995; Houle

700 2001; Flatt and Heyland 2011). Since the 1990s, a great deal has been learned
701 about the genetic, developmental and physiological effects of this pathway in model
702 organisms. This work has shown that IIS mutants affect major fitness-related traits,
703 and this in turn has illuminated our understanding of the molecular underpinnings of
704 growth, size, lifespan and trade-offs (Partridge and Gems 2002; Tatar et al. 2003;
705 Flatt et al. 2005; Flatt and Heyland 2011; Flatt et al. 2013). In particular, these
706 studies have revealed that the IIS pathway plays an evolutionarily conserved role in
707 the physiological regulation of longevity (Partridge and Gems 2002; Tatar et al.
708 2003); they have also given us some of the clearest examples of alleles exhibiting
709 antagonistic pleiotropy (Williams 1957; Flatt and Promislow 2007; and references
710 above).
711 The functional characterization of this pathway therefore promised an opportunity
712 for evolutionary geneticists to identify natural variants involved in life-history evolution
713 (de Jong and Bochdanovits 2003). Yet, 'life history loci' identified via functional
714 genetic analysis need not necessarily contribute to standing variation for these traits
715 in the wild (Flatt 2004; Flatt and Schmidt 2009; Fabian et al. 2018). For some time, it
716 thus remained unclear whether natural variation in this pathway impacts variation in
717 fitness-related traits in natural populations (see Reznick 2005; Fabian et al. 2018).
718 Today, we have growing evidence that variation in IIS indeed can make an
719 important contribution to life-history variation in flies and other insects, worms, fish,
720 reptiles and mammals, including effects on longevity in humans (e.g., de Jong and
721 Bochdanovits 2003; Williams et al. 2006; Flachsbart et al. 2008; Suh et al. 2008;
722 Willcox et al. 2008; Alvarez-Ponce et al. 2009; Sparkman et al. 2009, 2010; Paaby et
723 al. 2010; Stuart and Page 2010; Dantzer and Swanson 2012; Jovelin et al. 2014;
724 Paaby et al. 2014; Swanson and Dantzer 2014; McGaugh et al. 2015; Schwartz and

725 Bronikowski 2016; Zhao et al. 2016; and references therein). On the other hand,
726 'evolve and resequence' studies of *Drosophila* longevity have failed to find a major
727 contribution of standing variation in IIS to evolved changes in life history and lifespan,
728 perhaps suggesting that the IIS pathway might be selectively constrained, at least
729 with regard to the evolution of certain traits (e.g., Remolina et al. 2012; Fabian et al.
730 2018; Flatt and Partridge 2018). In sum, this body of work illustrates how one might
731 be able to connect genotypes to molecular mechanisms to components of fitness by
732 studying a fundamentally important physiological pathway from multiple angles
733 (Finch and Rose 1995; Houle 2001; Flatt and Heyland 2011; Flatt et al. 2013).
734

735 *Conclusions*

736 Here we have found that a strongly clinal polymorphism at the *foxo* locus (which
737 might be viewed as a marker for functionally significant alleles) has effects on several
738 fitness components known to vary across latitude, including viability, size-related
739 traits, starvation resistance and fat content. The directionality of most of these effects
740 matches overall phenotypic clines observed along the North American east coast,
741 especially with regard to size (e.g., Coyne and Beecham 1987; Schmidt et al. 2005a,
742 2005b; Schmidt and Paaby 2008; Durmaz et al. 2018). Together, our results thus
743 suggest that standing variation in the IIS pathway makes an important and – at least
744 partly – predictable contribution to life-history clines in *Drosophila*.
745

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761

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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.

1311 **Table 1.** Summary of ANOVA results for viability; femur length; wing area:thorax

1312 length ratio; female starvation resistance (also cf. Table S5). White and grey cells

1313 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*	

1314

1315 **Table 2.** ANOVA results for female fat loss upon starvation. *$p < 0.05$; **$p < 0.01$;

1316 ***$p < 0.001$. The fixed factor 'Treatment' has two levels: fed vs. starved; interactions

1317 involving the factors 'Allele' and 'Treatment' test for allelic differences in fat

1318 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)	$F_{4,32}=9.46^{***}$	$F_{4,32}=1.44$

1319

1320 **FIGURE CAPTIONS**

1321 **Figure 1.** Clinal candidates in the insulin/TOR signaling pathway. Overview of the
1322 insulin/insulin-like growth factor signaling (IIS)/target of rapamycin (TOR) pathway in
1323 *Drosophila melanogaster* (Oldham and Hafen 2003; Giannakou and Partridge 2007;
1324 Teleman 2010). Genes that harbor strongly clinally varying SNPs across latitude,
1325 identified by Fabian et al. (2012), are highlighted in red; arrows indicate activation
1326 and bar-ended lines represent inhibitory effects. In response to nutrients, IIS is
1327 activated by binding of ligands, called *Drosophila* insulin-like peptides (dilps 1-8), to
1328 the insulin-like receptor (InR) at the cell membrane. Inside the cell, signaling is
1329 transduced by an insulin receptor substrate (IRS) protein called chico. This activates
1330 phosphoinositide-3-kinase (PI3K) which converts phosphatidylinositol (3,4)-
1331 bisphosphate (PIP2) into phosphatidylinositol (3,4,5)-trisphosphate (PIP3). In turn,
1332 PIP3 stimulates pyruvate dehydrogenase kinase (PDK) and activates protein kinase
1333 B (AKT/PKB). The action of PI3K is antagonized by phosphatase and tensin
1334 homologue (PTEN) which converts PIP3 back to PIP2. AKT/PKB suppresses the
1335 forkhead (FKH) box O transcription factor FOXO by phosphorylating it; upon reduced
1336 IIS, FOXO becomes dephosphorylated and moves into the nucleus where it
1337 regulates the expression of hundreds of target genes. Target genes of FOXO include
1338 *InR*, controlled via a transcriptional feedback loop, and *initiation factor 4E-binding*
1339 *protein* (*4E-BP*); another target gene of IIS is *target of brain insulin* (*Tobi*), which
1340 encodes a glucosidase, but the details of its regulation remain poorly understood.
1341 FOXO is antagonized by 14-3-3ε. AKT/PKB antagonizes the activity of the tuberous
1342 sclerosis complex 1/2 (TSC1/TSC2); TSC1/2 in turn inactivates RAS homologue
1343 enriched in brain (RHEB). The inactivation of RHEB disinhibits, i.e. activates, target
1344 of rapamycin (TOR). TOR then activates the effector gene *S6 kinase* (*S6K*) and

1345 inhibits the negative regulator 4E-BP. The phenotypic effects of naturally occurring
1346 alleles of the genes in the IIS/TOR pathway remain poorly understood, but clinal
1347 polymorphisms in *InR* (Paaby et al. 2010; Paaby et al. 2014) and *foxo* (this study)
1348 have pleiotropic effects on life history in *Drosophila*.

