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*

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

53 Much has been learned about the genetics of fitness traits (e.g., size, lifespan),
54 mainly from studies of large-effect mutants and transgenes in yeast, *C. elegans*,
55 *Drosophila* and the mouse (Finch and Rose 1995; Oldham and Hafen 2003; Tatar et
56 al. 2003; Fielenbach and Antebi 2008; Kenyon 2010; Flatt and Partridge 2018), but
57 loci identified in such laboratory analyses do not necessarily harbor segregating
58 alleles that would contribute to genetic variance for traits in natural populations (Flatt
59 2004; Flatt and Schmidt 2009; Vonesch et al. 2016; Birney 2016; Fabian et al. 2018).
60 In particular, the identity and presumably subtle effects of naturally occurring life-
61 history polymorphisms are poorly known (Flatt and Schmidt 2009; Paaby and
62 Schmidt 2009; Flatt and Heyland 2011). While adaptation genomics can in principle
63 quite readily identify such candidate polymorphisms, a major – but rarely
64 accomplished – objective is to experimentally validate these candidates as genic
65 targets of selection (Barrett and Hoekstra 2011; Turner 2014; Flatt 2016; Siddiq et al.
66 2017). Thus, with a few exceptions, examples of causative life-history variants
67 remain rare (Schmidt et al. 2008; McKechnie et al. 2010; Paaby et al. 2010; Jones et
68 al. 2012; Johnston et al. 2013; Méndez-Vigo et al. 2013; Paaby et al. 2014; Barson et
69 al. 2015; Catalán et al. 2016; reviewed in Mackay et al. 2009; Barrett and Hoekstra
70 2011).
71 Despite conceptual and methodological limitations of the so-called quantitative trait
72 nucleotide (QTN) program (Rockman 2012), the identification of life-history
73 polymorphisms allows addressing fundamental questions about the genetic basis of
74 adaptation, including: (1) Which pathways and molecular functions underpin variation
75 in fitness-related traits? (2) Are these mechanisms evolutionarily conserved? (3)
76 What are the phenotypic effects of naturally segregating life-history variants? (4)
77 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

102 al. 2005a, 2005b; Hoffmann and Weeks 2007; Schmidt and Paaby 2008; Adrion et al.
103 2015; Fabian et al. 2015; Kapun et al. 2016a).
104 To begin to identify the genetic basis of adaptive life-history clines in *D.*
105 *melanogaster*, we have previously performed genome-wide analyses of latitudinal
106 differentiation along the North American cline (Fabian et al. 2012; Kapun et al.
107 2016b) (also see Turner et al. 2008; Bergland et al. 2014; Reinhardt et al. 2014;
108 Machado et al. 2018). Our analysis based on SNP F_{ST} outliers uncovered pervasive
109 genome-wide patterns of clinality, with hundreds of clinally varying SNPs mapping to
110 loci involved in the insulin/insulin-like growth factor signaling (IIS)/target of rapamycin
111 (TOR), ecdysone, torso, EGFR, TGFβ/BMP, JAK/STAT, lipid metabolism, immunity
112 and circadian rhythm pathways (Fabian et al. 2012). Many of the identified variants
113 also exhibit parallel differentiation in Australia (Fabian et al. 2012; Kapun et al.
114 2016b; also cf. Kolaczkowski et al. 2011; Reinhardt et al. 2014; Machado et al.
115 2016), thereby strengthening the case for clinal adaptation. However, while many
116 clinal variants might be shaped by selection, some of the observed differentiation
117 might be due to non-adaptive factors, including population structure, demography,
118 admixture or hitchhiking with causative sites (Endler 1977; Duchen et al. 2013; Kao
119 et al. 2015; Bergland et al. 2016). Unambiguously identifying adaptive clinal variants
120 as causal targets of selection thus requires comparing clinal patterns against neutral
121 expectations and – optimally – functional genetic testing (Barrett and Hoekstra 2011;
122 Kapun et al. 2016a, 2016b; Flatt 2016). To date, however, functional analyses and
123 experimental confirmations of clinal polymorphisms that are potentially subject to
124 spatially varying selection remain scarce (for some exceptions see e.g. Schmidt et al.
125 2008; McKechnie et al. 2010; Paaby et al. 2010; Lee et al. 2013; Paaby et al. 2014;
126 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 *14-3-3*ε, *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

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

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

172 From our previous population genomic data based on three population along the
173 North American cline (Fabian et al. 2012) we identified two strongly clinally varying
174 alternative *foxo* alleles, as defined by 2 focal SNPs, whose frequencies change
175 across latitude by ~60% between Florida and Maine (this paper; also see analyses in
176 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

202 expected phenotypic effects of the *foxo* variant in terms of clinality, plasticity and the
203 physiology of IIS in the Methods section below. In brief, our results show that the *foxo*
204 polymorphism affects multiple components of fitness according to these predictions,
205 in particular the clinality of size-related traits; we also observe that two alternative
206 *foxo* alleles respond plastically to changes in temperature and diet but overall we find
207 little evidence for G x E interactions.

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

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

219

220 *Methods*

221 IDENTIFICATION AND ISOLATION OF THE *FOXO* POLYMORPHISM

222 We identified two strongly clinal SNPs in *foxo* in the genomic data of Fabian et al.
223 (2012) by using an F_{ST} outlier approach: an A/G polymorphism at position *3R*:
224 9892517 (position in the *D. melanogaster* reference genome v.5.0; F_{ST} = 0.48
225 between Florida and Maine) and a T/G polymorphism at position *3R*: 9894559 (F_{ST} =
226 0.42 between Florida and Maine) (Fig. S1A, Supporting Information; cf. Fabian et al.

227 2012 for details of outlier detection; also see Betancourt et al. 2018). The A/G
228 polymorphism is a synonymous coding SNP, predicted to be located in the PEST
229 region of the FOXO protein, which serves as a protein degradation signal (analysis
230 with ExPASy [Artimo et al. 2012]; Fig. S2, Supporting Information). The T/G SNP is
231 located in the first intron of *foxo*, with no biological function attributed to this position
232 (Attrill et al. 2016).

233 While our initial identification of these SNPs was based on only three populations
234 (Florida, Pennsylvania, and Maine; see Fabian et al. 2012 for details), both SNPs are
235 also strongly clinal in a more comprehensive dataset based on 10 populations along
236 the cline, analyzed in the companion study by Betancourt et al. (2018) and collected
237 by the *Drosophila* Real Time Evolution Consortium (DrosRTEC; Bergland et al. 2014;
238 Kapun et al. 2016b; Machado et al. 2018). The frequency of the high-latitude [HL]
239 allele (A, T) for this 2-SNP variant ranges from ~10% in Florida to ~70% in Maine;
240 conversely, the alternative low-latitude [LL] allele (G,G) is prevalent in Florida but at
241 low frequency in Maine (Fig. S1A, Supporting Information). Because the two *foxo*
242 SNPs are located relatively closely to each other (~2 kb apart; Fig. S1A, Supporting
243 Information), we decided to study them experimentally in combination, as alternative
244 2-SNP alleles. Indeed, as shown in Fig. S1B (Supporting Information), the two focal
245 *foxo* SNPs are in perfect linkage disequilibrium (LD; $r^2 = 1$), without any significant LD
246 in-between the two sites. Importantly, our population genomic analyses in Betancourt
247 et al. (2018) suggest that this polymorphism exhibits much stronger clinality than the
248 majority of other *foxo* SNPs and that it is likely maintained non-neutrally by spatially
249 varying selection when compared to a genome-wide panel of 20,000 SNPs in short
250 introns that presumably evolve neutrally.

251 To isolate the two alternative *foxo* alleles for experiments we used whole-genome
252 sequenced inbred lines from the *Drosophila* Genetic Reference Panel (DGRP;
253 Mackay et al. 2012) to reconstitute outbred populations either fixed for the LL (G,G)
254 and the HL (A,T) alleles. This 'reconstituted or recombinant outbred population'
255 (ROP) or 'Mendelian randomization' approach produces populations that are
256 consistently and completely fixed for the two alternative allelic states to be compared,
257 with the rest of the genetic background being randomized (see Behrman et al. 2018
258 and Lafuente et al. 2018 for recent examples using this method). For each allele we
259 used two independent sets of DGRP lines (sets A and B for HL; sets C and D for LL;
260 each set consisting of 20 distinct lines) and two replicate population cages per set,
261 giving a total of 8 population cages (Fig. S3, Table S1, Supporting Information). ROP
262 cages were established from the DGRP lines at the University of Pennsylvania in
263 Philadelphia (Betancourt et al. 2018); F2 flies from these cages were transferred to
264 the University of Lausanne for establishing population cages at our laboratory (see
265 below).
266 By analyzing the genomes of the DGRP lines used to set up the experimental
267 populations we confirmed in Betancourt et al. (2018) that sets A and B versus sets C
268 and D were completely fixed ($F_{ST} = 1$) for the HL and LL alleles, respectively. This
269 analysis also showed that there was no systematic genomic differentiation (F_{ST}) in
270 the genome-wide background of the two focal SNPs: even though there exist other
271 SNPs that are strongly differentiated ($F_{ST} > 0.5$) between the HL and LL populations,
272 the majority of these SNPs are different between the independently replicated sets
273 (blocks) of DGRP lines that were used to make the HL vs. LL contrast. Thus, strongly
274 differentiated SNPs that are specific ('private') to a given set of lines do not make a
275 consistent contribution to the overall HL vs. LL contrast.

