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Allelic polymorphism at *foxo* contributes to local adaptation in *Drosophila melanogaster*

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Abstract

The insulin/insulin-like growth factor signalling pathway has been hypothesized as a major determinant of life-history profiles that vary adaptively in natural populations. In *Drosophila melanogaster*, multiple components of this pathway vary predictably with latitude; this includes *foxo*, a conserved gene that regulates insulin signalling and has pleiotropic effects on a variety of fitness-associated traits. We hypothesized that allelic variation at *foxo* contributes to genetic variance for size-related traits that vary adaptively with latitude. We first examined patterns of variation among natural populations along a latitudinal transect in the eastern United States and show that thorax length, wing area, wing loading, and starvation tolerance exhibit significant latitudinal clines for both males and females but that development time does not vary predictably with latitude. We then generated recombinant outbred populations and show that naturally occurring allelic variation at *foxo*, which exhibits stronger clinality than expected, is associated with the same traits that vary with latitude in the natural populations. Our results suggest that allelic variation at *foxo* contributes to adaptive patterns of life-history variation in natural populations of this genetic model.

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AUTHOR CONTRIBUTIONS

Paul Schmidt and Thomas Flatt conceived the project. Daniel K. Fabian and Martin Kapun identified the *foxo* SNPs and performed genomic analyses. Paul Schmidt, Subhash Rajpurohit, Esra Durmaz and Thomas Flatt designed the experiments. Nicolas J. Betancourt and Subhash Rajpurohit established populations and performed the experiments. Paul Schmidt, Nicolas J. Betancourt, Esra Durmaz, Martin Kapun, Subhash Rajpurohit and Thomas Flatt analysed the data. Nicolas J. Betancourt, Thomas Flatt and Paul Schmidt wrote the paper, with input from the other authors.

DATA AVAILABILITY STATEMENT

The raw phenotypic data have been made available at Dryad at: https://doi.org/10.5061/dryad.hhmgqnkgm.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Keywords

body size; cline; *foxo* ; genetic architecture; starvation tolerance

1 | INTRODUCTION

Elucidating the mechanistic basis of adaptive differentiation for complex traits in natural populations remains a fundamental goal in evolutionary biology. Fitness traits often exhibit a highly polygenic architecture (e.g., Arnegard et al., 2014; McCown et al., 2014; Savolainen et al., 2013). The likelihood of effectively mapping complex traits to causative polymorphism may depend on architecture of these quantitative traits (Barton et al., 2017; Boyle et al., 2017; Rockman, 2012; Roff, 2007; Wellenreuther & Hansson, 2016). Many empirical advances in understanding the mechanistic basis of adaptation in sexual, outbred populations include both a clear identification of traits that drive local adaptation as well as an apparently simple genetic architecture, with at least one locus that demonstrates a strong statistical association between allelic and phenotypic variation (e.g., Colosimo et al., 2005; Comeault et al., 2015; van't Hof et al., 2016; Jones et al., 2018; Lamichhaney et al., 2016). In such examples, alleles segregating at identified candidate loci can then be directly examined for functional differences that affect performance and fitness (e.g., Chakraborty & Fry, 2016; Cheviron et al., 2012; Laurie & Stam, 1988; Manceau et al., 2011).

Body size is a trait commonly associated with fitness in a variety of taxa (Blanckenhorn, 2000; Bonnet et al., 2017; Brown et al., 1993) including *Drosophila melanogaster* (e.g., Promislow et al., 1998; Reeve et al., 2001; reviewed in Flatt, 2020). Size often varies predictably across environmental gradients such as those associated with latitude (Ashton, 2002; Blanckenhorn & Demont, 2004; Huey et al., 2000; James et al., 1995; Stillwell et al., 2007); such clines suggest that body size is affected by spatially varying selection and reflects local adaptation (Partridge & Coyne, 1997; Stillwell, 2010). While size-related traits are in general highly polygenic (e.g., Boyle et al., 2017), individual loci can have large effects on size (e.g., Sutter et al., 2007). In particular, multiple components of the insulin/insulin-like growth factor signalling pathway (IIS) can regulate size (e.g., Colombani et al., 2005; Sutter et al., 2007); the forkhead box-O transcription factor gene *foxo* is a major regulator of IIS and impacts size as well as a variety of other traits associated with fitness (Fielenbach & Antebi, 2008; Hwangbo et al., 2004; Kramer et al., 2003, 2008; Libina et al., 2003; Mattila et al., 2009). Thus, the analysis of variation in body size offers an excellent system in which to examine the translation between genotype, phenotype and fitness in natural populations.