1349

1350 **Figure 2.** Viability (egg-to-adult survival). Effects of the clinal *foxo* variant on the
1351 proportion viability (egg-to-adult survival). (A) Dietary reaction norms at 18°C. (B)
1352 Dietary reaction norms at 25°C. (C) Thermal reaction norms measured on sucrose
1353 diet. (D) Thermal reaction norms measured on molasses diet. Data in (A, B) are the
1354 same as those shown in (C, D). Shown are means and standard errors. Red lines:
1355 low-latitude (LL) allele, blue lines: high-latitude (HL) allele.

1356

1357 **Figure 3.** Femur length. Effects of the *foxo* polymorphism on femur length (mm) in
1358 females and males. (A) Dietary reaction norms at 18°C. (B) Dietary reaction norms at
1359 25°C. (C) Thermal reaction norms measured on sucrose diet. (D) Thermal reaction
1360 norms measured on molasses diet. Data in (A, B) are the same as those shown in
1361 (C, D). Shown are means and standard errors. Red lines: low-latitude (LL) allele, blue
1362 lines: high-latitude (HL) allele.

1363

1364 **Figure 4.** Wing:thorax ratio. Effects of the *foxo* variant on the ratio of wing
1365 area:thorax length (mm) in females and males. (A) Dietary reaction norms at 18°C.
1366 (B) Dietary reaction norms at 25°C. (C) Thermal reaction norms measured on
1367 sucrose diet. (D) Thermal reaction norms measured on molasses diet. Data in (A, B)
1368 are the same as those shown in (C, D). Shown are means and (propagated)
1369 standard errors. Red lines: low-latitude (LL) allele, blue lines: high-latitude (HL) allele.

1370

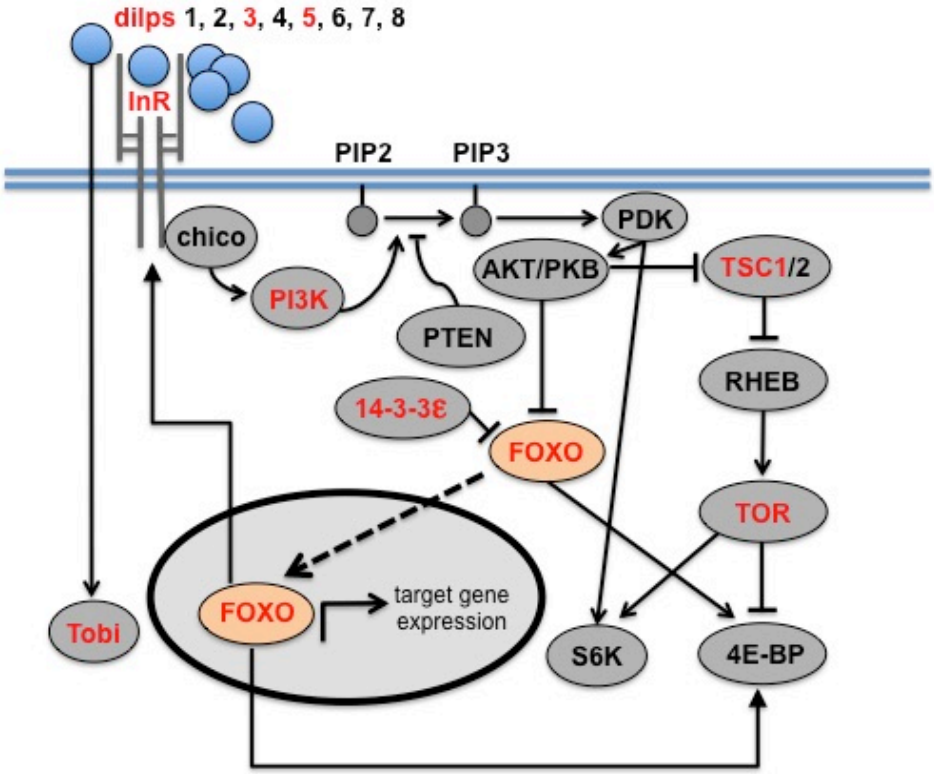
1371 **Figure 5,** Starvation resistance. Effects of the clinal *foxo* polymorphism on the
1372 duration of survival (in hrs) upon starvation in females. (A) Dietary reaction norms at
1373 18°C. (B) Dietary reaction norms at 25°C. (C) Thermal reaction norms measured on
1374 sucrose diet. (D) Thermal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