276 The most parsimonious interpretation of our results is therefore that the effects
277 reported below are caused by the two *foxo* SNPs which we have studied. However,
278 we cannot completely rule out that other (causative) sites are potentially in long-
279 range LD with our focal SNPs (see Fig. S1B, Supporting Information). A conservative
280 interpretation of our results is thus to view the two focal *foxo* SNPs as representing
281 'tags' or markers for functionally significant variants segregating at the *foxo* locus that
282 are in LD with the causative site(s), similar to those used in genome-wide association
283 studies (GWAS; e.g., Wang et al. 2010).
284
285 **POPULATION CAGES**
286 Population cages in our laboratory in Lausanne were maintained at 25ºC, 12:12 h
287 light:dark, 60% relative air humidity and controlled larval density. Larval density was
288 kept constant via egg collections (200-300 eggs per bottle [6 oz. = 177 mL]; 10
289 bottles per cage), with eclosing adults being released into cages (17.5 x 17.5 x 17.5
290 cm; BugDorm®) at a density of ~2000-2500 adults per cage. Prior to the phenotypic
291 assays population cages were kept for 10 generations to allow for free recombination
292 among lines within each cage and allelic state and to homogenize (randomize)
293 differences in genomic background between the two allelic states to be compared.
294 Before setting up assays, we kept cages for 2 generations under common garden
295 conditions (room temperature: ~22ºC, ~10:14 h light:dark, ~50% humidity). Thus,
296 phenotypes were measured after a total of 12 generations of recombination.
297
298 **PHENOTYPE ASSAYS**
299 All assays reported here were performed in our previous laboratory in Lausanne; in
300 our companion study we report independent assays performed under constant

301 environmental conditions in Philadelphia (Betancourt et al. 2018), allowing us to
302 assess the reproducibility of the allelic effects and to account for potential variation in
303 life-history traits due to potential differences in local laboratory assay conditions (cf.
304 Ackermann et al. 2001).

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

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

324 Body size was examined by measuring three proxies: wing area, thorax length and
325 femur length (*N* = 26-30 wings, 9-15 thoraces, and 19-21 femurs per cage, treatment,

326 and sex). Right wings and femurs were mounted on slides with CC/Mount™ tissue
327 mounting medium (Sigma Aldrich) and slides sealed with cover slips. Thorax length
328 was defined as the lateral distance between the upper tip of the thorax and the end of
329 the scutellar plate (*N* = 10-15 individuals per cage, treatment, and sex). Images for
330 morphometric measurements were taken with a digital camera (Leica DFC 290)
331 attached to a stereo dissecting microscope (Leica MZ 125; Leica Microsystems
332 GmbH, Wetzlar, Germany). We used ImageJ software (v.1.47) to measure femur and
333 thorax length (mm) and to define landmarks for calculating wing area (mm^2). To
334 measure wing area we defined 12 landmarks located at various vein intersections
335 along the wing; the total area encompassed by these landmarks was estimated using
336 a custom-made Python script (available upon request). In brief, we split the polygon
337 defined by the landmarks up into triangles and summed across their areas (Fig. S4,
338 Supporting Information). Thorax and femur (but not wing area) measurements were
339 repeated three times per individual (see below for estimates of 'repeatability'). From
340 these data, we calculated the ratio of wing area:thorax length, which is inversely
341 related to ‘wing loading’ (Azevedo et al. 1998; Gilchrist et al. 2000); reduced wing
342 loading (i.e., increased wing dimensions relative to body size) can improve flight
343 performance at low temperature (Frazier et al. 2008).
344 To measure starvation resistance (i.e., survival upon starvation) we placed flies
345 into vials containing 0.5% agar/water medium and scored the duration of survival (h)
346 upon starvation every 6 h until all flies had died (*N* = 5 vials × 10 flies per vial × 2
347 sexes × 8 cages × 4 conditions = 320 vials or 3200 flies).
348 Since there is typically a positive correlation between starvation resistance and
349 lipid content (Hoffmann and Harshman 1999), we also determined whole-body
350 triacylglyceride (TAG) content (in *μ*g per fly) using a serum triglyceride determination

351 kit (Sigma Aldrich; Tennessen et al. 2014). For each cage and treatment, triglyceride
352 content was estimated from 5-7-day-old females, either kept under fed or starved (24
353 h) conditions, by preparing 10 replicate homogenates, each made from 2 flies (8
354 cages × 4 conditions × 2 treatments × 10 replicates = 640 homogenates). To
355 estimate fat loss upon starvation we calculated the difference between fat content
356 under fed versus starved conditions, using treatment (fed vs. starved) means from
357 each population cage (mean fat loss per fly, in *μ*g).
358
359 **QRT-PCR ANALYSIS OF INSULIN SIGNALING STATE**
360 A well-established transcriptional read-out of FOXO signaling is the insulin-like
361 receptor InR: under conditions of high insulin (e.g., after a meal), InR synthesis is
362 repressed by a feedback mechanism controlled by FOXO; conversely, under
363 conditions of low insulin, activation of FOXO leads to upregulation of *InR* mRNA
364 (Puig et al. 2003; Puig and Tjian 2005). To test whether the *foxo* alleles differ in IIS
365 state we performed qRT-PCR, measuring *InR* mRNA abundance. For each cage and
366 treatment, we extracted total RNA from 5-7-day-old snap-frozen females in triplicate,
367 with each replicate prepared from 5 flies. RNA was extracted with the RNeasy kit
368 (Qiagen) and reverse transcribed with the GoScript Reverse Transcription System
369 (Promega). From each triplicate biological sample we prepared 3 technical replicates
370 (8 cages × 4 conditions × 3 biological replicates × 3 technical replicates = 288
371 samples). Relative transcript abundance was normalized by using *Actin5C* as an
372 endogenous control (Ponton et al. 2011). qRT-PCR was carried out using a
373 QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) and SYBR Green
374 GoTaq qPCR Master Mix (Promega). Thermal cycling was conducted at 95°C for 2
375 min, followed by 42 cycles of amplification at 95°C for 15 s and 60°C for 1 min, and

376 using the following melting curve: 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s.
377 Quantification of relative abundance for each sample was based on the Δ*CT* method.
378 We used the following primer sequences (Casas-Tinto et al. 2007; Ponton et al.
379 2011): *Actin5C forward*, 5'-GCGTCGGTCAATTCAATCTT-3'; *Actin5C reverse*, 5'-
380 AAGCTGCAACCTCTTCGTCA-3'; *InR forward*, 5'-CACAAGCTGGAAAGAAAGTGC-
381 3'; *InR reverse*, 5'- CAAACACGTTTCGATAATATTTTTCT-3'.
382
383 **STATISTICAL ANALYSIS**
384 Analyses were performed with JMP (SAS, Raleigh, NC, USA; v.11.1.1). Data were
385 analyzed with analysis of variance (ANOVA), testing the fixed effects of allele (*A*; HL
386 vs. LL), temperature (*T*; 18°C vs. 25°C), diet (*D*; sucrose vs. molasses), set (*S*;
387 independent blocks of DGRP lines) nested within *A*, replicate cage (*C*) nested within
388 the combination of *A* and *S*, and all 2- and 3-way interactions: $y = A + T + D + A \times T$
389 $+ A \times D + T \times D + A \times T \times D + S(A) + C(A,S)$, where *y* denotes the response variable
390 (trait). For simplicity, the sexes were analyzed separately (i.e., to reduce the number
391 of higher-order interactions).
392 For starvation resistance we measured age at death from multiple individuals per
393 replicate vial; we thus estimated and accounted for the random effect of vial (*V*),
394 nested within the combination of *A*, *S* and *C*, using restricted maximum likelihood
395 (REML) (see Supporting Information for these estimates).
396 Viability (proportion) data were arcsine square-root transformed prior to analysis.
397 ANOVA on thorax and femur length data was performed using means across 3
398 measures per individual. From the repeat measurements of these traits on the same
399 individuals, we estimated the 'repeatability' of our measurements (i.e., the intraclass
400 correlation; see Whitlock and Schluter 2009) by performing random-effects ANOVAs

401 with REML. Overall, repeatibility was very high for femur length (~91.9% for females;
402 94.4% for males) but less so for thorax length (~29.9% for females; 36.6% for males)
403 (details not shown). Because wings and thoraces were measured on separate
404 individuals, analysis of wing:thorax ratio was performed on population (cage) means.
405 For fat content, we included the fixed effect of starvation treatment (*Tr*; fed vs.
406 starved); interactions involving *A* and *Tr* (i.e., $A \times Tr$; $A \times D \times Tr$) test for allelic
407 differences in fat loss upon starvation. For simplicity, this analysis was performed
408 separately for the two rearing temperatures.
409 To estimate the magnitude of the allelic effects of the *foxo* polymorphism upon the
410 assayed fitness components we calculated Cohen's *d* (Table S3, Supporting
411 Information), a standardized measure of effect size (i.e., a signal to noise ratio,
412 defined as the difference between two means divided by their pooled standard
413 deviation) (Cohen 1988; Sawilowsky 2009). Low values of Cohen's *d* (e.g., 0.01) are
414 commonly interpreted as representing very small effect sizes, whereas effect sizes
415 >0.8 are interpreted as being qualitatively large to very large (Sawilowsky 2009).
416 We also estimated the relative contribution of the clinal *foxo* polymorphism to the
417 overall phenotypic cline for wing area, a trait which we have recently measured
418 across 6 populations along the North American east coast (Betancourt et al. 2018).
419 This analysis was performed for flies raised on a molasses diet at 25°C, i.e. using
420 similar assay conditions as those used by Betancourt et al. (2018) for clinal wing area
421 measurements. We calculated the proportional contribution of the *foxo* polymorphism
422 to the overall cline as follows: $\Delta_{foxo} \times \Delta_{\text{frequency}} / \Delta_{\text{cline}}$, where Δ_{foxo} is the difference in
423 mean wing area between the HL and LL allelic states, $\Delta_{\text{frequency}}$ is the allele frequency
424 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 $\times$ 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.