In *Drosophila melanogaster*, body size increases with increasing latitude on multiple continents (Coyne & Beecham, 1987; James et al., 1995, 1997; Karan et al., 1998). Such latitudinal patterns are mirrored by altitudinal clines where size increases with increasing altitude (Fabian et al., 2015; Lack et al., 2016). These parallel and replicated patterns suggest that patterns of size variation are adaptive and associated with thermally mediated selection (e.g., Partridge et al., 1994; Stillwell, 2010). However, it remains unknown why small body size is associated with higher fitness at low latitudes and large size with higher

fitness at high latitudes. De Jong and Bochdonavits (2003) hypothesized that adaptive patterns of size variation are driven primarily by one or more components of the IIS pathway; the simple prediction is that any causative variants would also exhibit pronounced and replicated allele frequency clines. Analysis of PoolSeq data has shown that multiple IIS genes (e.g., *Pi3K*, *foxo*, *InR*) are segregating for many alleles that vary predictably with latitude in *D. melanogaster* (Bergland et al., 2014; Fabian et al., 2012; Kapun et al., 2016; Kolaczkowski et al., 2011; Machado et al., 2021). The question is whether these clinal alleles are distinct with respect to gene function and, at least in part, underlie the observed patterns of local adaptation in size (Paaby et al., 2014).

Here, we examine patterns of variation among natural populations for two measures of body size (thorax length, wing area and their ratio) as well as two additional complex traits associated with fitness and correlated with size, development time and starvation tolerance. We evaluate patterns of clinality for SNPs at the *foxo* locus and test the functional significance of naturally occurring allelic variation; we specifically assess whether the inferred effects of allelic variation at *foxo* are consistent with patterns of trait variation among natural populations. If size in *D. melanogaster* is highly polygenic with no loci of major effect, then alternative alleles segregating at *foxo* should be effectively and functionally equivalent in their phenotypic effects in laboratory- or field-based functional assays. However, if *foxo* is a strong determinant of body size in this species, then differences among natural alleles may be of sufficient magnitude to be detectable in association studies. If allelic variation at *foxo* substantially contributes to phenotypic variation and local adaptation, then the effects of *foxo* alleles on phenotype should be concordant with patterns observed in natural populations.

2 | MATERIALS AND METHODS

2.1 | Identification of SNPs/alleles that vary predictably with latitude

Fabian et al. (2012) identified a series of SNPs in *foxo* that exhibited high *FST* in pooled sequencing of natural populations derived from Florida (low latitude), Pennsylvania (mid-latitude), and Maine, USA (high latitude). From this analysis, which suggested that *foxo* might be a clinal outlier in *D. melanogaster* populations in eastern North America, we selected candidate *foxo* alleles to test whether allelic variation at this locus might be of functional significance and contribute to phenotypic clines for traits linked to *foxo* function. The selection of the *foxo* alleles was based on: (i) High *FST* in the Fabian et al. (2012) data, (ii) preliminary indication that latitudinal differences in allele frequencies were concordant with seasonal differences in allele frequencies (following the rationale and methods in Paaby et al., 2014), and (iii) alleles being at intermediate frequency and present in sufficient numbers in the Drosophila Genetic Reference Panel (DGRP, Mackay et al., 2012) so that we could establish multiple, independent sets of lines for functional analysis (see Constructing recombinant outbred populations below). The selected *foxo* allele, which satisfied the three criteria described above, was actually a haplotype defined by two SNPs (synonymous A/G at position 3R:9892517, *D. melanogaster* reference genome v.5.0, and intronic T/G at position 3R:9894559) spanning approximately 2 kb within the *foxo* gene (see Figure 1, Table S2). These sites were observed to be in perfect linkage disequilibrium (r^2 =1) in the DGRP, such