1377

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

1384

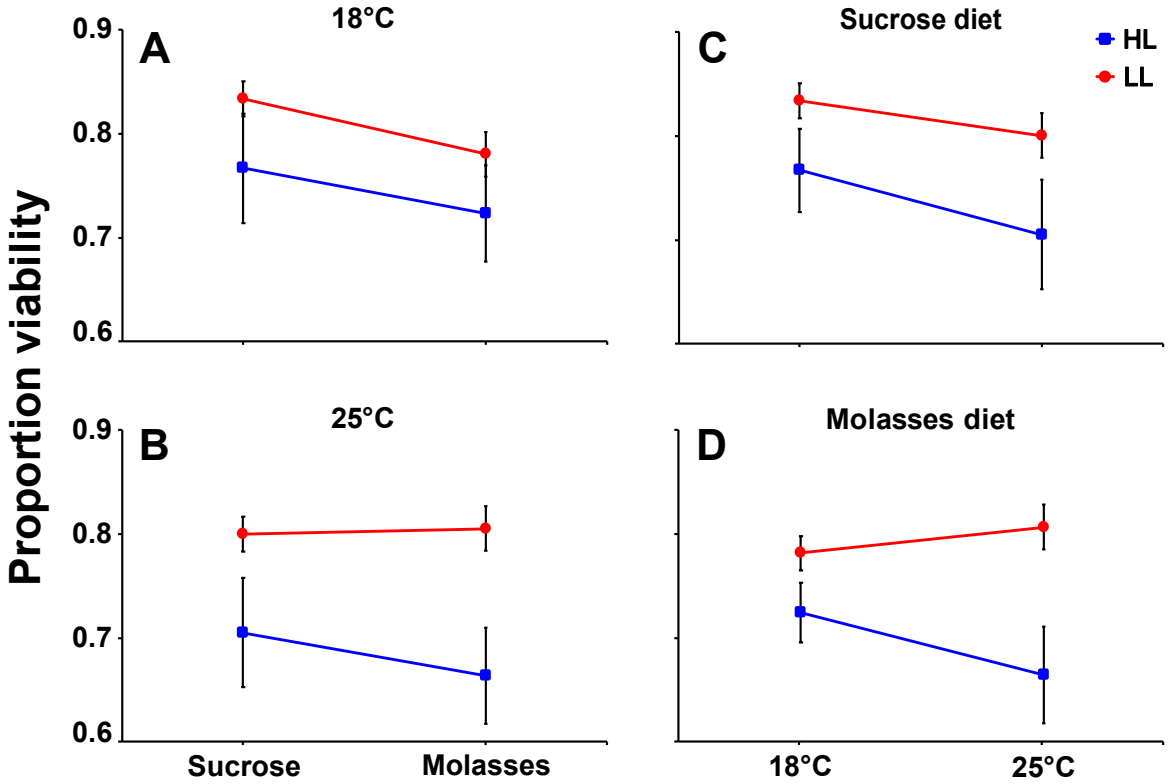
1385 **Figure 1**



1386

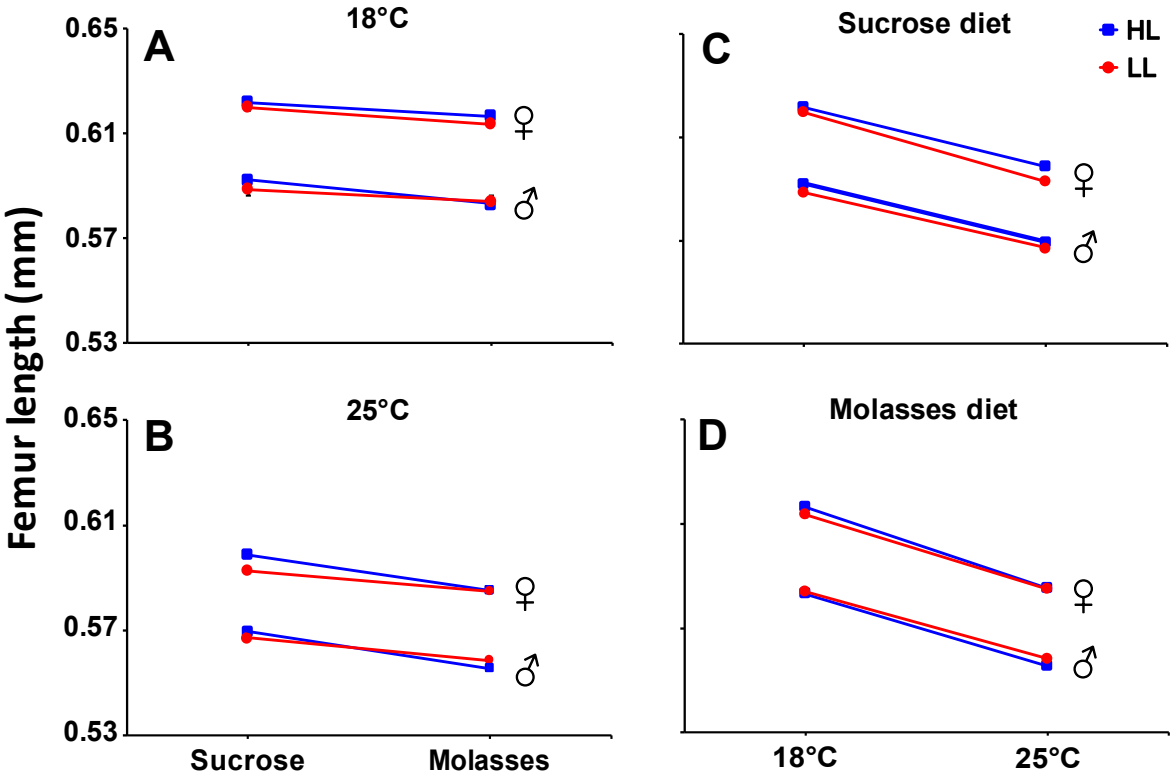
1387

1388 **Figure 2**



1389

1390 **Figure 3**



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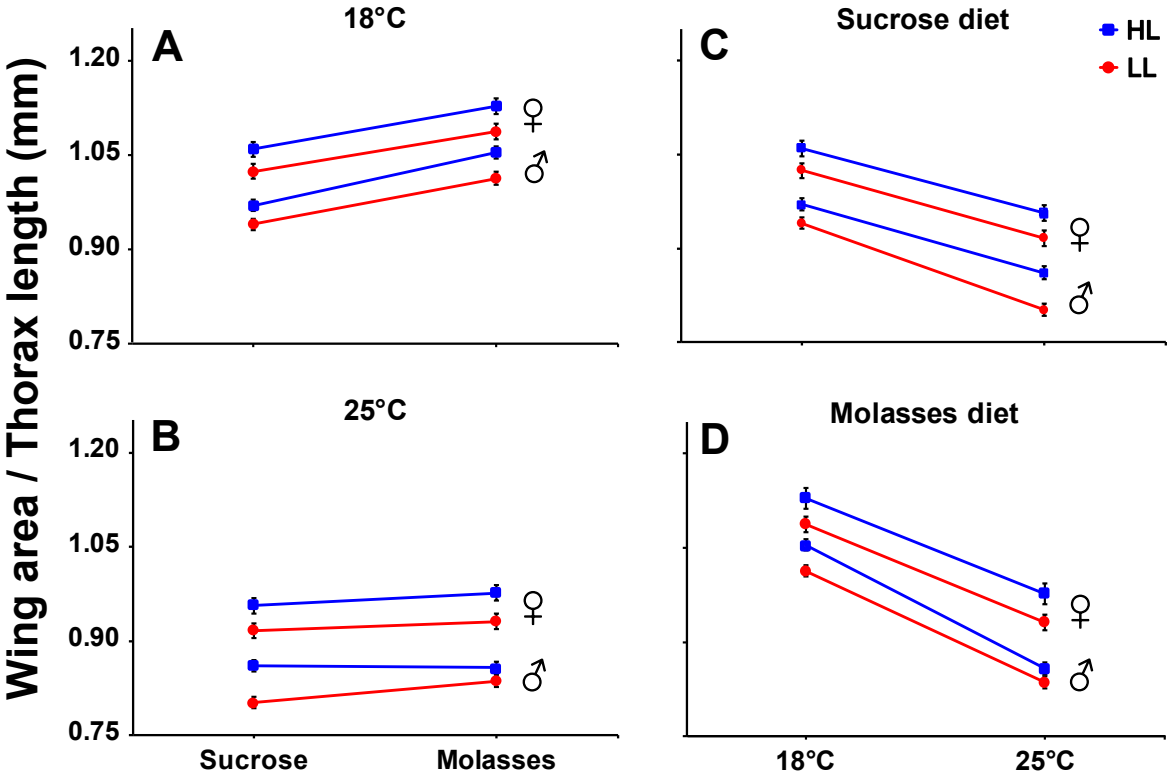
1393 **Figure 4**