499 2008; but see van ‘t Land et al. 1999). Diet – but not temperature – also had an
500 effect, with viability being higher on sucrose than on molasses diet (Fig. 2; Table 1).
501 We did not find any evidence for G × E interactions affecting this trait.
502
503 **CLINAL *FOXO* ALLELES DIFFER IN BODY SIZE**
504 Since both latitude and IIS affect size (de Jong and Bochdanovits 2003), we next
505 examined three proxies of body size (wing area, thorax and femur length). The HL
506 allele conferred larger femur length (Fig. 3; Table 1; in females but not males), wing
507 area (Fig. S5; Table S4, Supporting Information), and wing:thorax ratio than the LL
508 allele (Fig. 4; Table 1; for thorax data see Fig. S6; Table S4, Supporting Information).
509 These results are consistent with the positive size cline in North America (Coyne and
510 Beecham 1987; Betancourt et al. 2018) and with reduced wing loading at high
511 latitude (Azevedo et al. 1998; Bhan et al. 2014). Remarkably, with regard to wing
512 area, we estimate that the *foxo* polymorphism as measured in our experiments
513 makes a proportional contribution of ~14% to the total cline for wing area as
514 measured by Betancourt et al. (2018) (females: $\Delta_{foxo} \times \Delta_{\text{frequency}} / \Delta_{\text{cline}} \approx 0.017 \times 0.6 /$
515 $0.074 \approx 0.138$; males: $\Delta_{foxo} \times \Delta_{\text{frequency}} / \Delta_{\text{cline}} \approx 0.019 \times 0.6 / 0.083 \approx 0.137$) – this
516 represents a major contribution to the wing size cline along the North American east
517 coast (see Coyne and Beecham 1987; Betancourt et al. 2018).
518 For all size traits, females were larger than males (Fig. 3; Fig. 4; Table 1; Fig. S5;
519 Fig. S6; Table S4, Supporting Information), as is typically observed. With regard to
520 the plastic effects of temperature, femur length, thorax length and wing area were
521 larger at 18°C than at 25°C (Fig. 3; Fig. S5, Fig. S6, Supporting Information; Table 1;
522 Table S4, Supporting Information), as is expected based on previous work (David et
523 al. 1994; Partridge et al. 1994a). In terms of dietary plasticity, femur and thorax

524 length were larger on sucrose than on molasses diet (Fig. 3; Table 1; Fig. S6; Table
525 S4, Supporting Information), perhaps in line with the observation that more
526 carbohydrate-rich diets cause smaller size (Reis 2016); however, wing area and
527 wing:thorax ratio were larger on molasses than on sucrose diet (Fig. S5; Table S4,
528 Supporting Information; and Fig. 4; Table 1). Although we found a few G × E
529 interactions for size traits (Fig. 4; Fig. 5; Table 1; Fig. S5; Fig. S6; Table S4,
530 Supporting Information), the allelic reaction norms were overall remarkably parallel
531 across environmental conditions.
532
533 **POLYMORPHISM AT *FOXO* IMPACTS STARVATION AND FAT CATABOLISM**
534 The *foxo* alleles also differed in their effects on resistance to (survival of) starvation in
535 females (Fig. 5; Table 1), as might be expected based on the observation that *foxo*
536 mutants are more starvation sensitive than wildtype (Jünger et al. 2003; Kramer et al.
537 2003, 2008). However, contrary to clinal predictions (Schmidt and Paaby 2008;
538 Mathur and Schmidt 2017; Betancourt et al. 2018), LL females were more resistant
539 than HL females (Fig. 5; Table 1), suggesting a countergradient effect; in males,
540 there were no allelic differences in resistance (Fig. S7; Table S4, Supporting
541 Information; for estimates of the variance components of the random effect of vial
542 see Table S5, Supporting Information). Overall females were more resistant than
543 males (Fig. 5; Table 1; Fig. S7; Table S4, Supporting Information), consistent with
544 some but not other studies (Goenaga et al. 2010; but see Matzkin et al. 2009). For
545 both females and males, starvation resistance was higher at 18°C than at 25°C (Fig.
546 5; Table 1; Fig. S7; Table S4, Supporting Information), as previously reported
547 (Mathur and Schmidt 2017). Flies raised on molasses diet were more resistant than
548 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-

598 wide analyses of variation in size-related traits have identified novel regulators of
599 growth, several of which interact with the IIS/TOR pathway (Vonesch et al. 2016;
600 Strassburger et al. 2017). For example, in support of the idea that variation in IIS
601 contributes to clinal adaptation in *D. melanogaster,* Paaby and colleagues have
602 identified a clinal indel polymorphism in *InR* with pleiotropic effects on development,
603 body size, fecundity, lifespan, oxidative stress resistance, chill coma recovery, and
604 insulin signaling (Paaby et al. 2010, 2014). Our results on *foxo* lend further support to
605 the hypothesis of de Jong and Bochdanovits (2003).
606
607 **THE EFFECTS OF NATURAL VERSUS NULL ALLELES AT THE *FOXO* LOCUS**
608 Previous work with loss-of-function mutants and transgenes has uncovered a major
609 role of *foxo* in the regulation of growth, lifespan and resistance to starvation and
610 oxidative stress (Jünger et al. 2003; Puig et al. 2003; Kramer et al. 2003; Giannakou
611 et al. 2004; Hwangbo et al. 2004; Kramer et al. 2008; Slack et al. 2011), but nothing
612 is known yet about the effects of natural alleles at this locus. An important distinction
613 in this context is that null mutants, by definition, reveal the complete set of functions
614 and phenotypes of a given gene and may therefore be highly pleiotropic, whereas
615 'evolutionarily relevant' mutations or alleles might have much more subtle effects,
616 with little or no pleiotropy (Stern 2000). Based on our knowledge of the traits affected
617 by *foxo* in null mutants and transgenes (Jünger et al. 2003; Kramer et al. 2003, 2008;
618 Slack et al. 2011), we measured how the clinal 2-SNP variant affects size traits and
619 starvation resistance.
620 Although we could not predict with certainty the directionality and/or the degree of
621 pleiotropy of the allelic effects *a priori*, we found that the *foxo* polymorphism
622 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

648 references above) and might support the idea that the architecture of life-history
649 traits, which are connected via multiple trade-offs, is inherently pleiotropic (Williams
650 1957; Finch and Rose 1995; Flatt et al. 2005; Flatt and Promislow 2007; Flatt and
651 Schmidt 2009; Flatt et al. 2013; Paaby et al. 2014); it also provides a contrast to the
652 model from evo-devo which posits that most evolutionarily relevant mutations should
653 exhibit little or no pleiotropy (Stern 2011). In particular, the pleiotropic effects of the
654 *foxo* variant might explain why this polymorphism might be maintained, through some
655 form of balancing selection, in natural populations along the cline.
656

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

658 How does the *foxo* variant contribute to phenotypic clines observed across latitude?
659 High-latitude flies tend to be characterized, for example, by larger body size,
660 decreased fecundity, longer lifespan and improved stress resistance as compared to
661 low-latitude flies, and this differentiation is genetically based (Coyne and Beecham
662 1987; Schmidt et al. 2005a, 2005b; Schmidt and Paaby 2008; Mathur and Schmidt
663 2017; Durmaz et al. 2018). Do the allelic effects go in the same direction as the
664 latitudinal gradient, representing cogradient variation, or do certain allelic effects run
665 counter to the cline, representing countergradient variation (Levins 1968; Conover
666 and Schultz 1995)? Cogradient variation occurs when diversifying selection favors
667 different traits in different environments, as expected from selection along a cline,
668 whereas countergradient variation occurs when stabilizing selection favors similar
669 traits in different environments (Conover and Schultz 1995; Marcil et al. 2006).
670 Consistent with clinal expectation, the HL allele confers larger size (Coyne and
671 Beecham 1987; de Jong and Bochdanovits 2003); increased wing:thorax ratio, which
672 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

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

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1319

1320 **DATA ACCESSIBILITY**

1321 Phenotypic raw data are available from Dryad at doi to be added upon acceptance.

1322

1323 **AUTHOR CONTRIBUTIONS**

1324 T.F. and P.S. conceived the project. D.F. and M.K. identified the *foxo* SNPs and
1325 performed genomic analyses. T.F., P.S., E.D. and S.R. designed the experiments.
1326 SR and NB established reconstituted outbred populations. E.D., S.R. and N.B.
1327 performed the experiments. E.D., N.B., P.S. and T.F. analyzed the data. E.D., P.S.
1328 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

1360 **Table 2.** ANOVA results for female fat loss upon starvation. *$p < 0.05$; **$p < 0.01$;

1361 ***$p < 0.001$. The fixed factor 'Treatment' has two levels: fed vs. starved; interactions

1362 involving the factors 'Allele' and 'Treatment' test for allelic differences in fat