that they covary and are not independent. Further details regarding these polymorphic sites and the landscape of linkage disequilibrium at *foxo* are provided in our previous publication (Durmaz et al., 2019).

To examine clinal patterns associated with the *foxo* locus in general (i.e., *foxo* compared to the genome as a whole) and the focal low- and high-latitude *foxo* haplotypes in particular (i.e., the focal *foxo* SNPs as compared to other SNPs across the *foxo* locus), we analysed patterns of allele frequency variation in 10 populations collected at different latitudes in the eastern U.S. and sequenced as pools (Figure 1; Table S1; Bergland et al., 2014; Kapun et al., 2016; Machado et al., 2021). We restricted our analyses to high-confidence SNPs that were polymorphic in the DGRP data set (Langley et al., 2012; Mackay et al., 2012) and isolated a total of 1372 SNPs located inside or within 2 kbp up- and downstream of the annotated *foxo* gene. To provide a null genomic context for allele frequency differentiation at *foxo*, we isolated 20,000 SNPs located in short introns (<60 bp) and at least 100 kbp distance from the breakpoints of common cosmopolitan inversions (Corbett-Detig et al., 2012) that are consistent with patterns of neutrality (Clemente & Vogl, 2012; Parsch et al., 2010). For each of these neutral and *foxo*-associated SNPs, we tested for significant correlations between latitude and allele frequencies using generalized linear models (GLM) with a binomial error structure of the form: $y_i = L + \varepsilon_i$, where y_i is the allele frequency of the i^{th} SNP, L is the continuous factor "Latitude" and ε_i is the binomial error of the i^{th} SNP (Figure 2a,b). We further assessed whether allele frequency changes of the two candidate SNPs that constitute our focal haplotype were more clinal than neutral SNPs or other SNPs located within or in the proximity of *foxo* (Figure 2c,d). To this end, we compared the $-\log_{10}(p)$-values from the GLMs for each of the two focal SNPs to distributions of $-\log_{10}(p)$-values from GLMs of either neutral SNPs or noncandidate SNPs associated with *foxo*. We subsequently calculated empirical cumulative distribution functions (ECDF; with total area = 1) based on $-\log_{10}(p)$-values from all neutral or noncandidate *foxo* SNPs in *R* (R Development Core Team, 2009). To test if the significance values associated with the two focal SNPs were greater than the 95 percentiles of each distribution, we integrated over the area under each ECDF with values larger than the significance of each candidate SNP and subtracted the integral value from 1, which represents the total area of the ECDF.

To investigate and visualize the relative patterns of allele frequency change for the two focal SNPs with respect to all other SNPs located inside or in close proximity to *foxo*, we conditioned the alleles to have lower frequencies in Florida compared to the population in Maine for each *foxo*-associated SNP. Allele frequencies in Florida were set to zero and we then calculated the allele frequency differences relative to Florida for all other populations (Figure 1).