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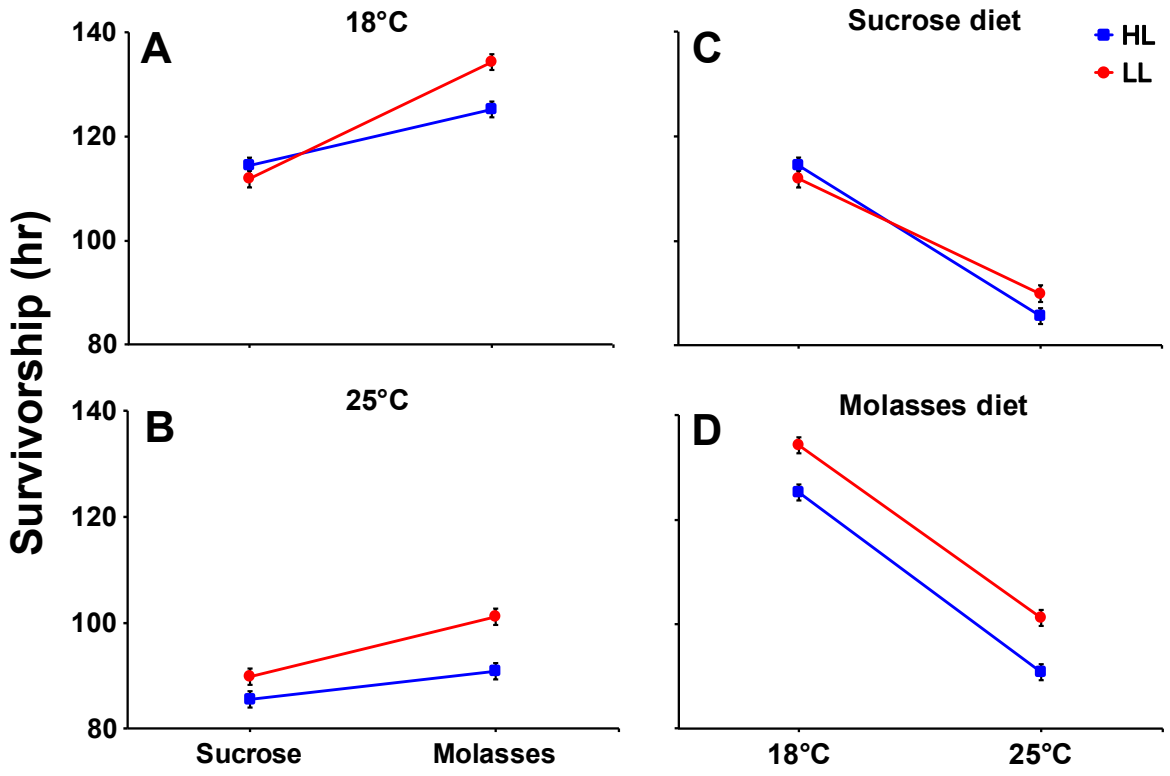
1396 **Figure 5**



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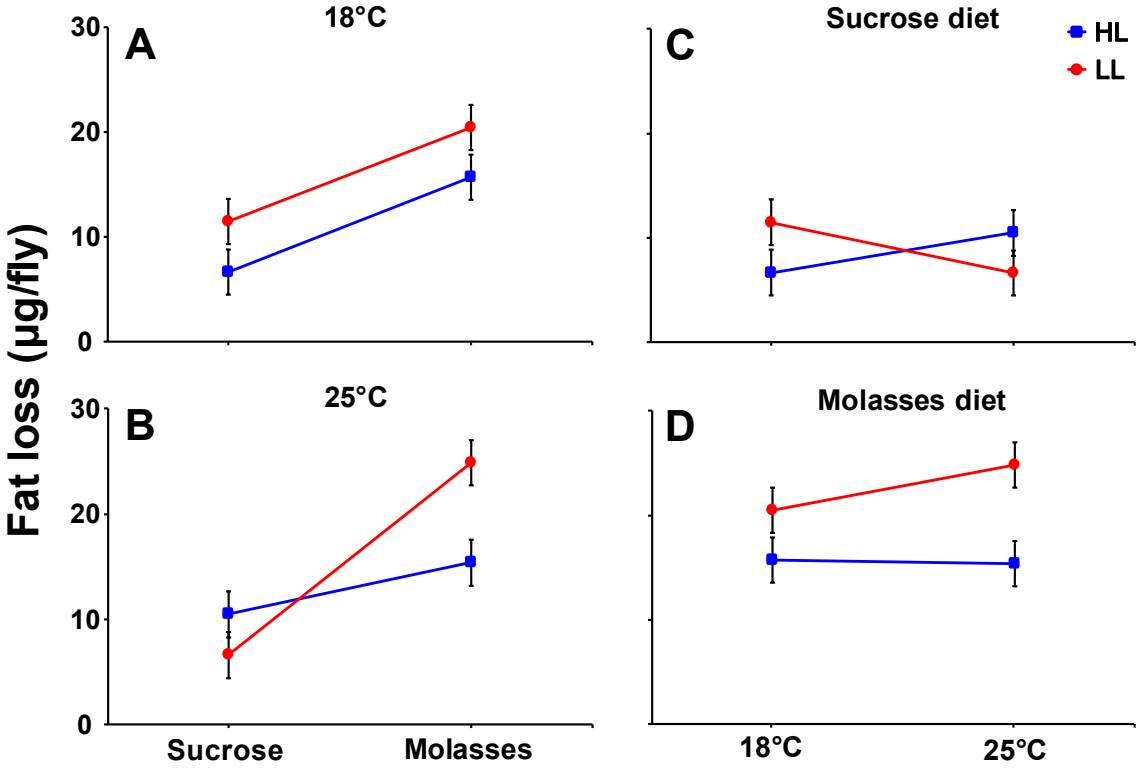
1399 **Figure 6**