1363 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

1364

1365 **FIGURE CAPTIONS**

1366 **Figure 1.** Clinal candidates in the insulin/TOR signaling pathway. Overview of the
1367 insulin/insulin-like growth factor signaling (IIS)/target of rapamycin (TOR) pathway in
1368 *Drosophila melanogaster* (Oldham and Hafen 2003; Giannakou and Partridge 2007;
1369 Teleman 2010). Genes that harbor strongly clinally varying SNPs across latitude,
1370 identified by Fabian et al. (2012), are highlighted in red; arrows indicate activation
1371 and bar-ended lines represent inhibitory effects. In response to nutrients, IIS is
1372 activated by binding of ligands, called *Drosophila* insulin-like peptides (dilps 1-8), to
1373 the insulin-like receptor (InR) at the cell membrane. Inside the cell, signaling is
1374 transduced by an insulin receptor substrate (IRS) protein called chico. This activates
1375 phosphoinositide-3-kinase (PI3K) which converts phosphatidylinositol (3,4)-
1376 bisphosphate (PIP2) into phosphatidylinositol (3,4,5)-trisphosphate (PIP3). In turn,
1377 PIP3 stimulates pyruvate dehydrogenase kinase (PDK) and activates protein kinase
1378 B (AKT/PKB). The action of PI3K is antagonized by phosphatase and tensin
1379 homologue (PTEN) which converts PIP3 back to PIP2. AKT/PKB suppresses the
1380 forkhead (FKH) box O transcription factor FOXO by phosphorylating it; upon reduced
1381 IIS, FOXO becomes dephosphorylated and moves into the nucleus where it
1382 regulates the expression of hundreds of target genes. Target genes of FOXO include
1383 *InR*, controlled via a transcriptional feedback loop, and *initiation factor 4E-binding*
1384 *protein* (*4E-BP*); another target gene of IIS is *target of brain insulin* (*Tobi*), which
1385 encodes a glucosidase, but the details of its regulation remain poorly understood.
1386 FOXO is antagonized by 14-3-3ε. AKT/PKB antagonizes the activity of the tuberous
1387 sclerosis complex 1/2 (TSC1/TSC2); TSC1/2 in turn inactivates RAS homologue
1388 enriched in brain (RHEB). The inactivation of RHEB disinhibits, i.e. activates, target
1389 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.

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1416 **Figure 5,** Starvation resistance. Effects of the clinal *foxo* polymorphism on the
1417 duration of survival (in hrs) upon starvation in females. (A) Dietary reaction norms at
1418 18°C. (B) Dietary reaction norms at 25°C. (C) Thermal reaction norms measured on
1419 sucrose diet. (D) Thermal reaction norms measured on molasses diet. Data in (A, B)
1420 are the same as those shown in (C, D). Shown are means and standard errors. Red
1421 lines: low-latitude (LL) allele, blue lines: high-latitude (HL) allele.

1422

1423 **Figure 6.** Fat loss upon starvation. Effects of the clinal *foxo* variant on female
1424 triglyceride loss upon starvation (*μg*/fly). (A) Dietary reaction norms at 18°C. (B)
1425 Dietary reaction norms at 25°C. (C) Thermal reaction norms measured on sucrose
1426 diet. (D) Thermal reaction norms measured on molasses diet. Data in (A, B) are the
1427 same as those shown in (C, D). Shown are means and (propagated) standard errors.
1428 Red lines: low-latitude (LL) allele, blue lines: high-latitude (HL) allele.

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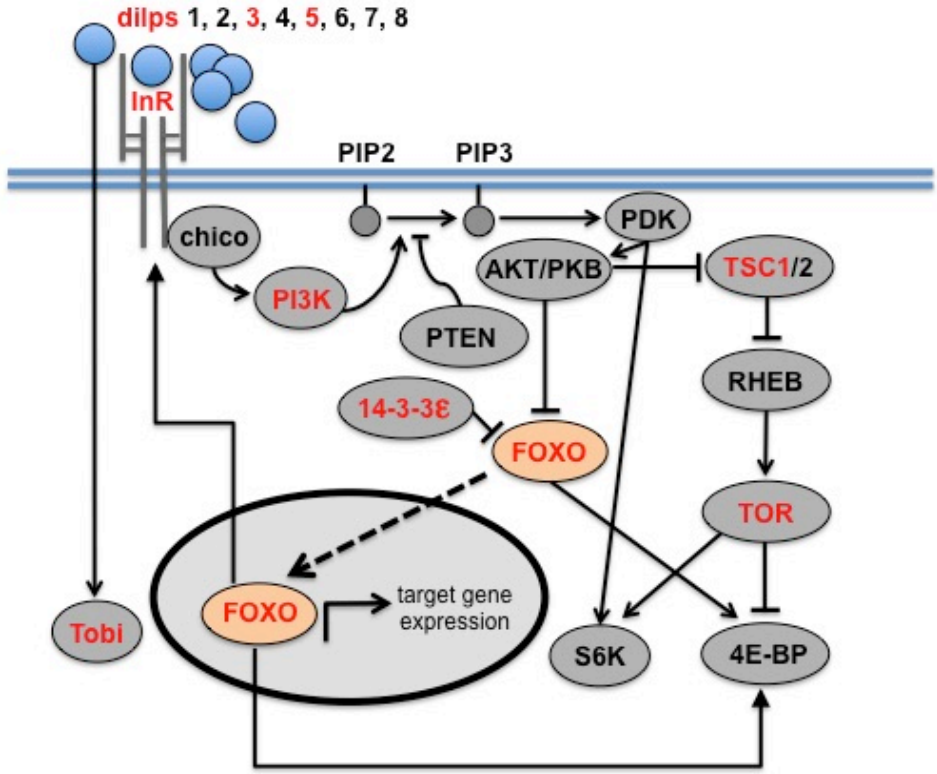
1430 **Figure 1**



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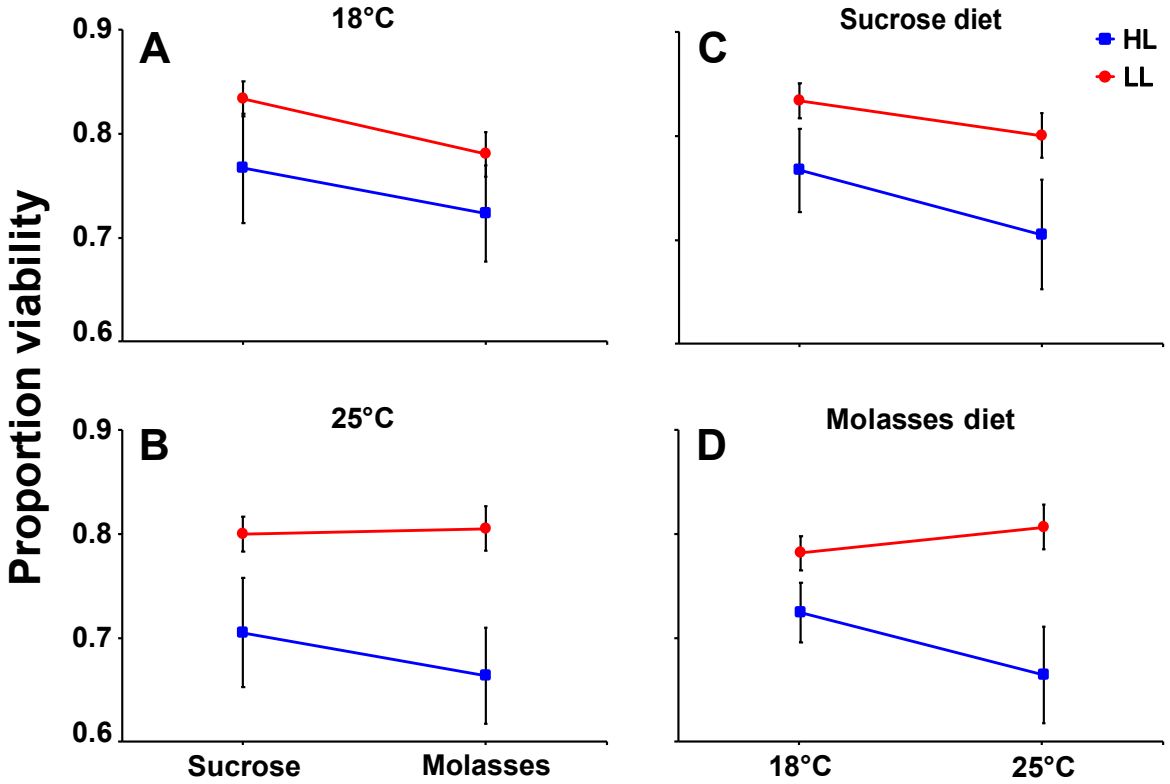
1433 **Figure 2**