2.2 | Constructing recombinant outbred populations

Based on the combination of the results of Fabian et al. (2012) and the DrosRTEC (*Drosophila* Real-Time Evolution Consortium) sequencing effort (Machado et al., 2021), we identified individual lines in the DGRP (Mackay et al., 2012) that were homozygous for the candidate *foxo* allele that was at high frequency in high-latitude populations [hereafter, the high-latitude (HL) allele], and lines that were fixed for the *foxo* allele that was at

high frequency in low-latitude samples [hereafter, the low-latitude (LL) allele]. Scripts for this filtering of the DGRP based on nucleotide state and locus are provided in Files S4 and S5. Two biological replicates were established using 18 independent (different and nonoverlapping) lines per cage per *foxo* allele; thus, each cage was constructed with a completely distinct and independent set of inbred lines (see Table S2). These biological replicates were then also replicated as technical replicates, resulting in a total of eight experimental population cages (Table S2). Each population cage was individually founded using 10 individuals of each sex, from age- and density-controlled cohorts, from each of the 18 inbred DGRP founding lines associated with each specific cage.

After establishment, each of the eight population cages was cultured at a population size of ~2000 adults on standard cornmeal-molasses medium for eight discrete generations of outcrossing under conditions of constant temperature (25°C) and photoperiod (12L:12D). Thus, at the end of the experimental period, we generated replicate population cages in which the focal *foxo* allele was homozygous and fixed for either the high- or low-latitude allele and the genomic background was randomized across the 18 inbred lines used to find each respective cage (Behrman, Howick, et al., 2018; Paaby et al., 2014). To test for potential genome-wide patterns of genetic differentiation among the recombinant outbred populations (ROP) fixed for either the high or low-latitude haplotypes, we used available genome sequence data for the inbred lines used to found experimental populations (Table S2) for sets A and B (representing the two biological replicates) to calculate SNP-wise F_{ST} based on the method of Weir and Cockerham (1984). This was done to evaluate whether any other position in the genome was highly differentiated among our experimental populations and could thus confound interpretation of results (Figure 3; Files S4 and S5). Note that our experimental approach makes two assumptions: (i) De novo mutational input at the *foxo* locus is insufficient to meaningfully affect experimental outcomes, and (ii) genetic drift and/or selection did not result in random fixation of any additional alleles in the construction of these populations. After eight generations of density-controlled culture under standardized environmental conditions, we established replicate density-controlled vial cultures (30 ± 10 eggs/vial) for subsequent phenotyping of the high-latitude and low-latitude *foxo* alleles.

2.3 | Isofemale lines from natural populations

Thirty isofemale lines were randomly selected from each of six natural populations along the east coast of the U.S. to serve as a latitudinal comparison to the recombinant *foxo* populations [described in Rajpurohit et al., 2017, 2018: Homestead, FL (HFL), Jacksonville, FL (JFL), Charlottesville, VA (CVA), Media, PA (MPA), Lancaster, MA (LMA), and Bowdoin, ME (BME)]. Individual lines from each population were maintained on a 21-day culture regime under the same environmental conditions as the *foxo* recombinant cages (25°C, 12L:12D). Prior to phenotyping, each isofemale line was cultured for two generations at low density (30 ± 10 eggs/vial) at 25°C, 12L:12D; in the third generation, freshly eclosed flies were collected in daily cohorts and used in the phenotypic assays described below.

2.4 | Phenotype assays

In all assays, *foxo* ROPs were tested simultaneously in three replicates (experimental blocks, conducted on different days) in the same generation since founding of the experimental cages. For assessment of starvation resistance, virus infection of the cages precluded running three independent blocks, and a single block was included in the analysis. For the natural populations, all lines from all populations were assayed simultaneously for all phenotypes using discrete 1d cohorts for each phenotype.

2.4.1 | Development time—For the *foxo* recombinant outbred populations, eggs were collected from each cage over a 3 h window using large Petri dishes containing standard medium supplemented with live yeast. The collected eggs were then counted and distributed in groups of 30 into three replicate vials per cage. For the natural populations, eggs from all isofemale lines were similarly collected over 3 h in small collection receptacles. Eggs were counted and distributed to new collection vials, with density also standardized at 30 eggs per vial. All experimental material was subsequently cultured under the standard conditions. Experimental vials were checked four times daily (9 a.m., 1 p.m., 5 p.m., 9 p.m.); eclosion events and sex were recorded.