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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

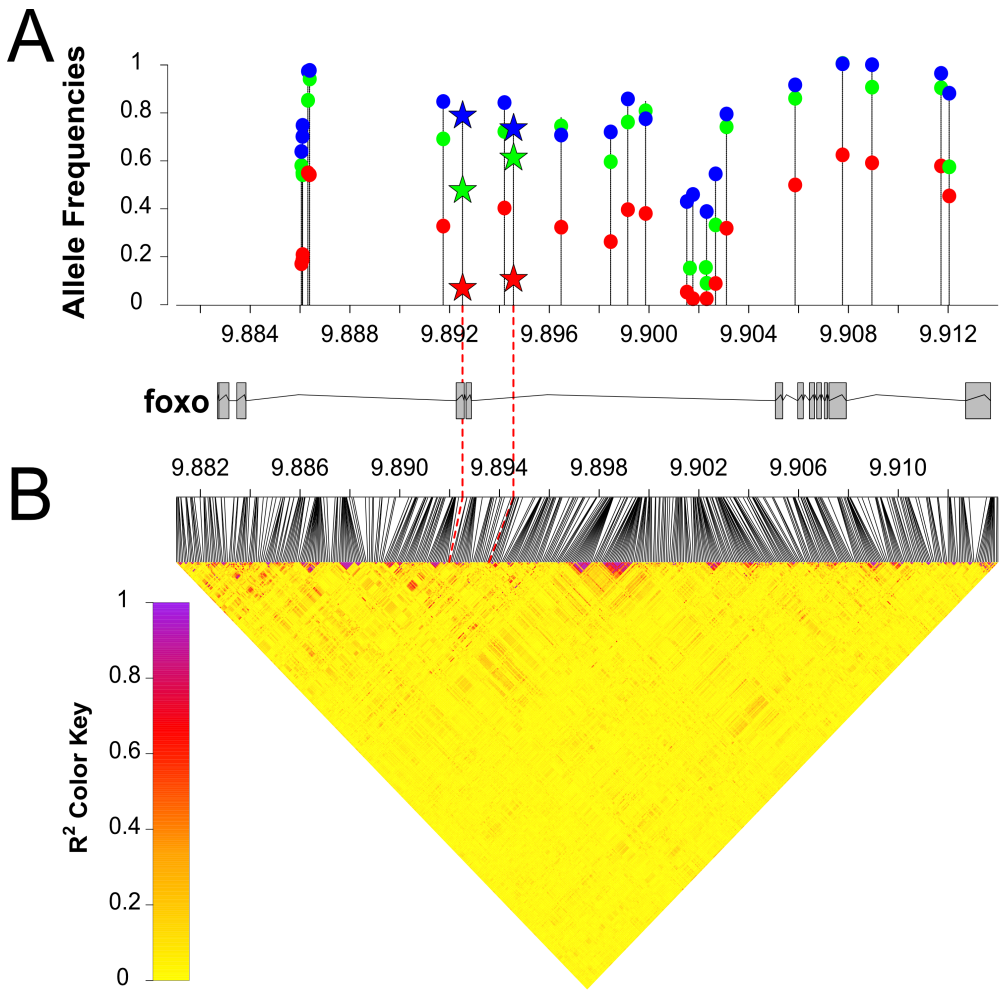
A
Allele Frequencies
1
0.8
0.6
0.4
0.2
0
9.884
9.888
9.892
9.896
9.900
9.904
9.908
9.912
foxo
B
9.882
9.886
9.890
9.894
9.898
9.902
9.906
9.910
R2 Color Key
1
0.8
0.6
0.4
0.2
0

Figure S2

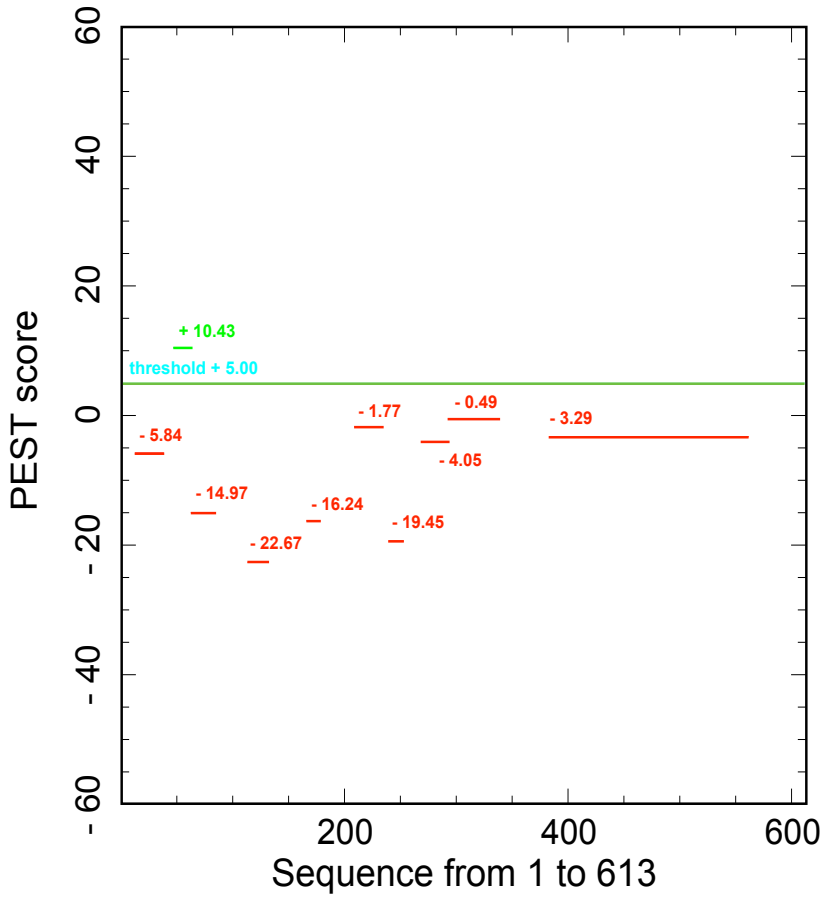
PEST score
60
40
20
0
- 20
- 40
- 60
200
400
600
Sequence from 1 to 613
+ 10.43
threshold + 5.00
- 5.84
- 14.97
- 22.67
- 16.24
- 1.77
- 19.45
- 4.05
- 0.49
- 3.29