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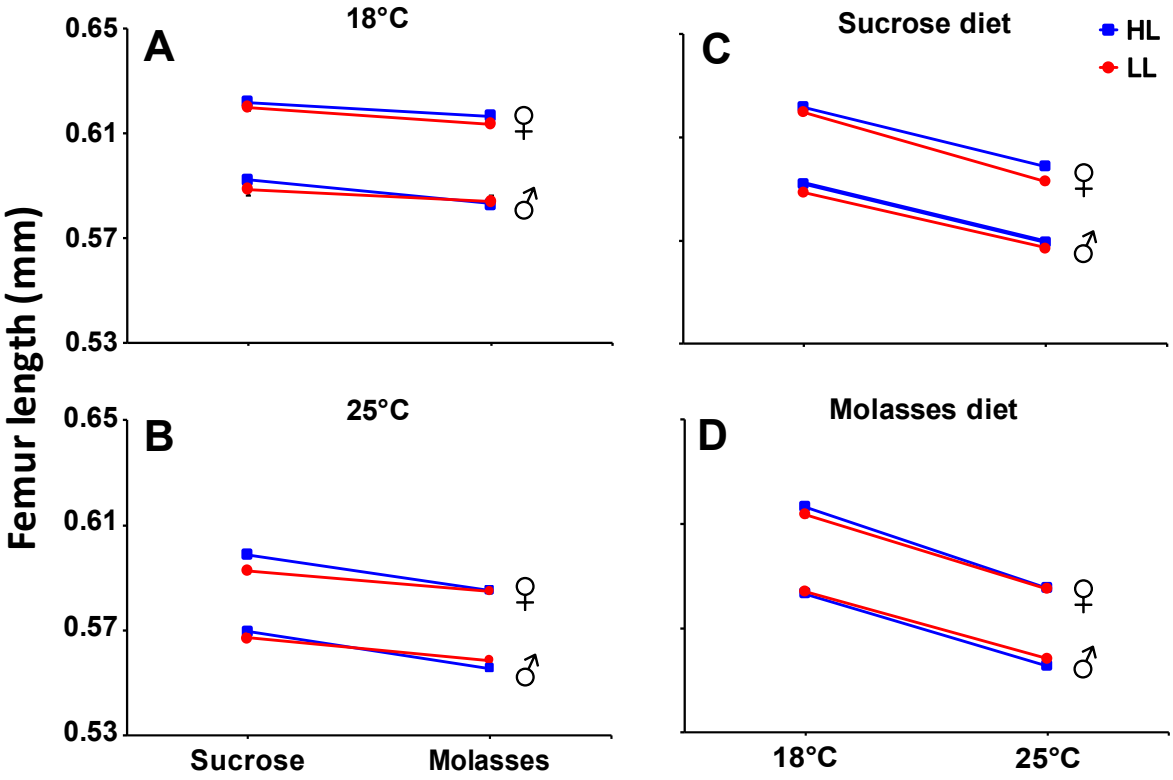
1435 **Figure 3**



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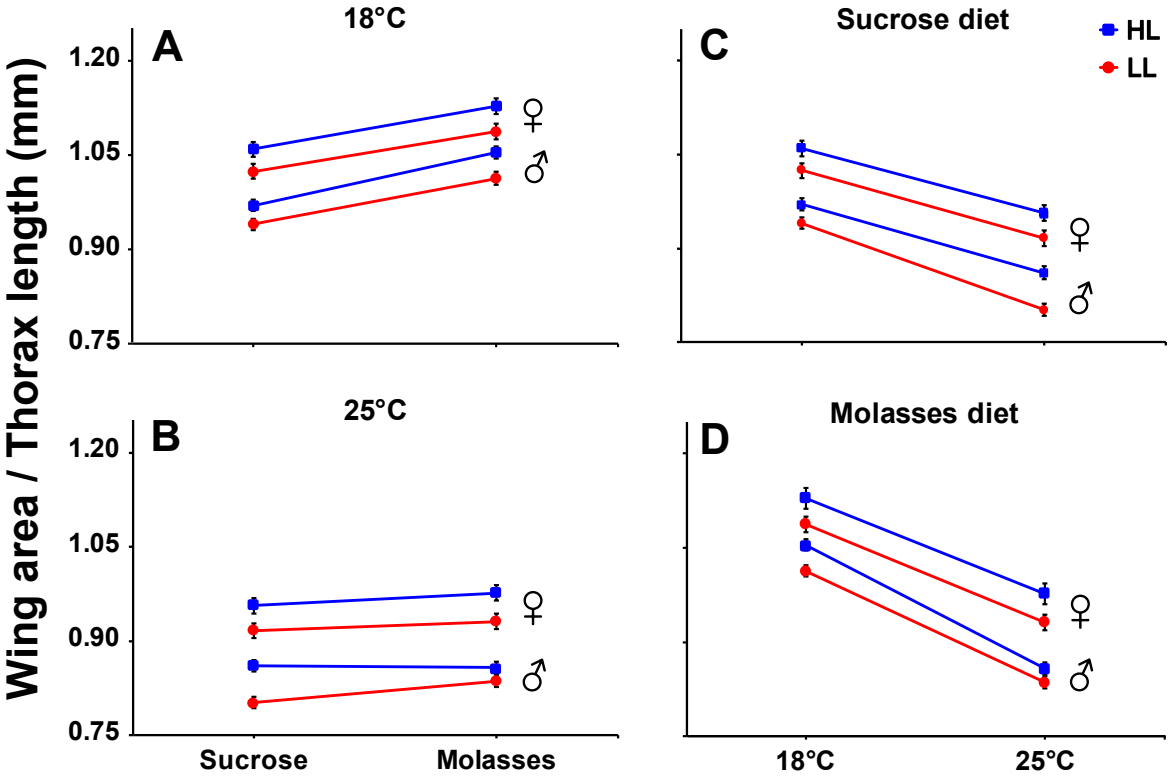
1438 **Figure 4**



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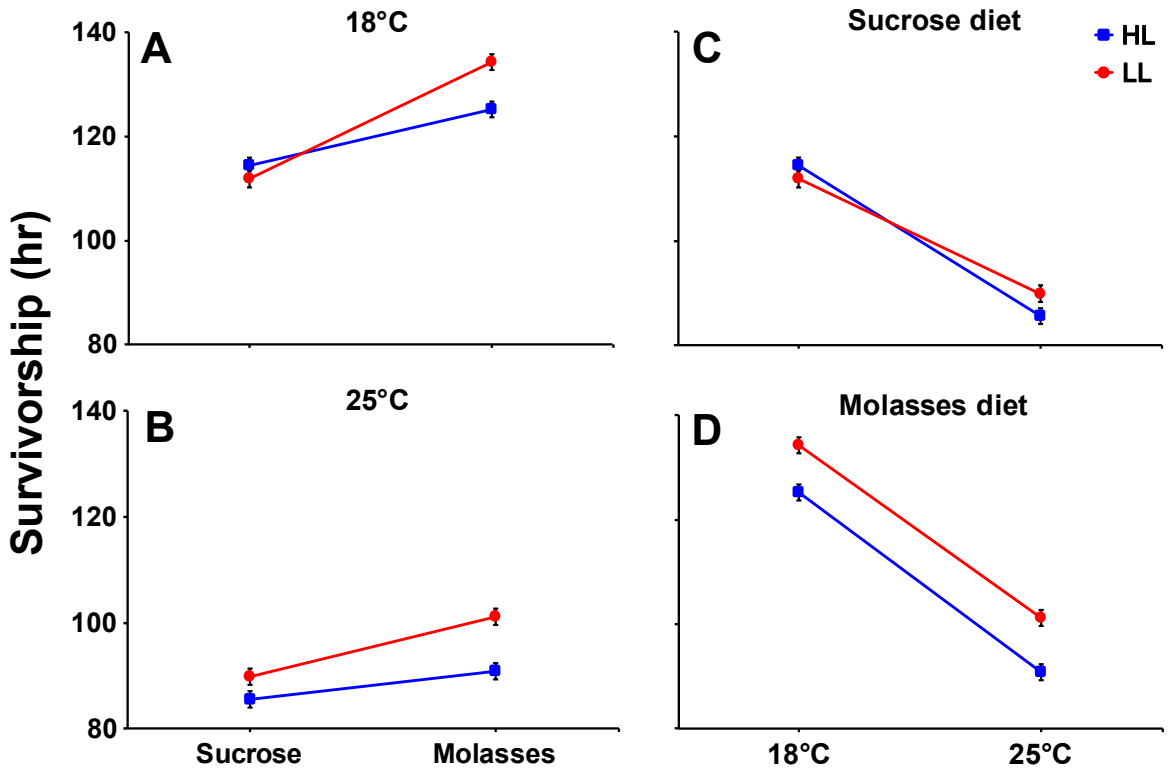
1441 **Figure 5**



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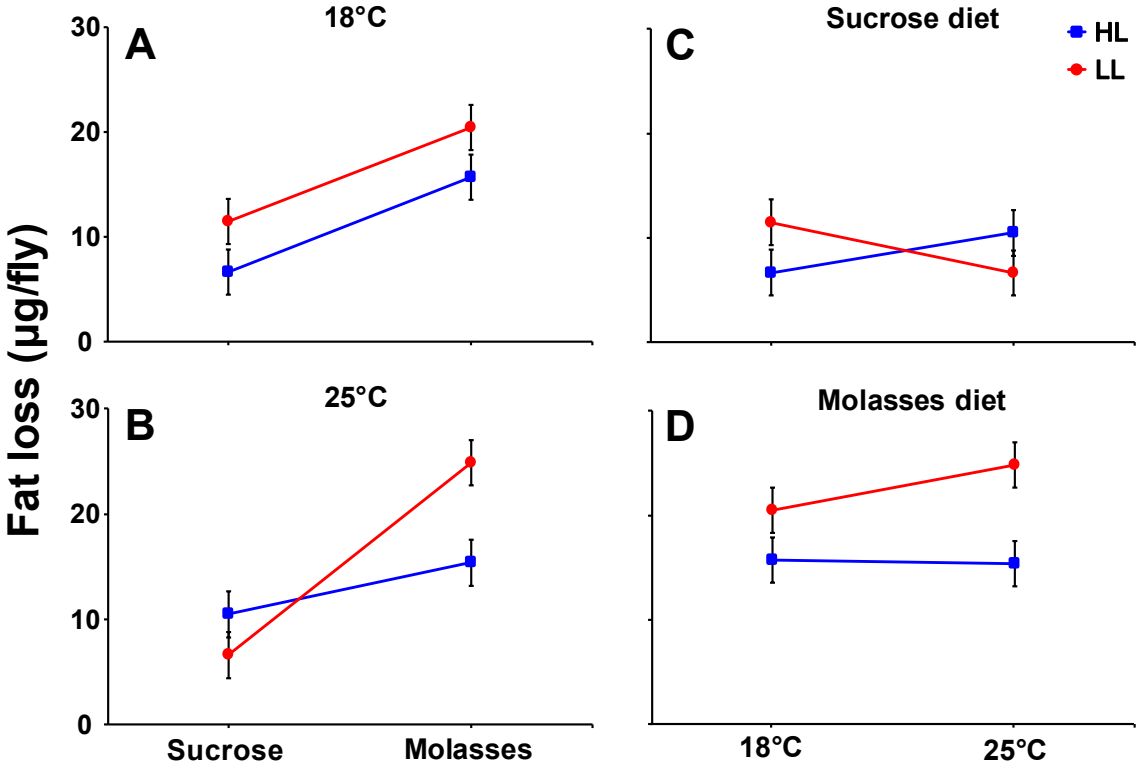
1444 **Figure 6**