2.4.2 | Body size—For both the *foxo* cages as well as the natural populations, flies from the development assay were transferred to new vials, allowed to mate and age for five days post eclosion, then were preserved in 95% ethanol for subsequent size measurements. A total of 10 flies of each sex were randomly sampled and measured from each *foxo* ROP cage and five flies for each sex were measured for each isofemale line from the natural populations. Body size measurements (thorax length and wing area) were recorded using a Leica MZ9.5 microscope mounted with an Olympus DP73 camera with CellSens standard measuring software. Thorax length was measured as the longest length across the dorsal shield in lateral view; wing area was defined as a polygon using a standardized series of veinous landmarks. We also calculated and analysed the ratio of total wing area to thorax length, indicative of (the inverse of) “wing loading”, a variable thought to be an important determinant of flight ability (Azevedo et al., 1998; Gilchrist et al., 2000).

2.4.3 | Starvation resistance—For the *foxo* recombinant outbred populations, embryos were collected from each cage in two replicate glass culture bottles and density was standardized at 150 ± 10 eggs per bottle. Isofemale lines from the natural populations were transferred into replicate vials and density standardized at 30 ± 10 embryos per vial. All culture was done under the standard conditions (25°C, 12L:12D). Upon eclosion, mixed sex daily cohorts were collected over 3 days and subsequently aged to 5 days. The flies were then separated by sex into replicate groups of 10 and placed into glass vials equipped with a small cotton ball saturated with 1 ml of water. Samples were placed in an incubator at 25°C, 12L:12D and mortality was recorded at four timepoints per day (9 a.m., 1 p.m., 5 p.m., and 9 p.m.) until all flies had died. The *foxo* cages were assayed in three replicates per cage population; all isofemale lines from each of the natural populations were also assayed.

2.5 | Statistical analysis

For the natural populations, data were analysed separately by sex. All data were analysed using a mixed-effects ANOVA model, using population as a fixed factor and isofemale line nested within population as a random factor (estimated with restricted maximum likelihood). For all traits other than starvation tolerance, experimental block ($N = 3$) was also included as an additional effect. For the *foxo* recombinant outbred populations, we analysed data separately for both sexes with nested ANOVA models, using *foxo* allele, DGRP set nested in allelic state, and experimental replicate (population cage) nested in a combination of set and allele as factors.

3 | RESULTS

3.1 | Alleles at *foxo* exhibit steep latitudinal clines

By analysing genome-wide Pool-Seq data from 10 populations sampled along the U.S. east coast generated by the DrosRTEC consortium (Kapun et al., 2016; Machado et al., 2021), we show that numerous SNPs associated with *foxo* exhibit steep latitudinal clines and extensive differentiation as a function of geography (Figure 1). Notably and consistent with previous observations by Fabian et al. (2012), the two focal *foxo* candidate SNPs that are denoted by black and red lines in Figure 1a,b, respectively, exhibit strong patterns of allele frequency change across latitudes. In fact, clinal patterns of the two candidate SNPs were more pronounced than 95% of neutrally evolving SNPs located in short introns (>95.94% for 3R:9,892,517 and >98% for 3R:9,894,559, respectively; Figure 2a,b) and 91% of all noncandidate SNPs located within or close to *foxo* (>91.22% for 3R:9,892,517 and >96% for 3R:9,894,559, respectively; Figure 2c,d).

In establishing the recombinant outbred populations (ROPs) using the DGRP panel of inbred lines, the goal was to use candidate SNPs as markers of functional effects for naturally occurring alleles or haplotypes at this locus (Berhman et al., 2018; Paaby et al., 2014). The utility of this method is predicated on using a sufficient number of independent inbred lines such that no other position in the genome, other that the candidate site(s), is fixed or highly differentiated between experimental sets. In Figure 3, *FST* between experimental cages (*foxo* allele AT vs. *foxo* allele GG) is plotted as a function of chromosomal position for all SNPs segregating in the biological replicate sets A (Figure 3a) and B (Figure 3b). While there are multiple sites on each chromosome arm with $FST > 0.4$ between the sets of lines used to construct the alternative *foxo* allelic cages, only the candidate sites are fixed between the cages that comprise the allelic states.