Figure S3

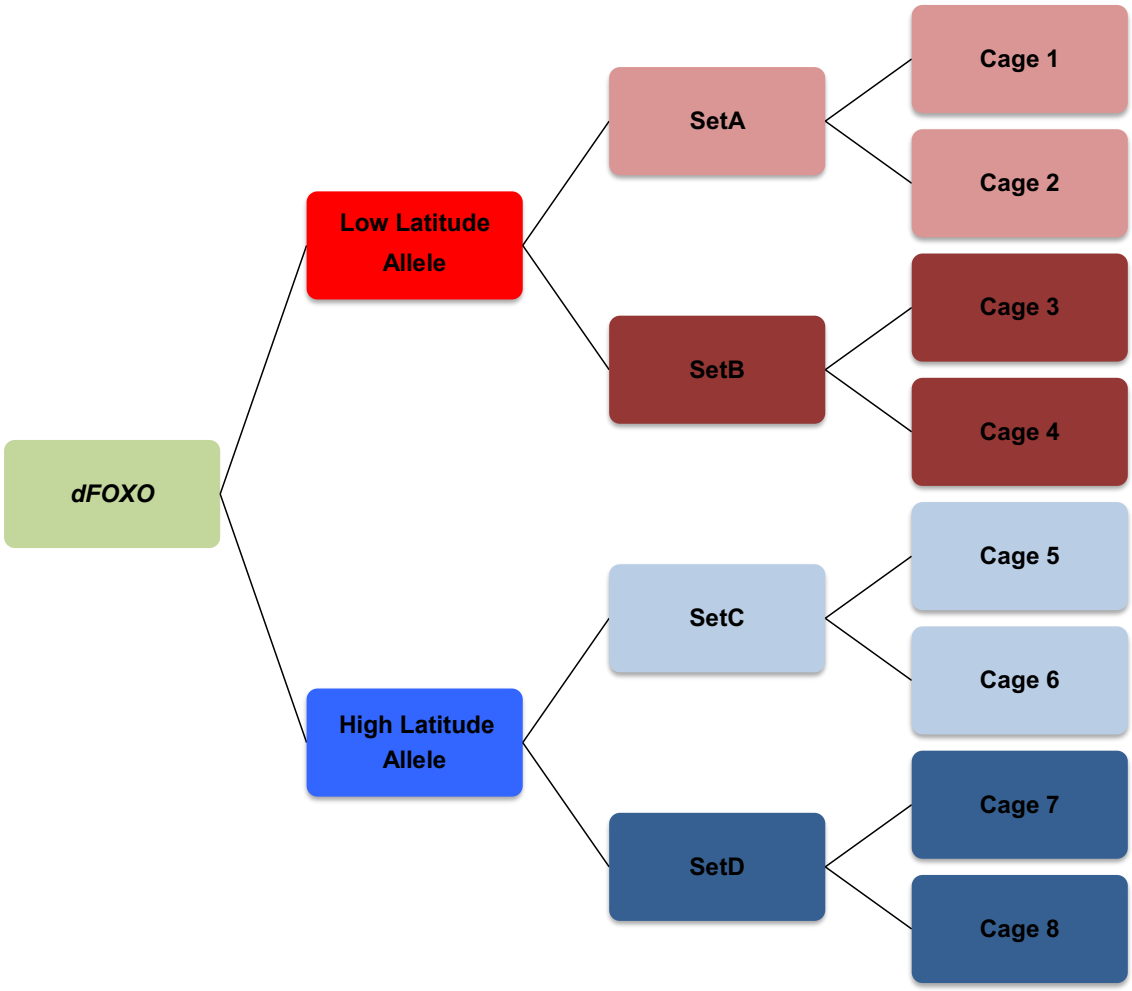
dFOXO
Low Latitude Allele
High Latitude Allele
SetA
SetB
SetC
SetD
Cage 1
Cage 2
Cage 3
Cage 4
Cage 5
Cage 6
Cage 7
Cage 8