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1458 **Supporting Information**

1459

1460 **Figure S1.** Clinal *foxo* candidate SNPs. (A) Allele frequencies of clinal *foxo* SNPs in
1461 Florida (red), Pennsylvania (green) and Maine (blue), identified by Fabian et al.
1462 (2012) and conditioned to raise in frequency from Florida to Maine. The two strongly
1463 clinal *foxo* SNPs studied here are marked with star symbols. Note that the SNP in-
1464 between the two focal SNPs is much less strongly clinal, with a much higher
1465 frequency in Florida than the 2 candidate SNPs. The x-axis shows the genomic
1466 position of the SNPs on chromosome *3R* in million base pairs (Mbp). The plot
1467 underneath the x-axis shows the gene model for *foxo*. (B) Linkage disequilibrium (LD;
1468 as measured by pairwise r^2) among all polymorphic *foxo* SNPs (minor allele
1469 frequency ≥ 0.1) in the DGRP lines used to set up experimental populations (see
1470 Materials and Methods section). The two focal SNPs are in perfect LD in the
1471 experimental populations (r^2 =1), but there is no significant LD among other, non-
1472 focal sites. Nonetheless, we cannot rule out with certainty that other SNPs are in LD
1473 with our two focal SNPs; a cautious interpretation would thus be to view our focal
1474 SNPs as representing "tag SNPs". Also see Fig. S3; also see analyses in Betancourt
1475 et al. (2018).

1476

1477 **Figure S2.** PEST motif prediction for FOXO. The T/G polymorphism in *foxo* at
1478 position *3R*: 9894559, is predicted to be located in the PEST region of the FOXO
1479 protein (analysis of *foxo* sequence using ExPASy [Artimo et al., 2012]); PEST motifs
1480 serve as protein degradation signals (Artimo et al., 2012). The potential PEST motif
1481 (RPENFVEPTDELDSTK) between amino acid positions 49 and 64 (shown in green)
1482 encompasses the *foxo* SNP at position 51 (E = glutamic acid).

1483

1484 **Figure S3.** Experimental design for reconstituted outbred *foxo* populations. We
1485 isolated the 2-SNP *foxo* variant by reconstituting outbred populations, fixed for either
1486 the low- or high-latitude allele, from lines of the *Drosophila* Genetic Reference Panel
1487 (DGRP). Each *foxo* allele was represented by two independent sets of distinct DGRP
1488 lines, with two replicate cages per set. See Materials and Methods section for details;
1489 also see Fig. S1B; also see analyses in Betancourt et al. (2018).

1490

1491 **Figure S4.** Coordinates of landmarks used to estimate wing area. We calculated the
1492 total wing area encompassed by 12 landmarks (in yellow) by splitting the polygon up
1493 into triangles (shown in different colors) and by summing across the areas defined by
1494 these triangles. See Materials and Methods section for details.

1495

1496 **Figure S5.** Effects of the *foxo* variant on total wing area. Effects of the clinal *foxo*
1497 variant on wing area (mm^2) in females and males. (A) Dietary reaction norms at
1498 18°C. (B) Dietary reaction norms at 25°C. (C) Thermal reaction norms on sucrose
1499 diet. (D) Thermal reaction norms on molasses diet. Shown are means and standard
1500 errors. Red lines: low-latitude (LL) allele, blue lines: high-latitude (HL) allele. See
1501 Results section for details.

1502

1503 **Figure S6**. Effects of the *foxo* variant on thorax length. Effects of the clinal *foxo*
1504 variant on thorax length (mm) in females and males. (A) Dietary reaction norms at
1505 18°C. (B) Dietary reaction norms at 25°C. (C) Thermal reaction norms on sucrose
1506 diet. (D) Thermal reaction norms on molasses diet. Shown are means and standard

1507 errors. Red lines: low-latitude (LL) allele, blue lines: high-latitude (HL) allele. See
1508 Results section for details.
1509
1510 **Figure S7.** Effects of the *foxo* variant on male survival upon starvation. Effects of the
1511 clinal *foxo* polymorphism on the duration of survival (in hrs) upon starvation in males.
1512 (A) Dietary reaction norms at 18°C. (B) Dietary reaction norms at 25°C. (C) Thermal
1513 reaction norms on sucrose diet. (D) Thermal reaction norms on molasses diet.
1514 Shown are means and standard errors. Red lines: low-latitude (LL) allele, blue lines:
1515 high-latitude (HL) allele. See Results section for details.
1516
1517 **Figure S8.** Effects of the *foxo* variant on relative abundance of insulin-like receptor
1518 (InR) transcription levels. (A) Low-latitude (LL) allele has higher level of InR
1519 transcription than the high-latitude (HL) allele. (B) Carbohydrate-rich molasses diet
1520 resulted in more InR transcripts than the sucrose diet. Shown are means and
1521 standard errors. See Results section for details.
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1532 **Figure S1**

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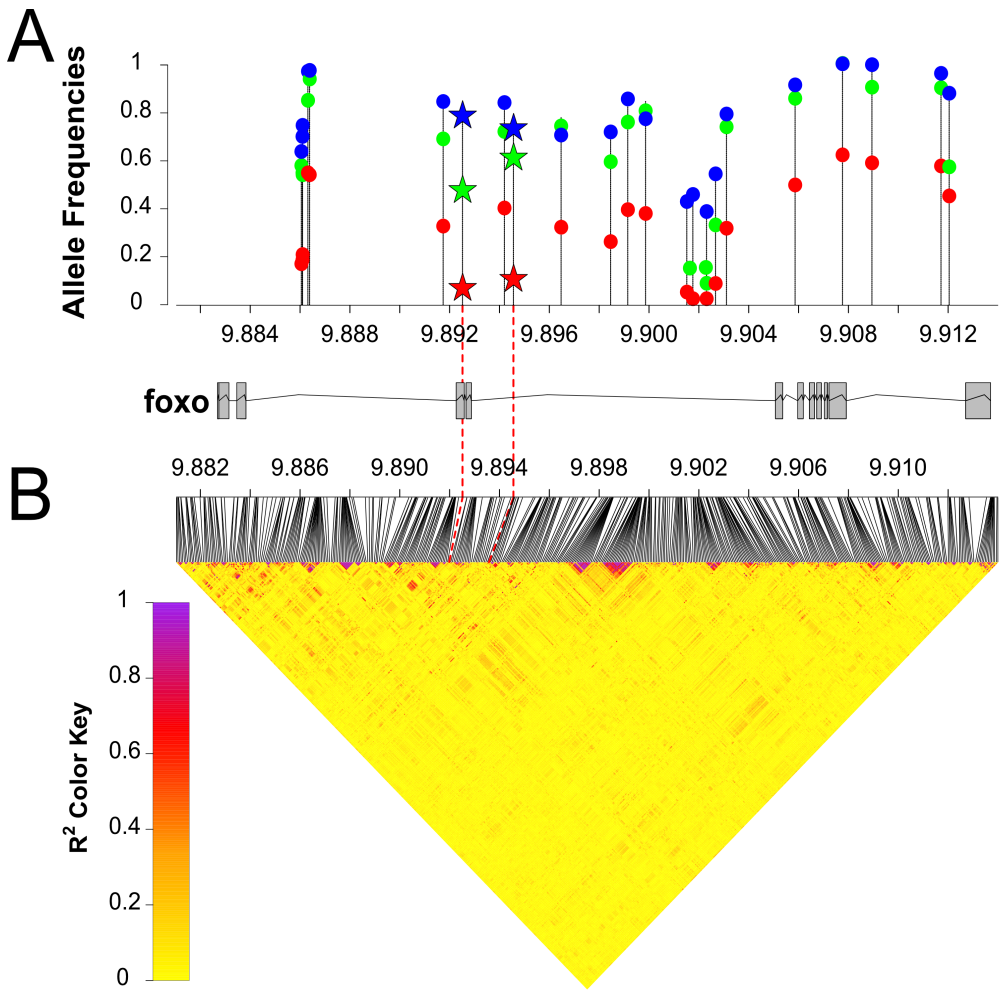