3.2 | Natural populations vary clinally for size-related traits

3.2.1 | Development time—Development time did not vary predictably with latitude. For both males and females, significant variation was observed among lines and among populations (Table 1) but there was no major association with latitudinal origin (Table S3, Figures 4a and 5a). We did, however, observe distinct patterns of development time among the replicate experimental blocks, despite controlling for density and culture conditions. This suggests that this trait is affected by additional environmental variables, measurement or other experimental error, or a combination of the two. It should be noted, however, that

in examination of the experimental blocks individually, no significant association between development time and latitudinal origin of the population was observed. This is in contrast to patterns of seasonal variation, which demonstrate predictable change in development time as a function of time of collection (Behrman et al., 2015).

3.2.2 | Body size—In the six sampled natural populations that were assayed for trait variation, all measures of size (thorax length, wing area, and the ratio of wing area to thorax length) were highly distinct among populations (Table 1), and these patterns of differentiation exhibited a positive association with latitude (Table S3) for both females (Figure 4b,d) and males (Figure 5b,c). Sexes were analysed separately due to dimorphism and the potential for differential allometry, but exhibited qualitatively similar patterns of size variation among populations and as a function of latitude. As expected, these results are consistent with previous associations between latitude and body size in *D. melanogaster* (e.g., Coyne & Beecham, 1987; James et al., 1995; de Jong & Bochdanovits, 2003).

3.2.3 | Starvation resistance—As with body size, starvation tolerance was highly variable among isofemale lines within populations yet exhibited a robust association with geography for both sexes (Table 1, Figures 4d and 5d). The patterns of increasing tolerance with increasing latitude is consistent with other aspects of stress tolerance in North American populations (Schmidt & Paaby, 2008), but opposite to what has been observed on the Indian subcontinent (Karan et al., 1998). Starvation tolerance does not appear to vary predictably with latitude in other assayed geographic regions (Hoffmann et al., 2001; Robinson et al., 2000). All raw data are available at https://doi.org/10.5061/dryad.hhmgqnkgm.

3.3 | *foxo* alleles may make a large contribution to body size clines

3.3.1 | Development time—Development time varied significantly across sets of lines and replicates; in contrast to the other traits measured, development time was not distinct between the high- and low-latitude *foxo* alleles for females but was for males (Table 2, Figures 4e and 5e). Differences in average development time between *foxo* genotypes, measured as time to eclosion, were small in magnitude for each sex (less than one hour difference in mean development time for females, slightly more than two hours for males; Figures 4e and 5e). Overall these results suggest that there are no major functional differences associated with these naturally occurring *foxo* alleles with respect to development time. This is somewhat congruent with data on Australian clines, where the relationship between body size and development time is inconsistent across latitudes (e.g., James et al., 1995, 1997). Development time was the most variable of the traits studied here, both with respect to experimental replication and variation among natural as well as reconstituted outbred populations.

3.3.2 | Body size—Thorax length and wing area were both highly distinct between the high- and low-latitude *foxo* genotypes (Table 2). Significant heterogeneity was present between the replicate sets A and B, as expected based on distinct composition of founding inbred lines, as well as among experimental replicates that were cultured independently since cage initiation. Despite these two sources of cage effects, the differences between

foxo genotypes were consistent with expectations based on geography: the genotypes homozygous for the high-latitude *foxo* allele were significantly larger than genotypes homozygous for the low-latitude allele (Figures 4f and 5f). The ratio of wing area to thorax length also demonstrated significant and predictable differences between the high and low-latitude *foxo* genotypes (Figures 4g and 5g). Furthermore, the observed differences between *foxo* genotypes are strikingly similar in effect size to the magnitude of trait differences observed between the populations sampled from the latitudinal extremes (Figure 4b,c vs. Figure 4f,g; Figure 5b,c vs. Figure 5f,g), suggesting that variation at the *foxo* locus makes a major contribution to body size clines.