Figure S4

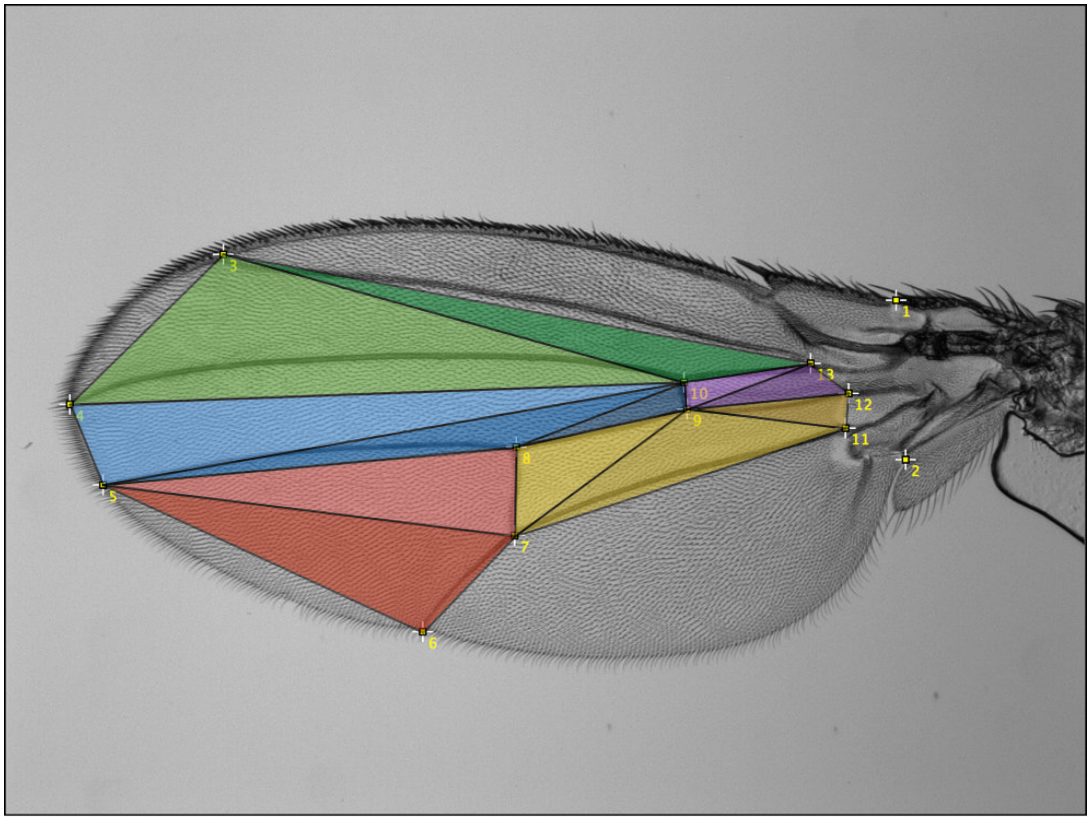

Figure S5

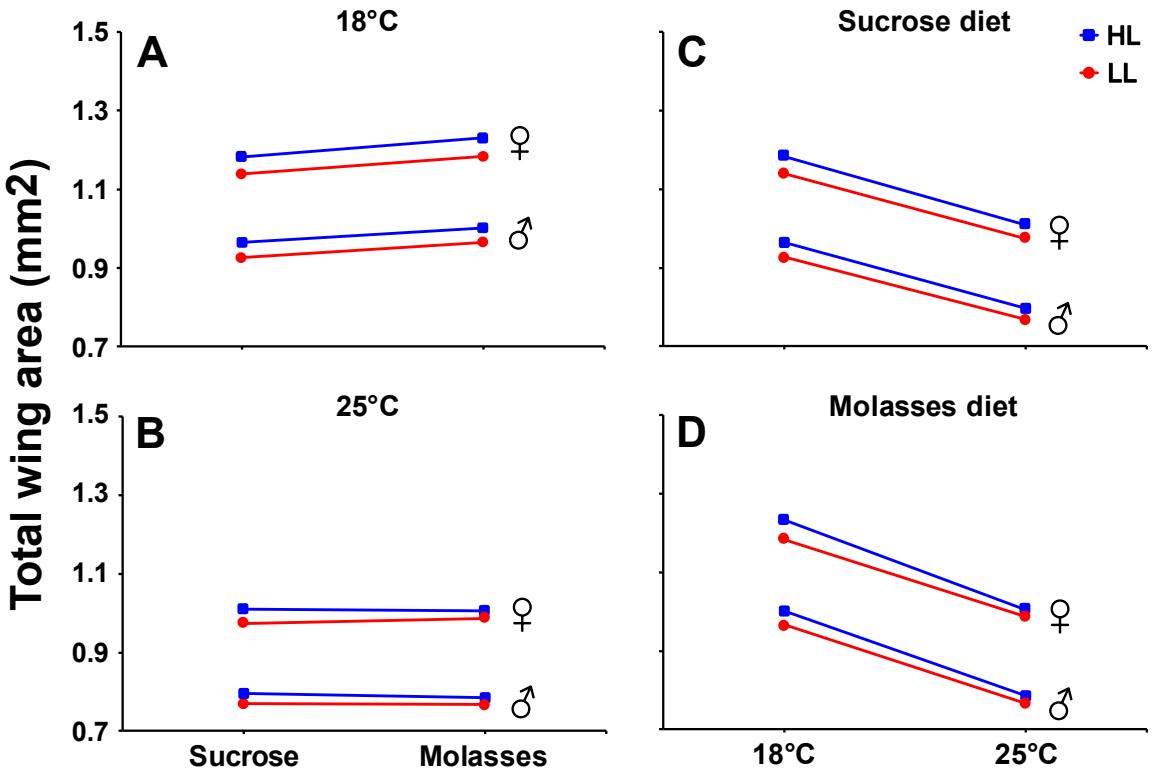
Total wing area (mm2)
1.5
1.3
1.1
0.9
0.7
A
18°C
B
25°C
C
Sucrose diet
D
Molasses diet
HL
LL
♀
♂
Sucrose
Molasses
18°C
25°C