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1557 **Figure S2**

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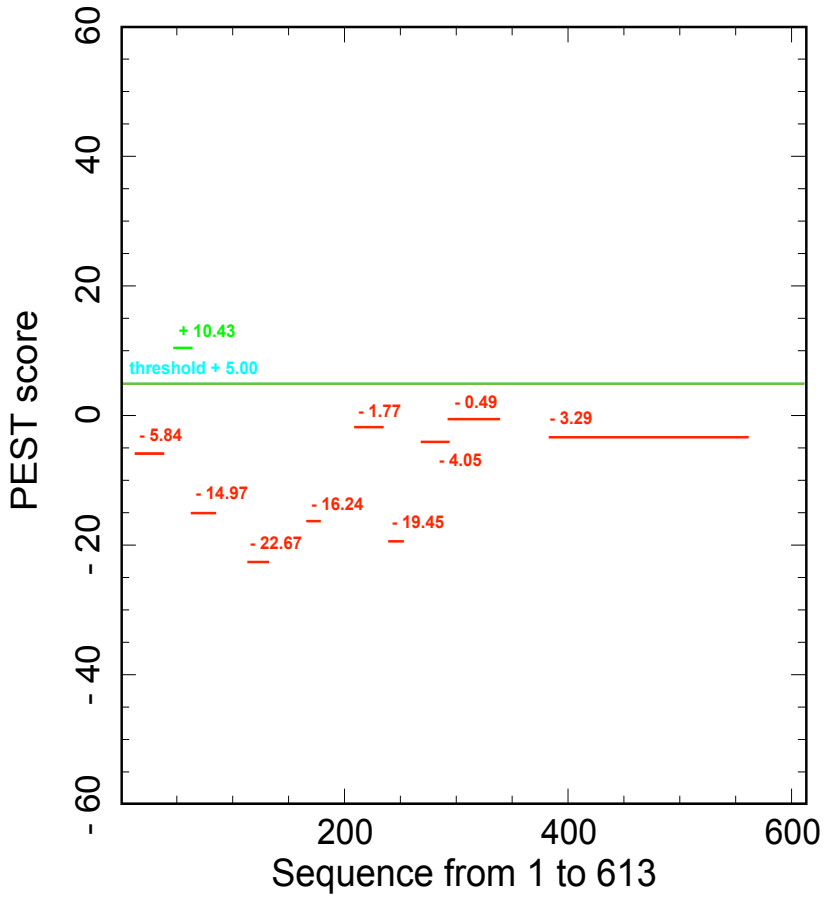

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1569 **Figure S3**

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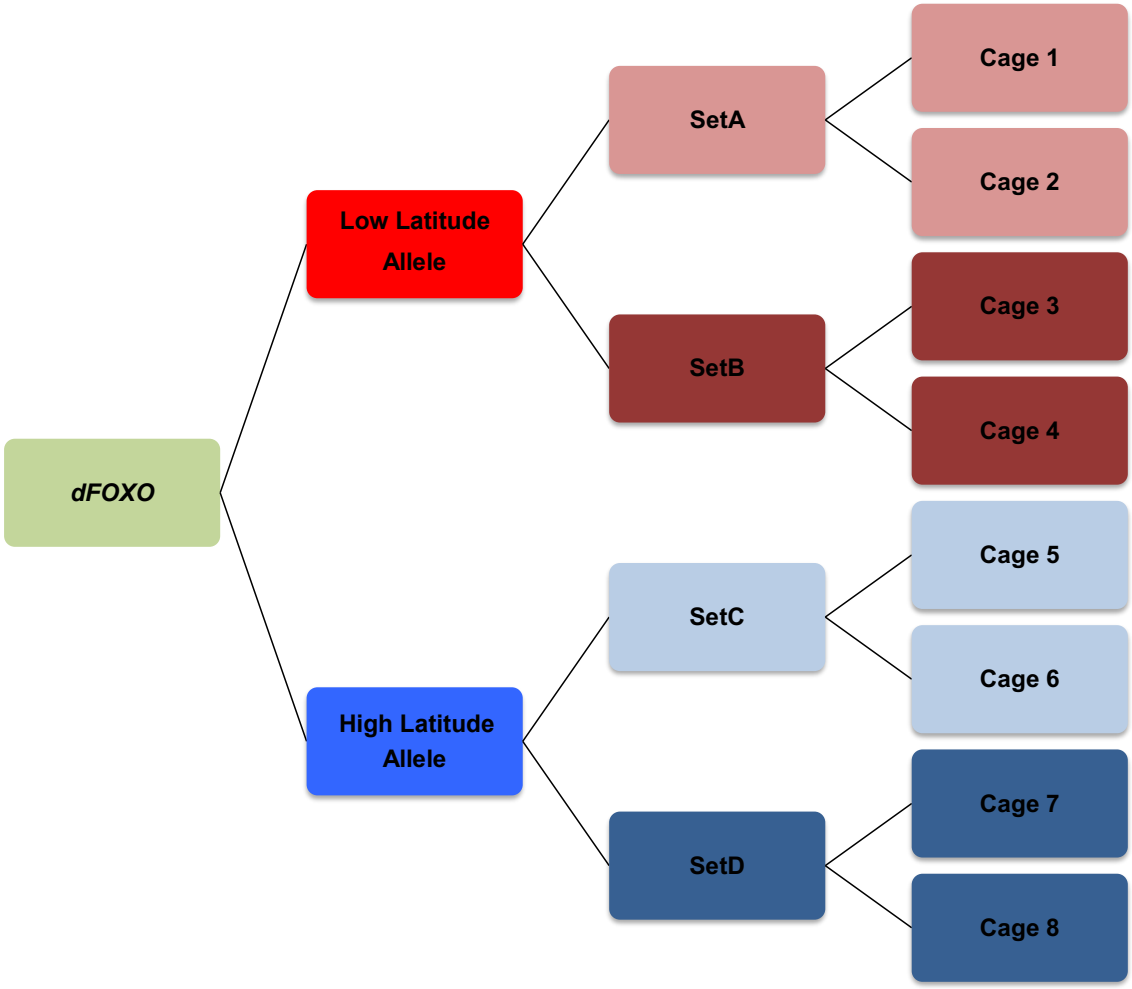