3.3.3 | Starvation resistance—Starvation resistance was also distinct between the *foxo* genotypes and varied predictably with geography (Table 2, Figures 4h and 5h). For both males and females, the genotype homozygous for the high-latitude *foxo* allele was associated with increased starvation tolerance, which may be associated with effects of the alleles on body size and/or lipid content (e.g., Chippindale et al., 1996). A significant amount of variance in starvation tolerance was also associated with cage effects for both sets of inbred lines and culture replicate (Table 2). The effect size associated with *foxo* genotype was approximately 10%, again similar to what was observed for associated differences in body size. However, unlike the patterns observed for size related traits, the differences between *foxo* genotypes appear to explain a small amount of the variance in this trait among natural populations across the sampled geographic range in the eastern U.S. (Figure 4d vs. Figure 4h; Figure 5d vs. Figure 5h). These distinct patterns also suggest that differences in starvation tolerance are not determined solely by differences in size.

4 | DISCUSSION

In *D. melanogaster*, there is abundant evidence for local adaptation. Natural populations exhibit rapid and predictable responses to environmental parameters that vary with season, both in terms of phenotypic (Behrman, Howick, et al., 2018; Behrman, Kawecki, et al., 2018; Behrman et al., 2015; Rajpurohit et al., ,2017, 2018; Schmidt & Conde, 2006) and allele frequency (Behrman, Kawecki, et al., 2018; Bergland et al., 2014; Cogni et al., 2014; Machado et al., 2021) change. Similarly, many fitness-associated traits have been shown to vary predictably with latitude, often in parallel across independent gradients (e.g., Oakeshott et al., 1982; Paaby et al., 2010; Yang & Edery, 2018). Latitudinal allele frequency clines at candidate loci (e.g., Cogni et al., 2017; Paaby et al., 2010; Schmidt et al., 2000; Sezgin et al., 2004) are now placed in a genomic context in which tens of thousands of SNPs are known to be clinal (e.g., Bergland et al., 2016; Fabian et al., 2012; Kapun et al., 2016; Kolackzowski et al., 2011). While allele frequency clines may be generated by demography (Bergland et al., 2016; Kao et al., 2015), at least some of the observed clines may be generated by spatially varying selection (Schmidt et al., 2008; Svetec et al., 2016). Two of the associated, major questions are: (i) how many, or what proportion of, allele frequency clines reflect spatially varying selection and thus local adaptation; and (ii) how are allele frequency and phenotypic clines integrated? Alleles exist in a genomic context, and complex traits are similarly correlated as well as affected by epistasis (Mackay, 2014); it is extremely unlikely that all allele and phenotypic clines are

independent and reflect selection on single variants or traits. However, the effect size of individual adaptive polymorphisms and the architecture of local adaptation are, arguably, not well resolved. There is a paucity of detailed, mechanistic and comprehensive investigations as to the functional significance of segregating polymorphisms in natural populations. It is infeasible to assess the functional impact of all polymorphisms across the genome. However, multiple, intersecting methodologies (e.g., direct mapping, expression analyses, mutant analysis, patterns of variation in natural populations) can be used to identify a subset of variants that may be examined for functional significance. Ideally, investigation of a sufficient number of outliers could generate an empirical distribution of genic or allelic effect sizes for specific fitness-associated traits. Such investigations are essential in resolving the genetic architecture and dynamics of local adaptation.