Figure S6

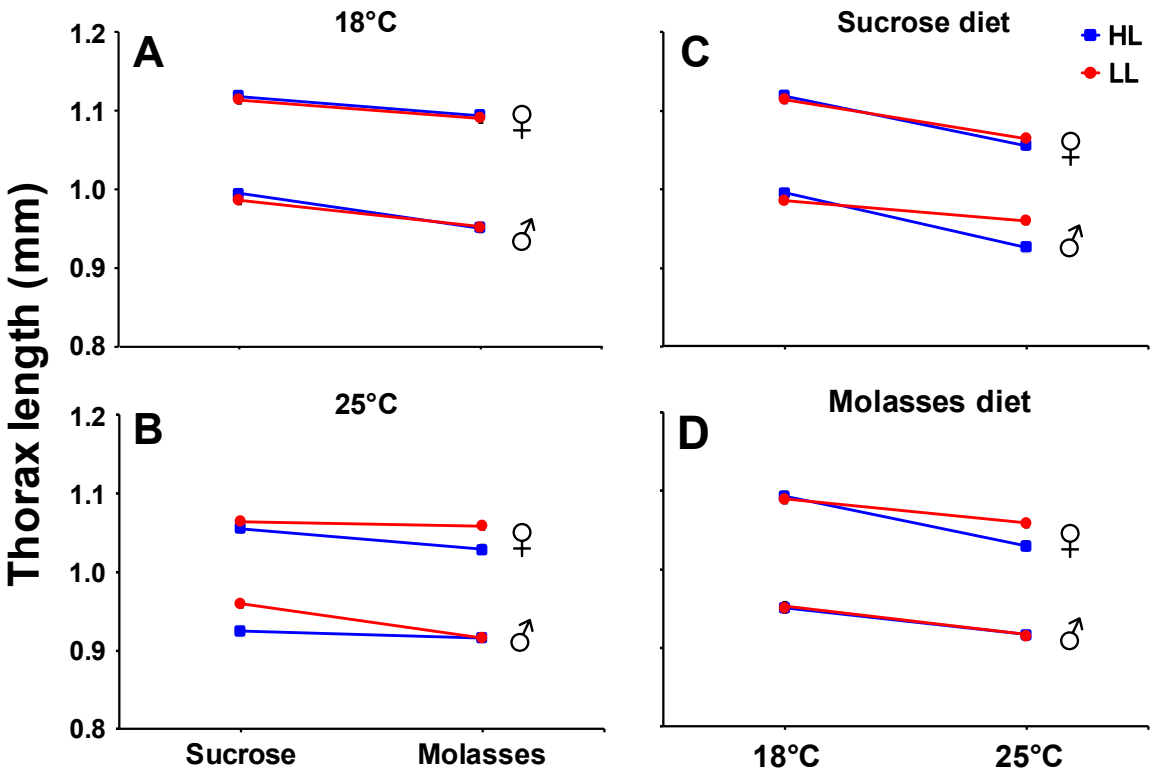
A
18°C
B
25°C
C
Sucrose diet
D
Molasses diet
HL
LL
♀
♂
Thorax length (mm)
1.2
1.1
1.0
0.9
0.8
Sucrose
Molasses
18°C
25°C

Figure S7

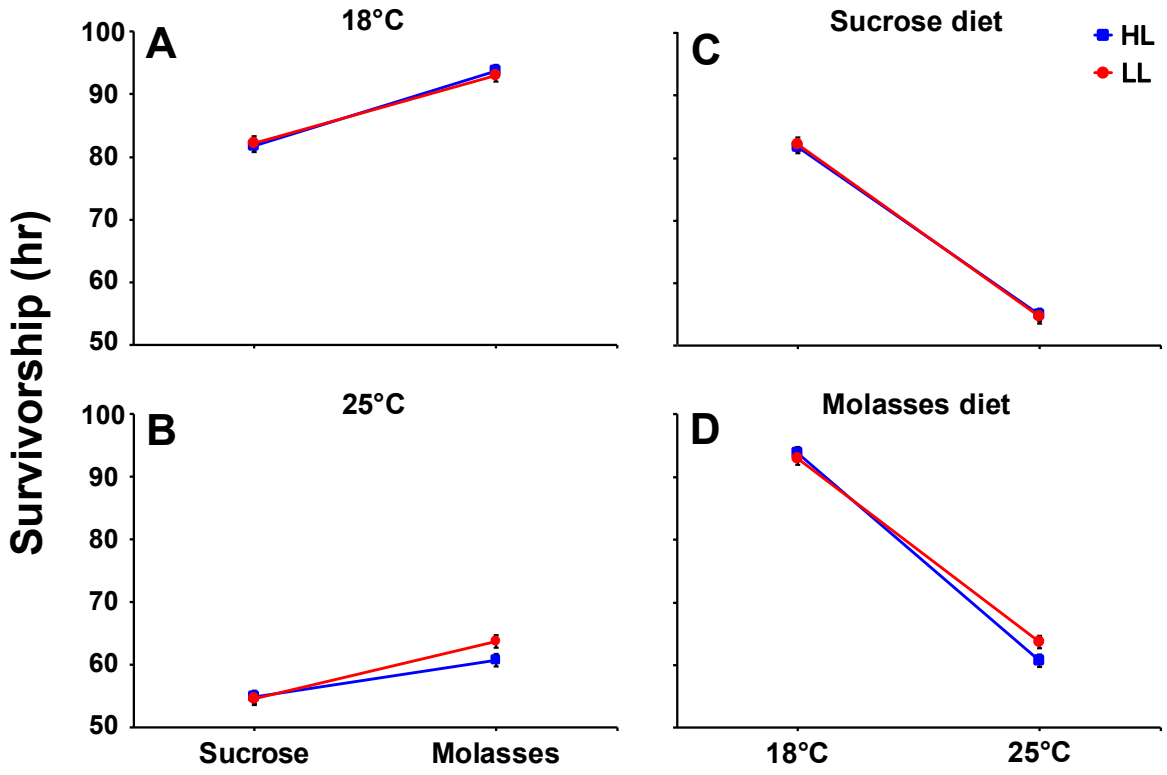
Survivorship (hr)
A
18°C
B
25°C
C
Sucrose diet
D
Molasses diet
HL
LL
100
90
80
70
60
50
Sucrose
Molasses
18°C
25°C

Figure S8

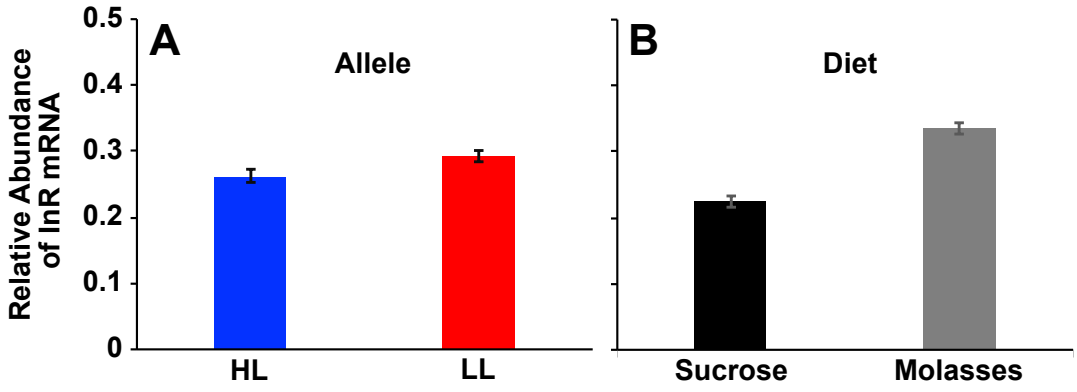
A
Allele
B
Diet
Relative Abundance of InR mRNA
0.5
0.4
0.3
0.2
0.1
0
HL
LL
Sucrose
Molasses

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