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1580 **Figure S4**

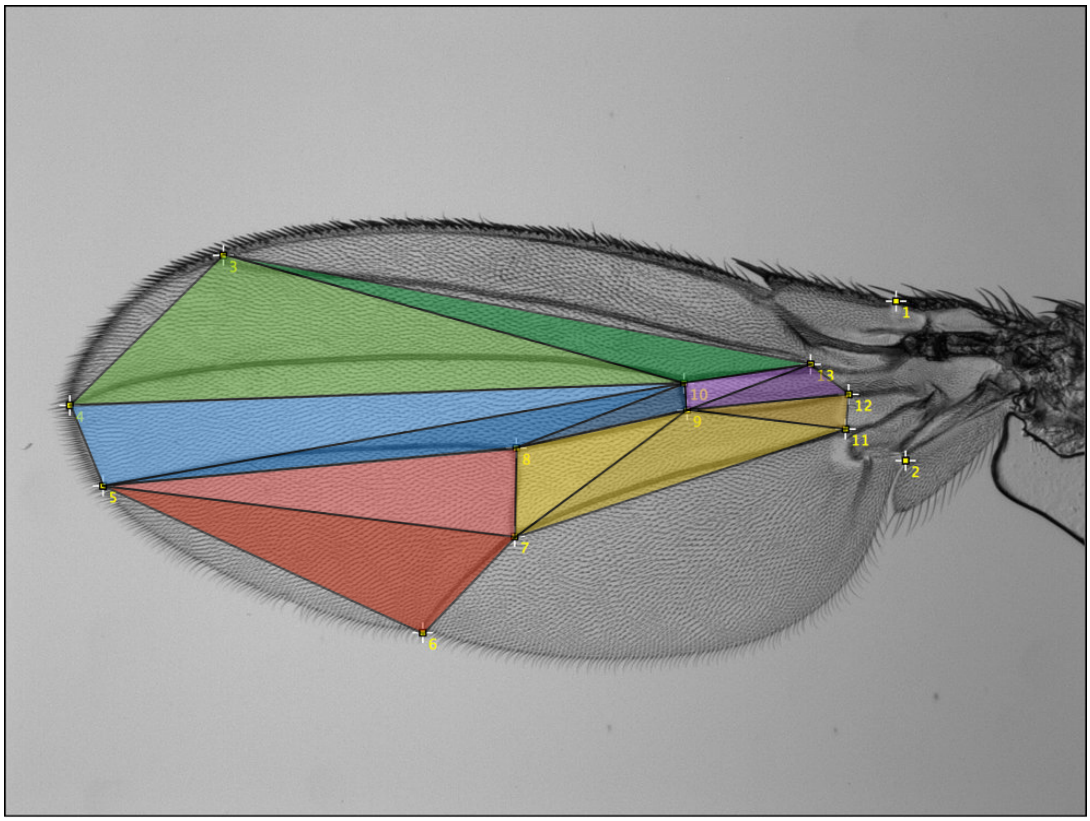

Figure S5

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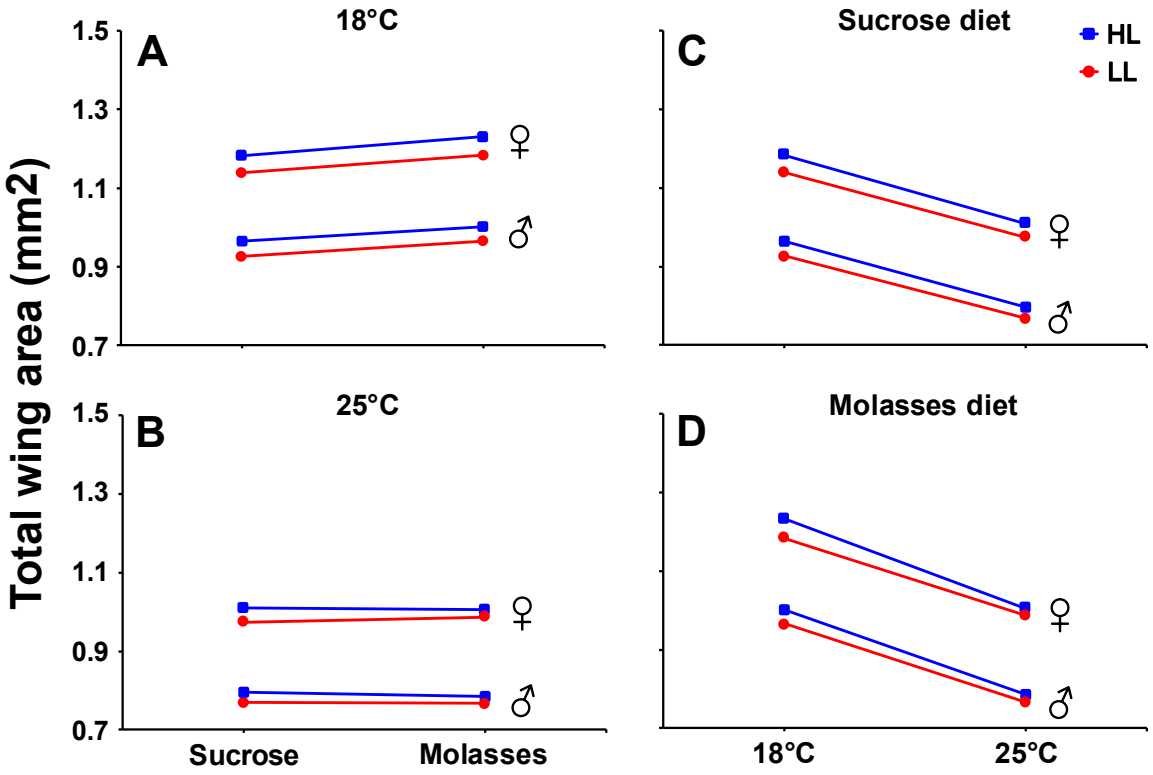

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1619 **Figure S6**

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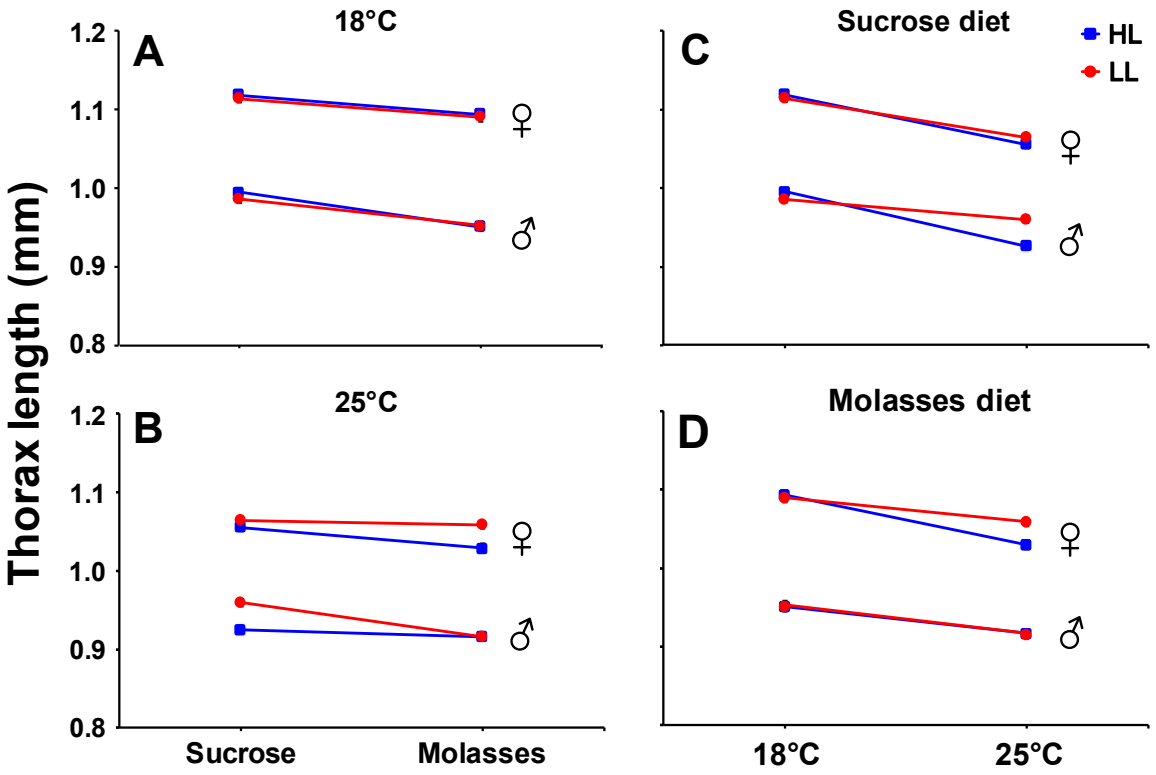

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1633 **Figure S7**

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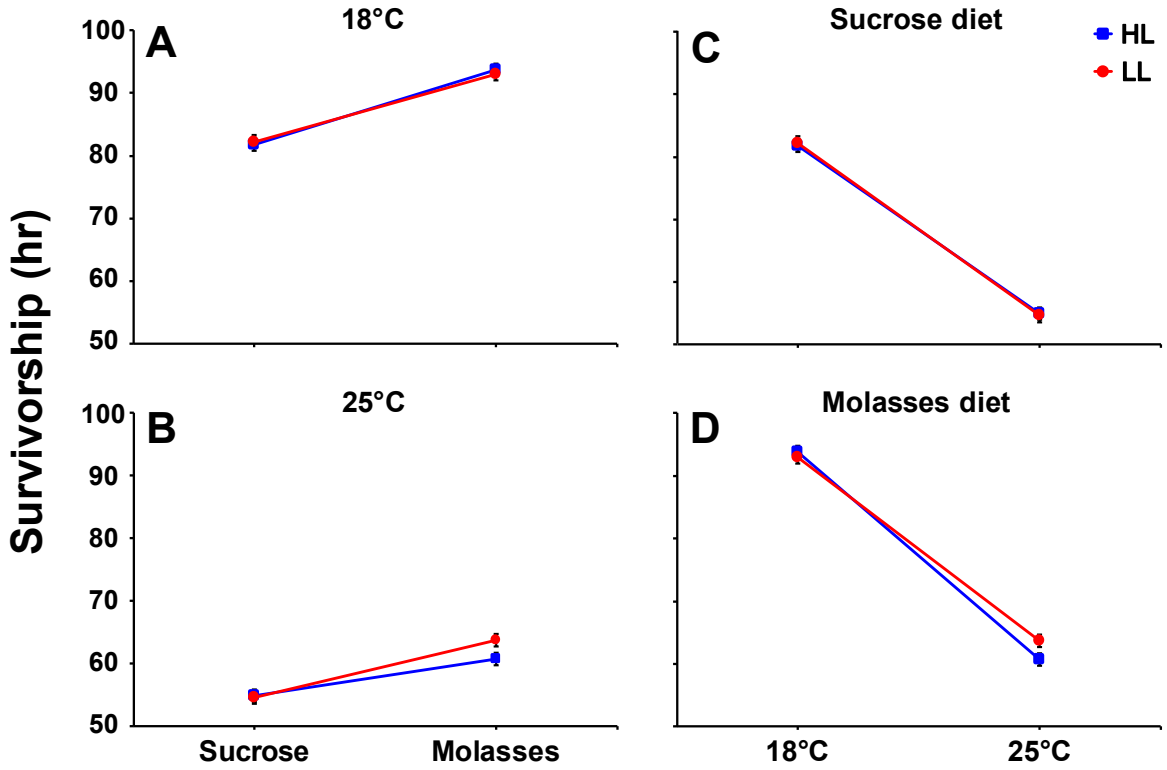

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1647 **Figure S8**

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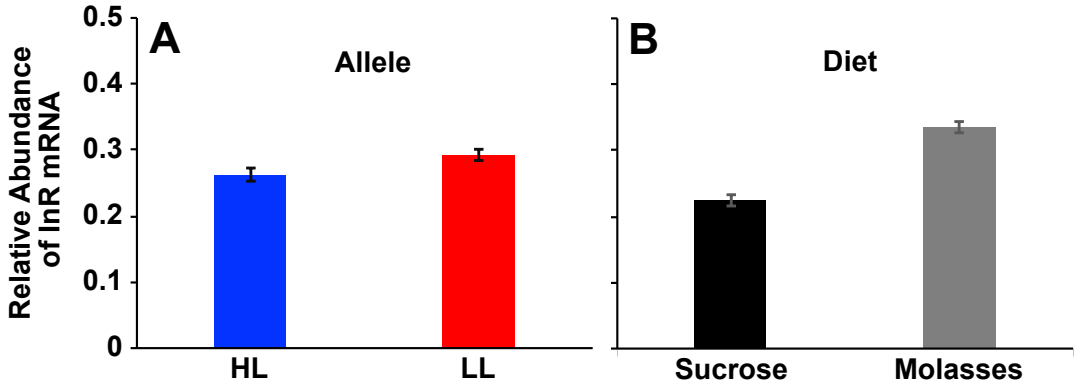

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Table S1. Details of design of reconstituted outbred population cages. HL: high-latitude *foxo* allele; LL: low-latitude *foxo* allele. See Materials and Methods section for details.

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

1 **Table S6.** Summary of ANOVA results for relative abundance of *insulin-like receptor*
2 (*InR*) transcript levels. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
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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)	$F_{4,80}=5.73^{***}$

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