The *foxo* locus is an example of a robust candidate suitable for such functional analysis and integration of genotype to phenotype to fitness. Genetic manipulations of the *foxo* gene have revealed pronounced effects on lifespan, multiple aspects of stress tolerance including starvation resistance, and growth phenotype (Giannakou et al., 2004; Hwangbo et al., 2004; Jünger et al., 2003; Kramer et al., 2003; Kramer et al., 2008; Puig et al., 2003; Slack et al., 2011). These traits vary with latitude in *D. melanogaster* (Coyne & Beecham, 1987; James et al., 1995; Schmidt et al., 2005); thus, the *foxo* gene is a logical candidate for determining variation for these traits in natural populations (de Jong & Bochdonavits, 2003). However, while it is clear that *foxo* laboratory mutants or transgenes have highly pleiotropic effects on life-history phenotypes, polymorphisms segregating in natural populations need not necessarily affect variance for traits related to gene function (e.g., Stern, 2011).

In previous work, we have performed independent assays of the natural *foxo* alleles studied here, with a focus on genotype by environment interactions (diet, temperature) and predictions regarding insulin signalling (Durmaz et al., 2019). Here, we extend this work by (i) demonstrating that these *foxo* alleles represent major clinal outlier loci probably subject, either directly or indirectly, to spatially varying selection; (ii) directly comparing the effects of the *foxo* alleles to new data on phenotypic clines and showing that they may make a significant contribution to these trait clines, particularly for body size; and (iii) by independently verifying the robustness of the allelic effects at *foxo* using different assay conditions in another laboratory.

Our present results demonstrate that allelic variation at *foxo* has large effects on body size and starvation tolerance. Durmaz et al. (2019) also show that these focal *foxo* variants are also associated with significant differences in viability, fat catabolism, and FOXO activity, as indicated by differences in transcript abundance of a FOXO target (*InR*). We show here that two measures of size, thorax length and wing area, as well as starvation tolerance, vary predictably with latitude in natural populations of *D. melanogaster*. Furthermore, the high-latitude *foxo* allele is associated with larger size and greater starvation resistance, whereas flies homozygous for the low-latitude *foxo* allele are smaller and less tolerant. Thus, the allelic effects parallel patterns of variation observed in natural populations, and are also consistent with predicted effects on phenotype due to genotypic differences in insulin/insulin-like signalling (Durmaz et al., 2019). This is distinct from the countergradient patterns that have been previously observed for allelic variants at the *Drosophila insulin*

receptor (*InR*) (Paaby et al., 2014). The effect size of the *foxo* alleles is seemingly large, particularly for body size: the difference between high-and low-latitude *foxo* alleles is approximately the same as the observed size difference between flies sampled from Florida and Maine. This suggests that allelic variation at *foxo* underpins, at least in part, variance in body size in these populations. It remains to be determined whether *foxo* is a major-effect locus for size in other taxa.

Despite the parallels we observed between the assayed *foxo* variants and the patterns in natural populations, we cannot conclude that it is these two focal SNPs (positions) that themselves cause the observed differences in size and starvation tolerance, or that they directly contribute to variance for these traits in natural populations. The linkage disequilibrium present in the founding inbred lines (DGRP) would decay to some extent by the eight generations of outcrossing but would remain pronounced; thus, without further characterization, these SNPs need to be interpreted as markers for functionally significant allelic variation segregating at this locus. Gene editing or similar techniques, in which the focal sites are manipulated in multiple common genetic backgrounds, would be essential in directly examining causality. Such investigations are the focus of future work.

5 | CONCLUSION

Here, we have shown that both starvation tolerance and two estimates of body size exhibit pronounced latitudinal clines in the sampled natural populations, whereas development time exhibited no clear association with latitude. Similarly, we find that allelic variation at *foxo* has predictable effects on body size and starvation tolerance but minor and sex-specific effects on development time. The assayed alleles at *foxo* explain a small amount of the variance among natural populations for starvation tolerance, but may have a large impact on genetic variance for size. Our results suggest a distinct genetic architecture for correlated fitness traits, and that allelic variation at the *foxo* locus underlies, in part, patterns of local adaptation in natural populations of this genetic model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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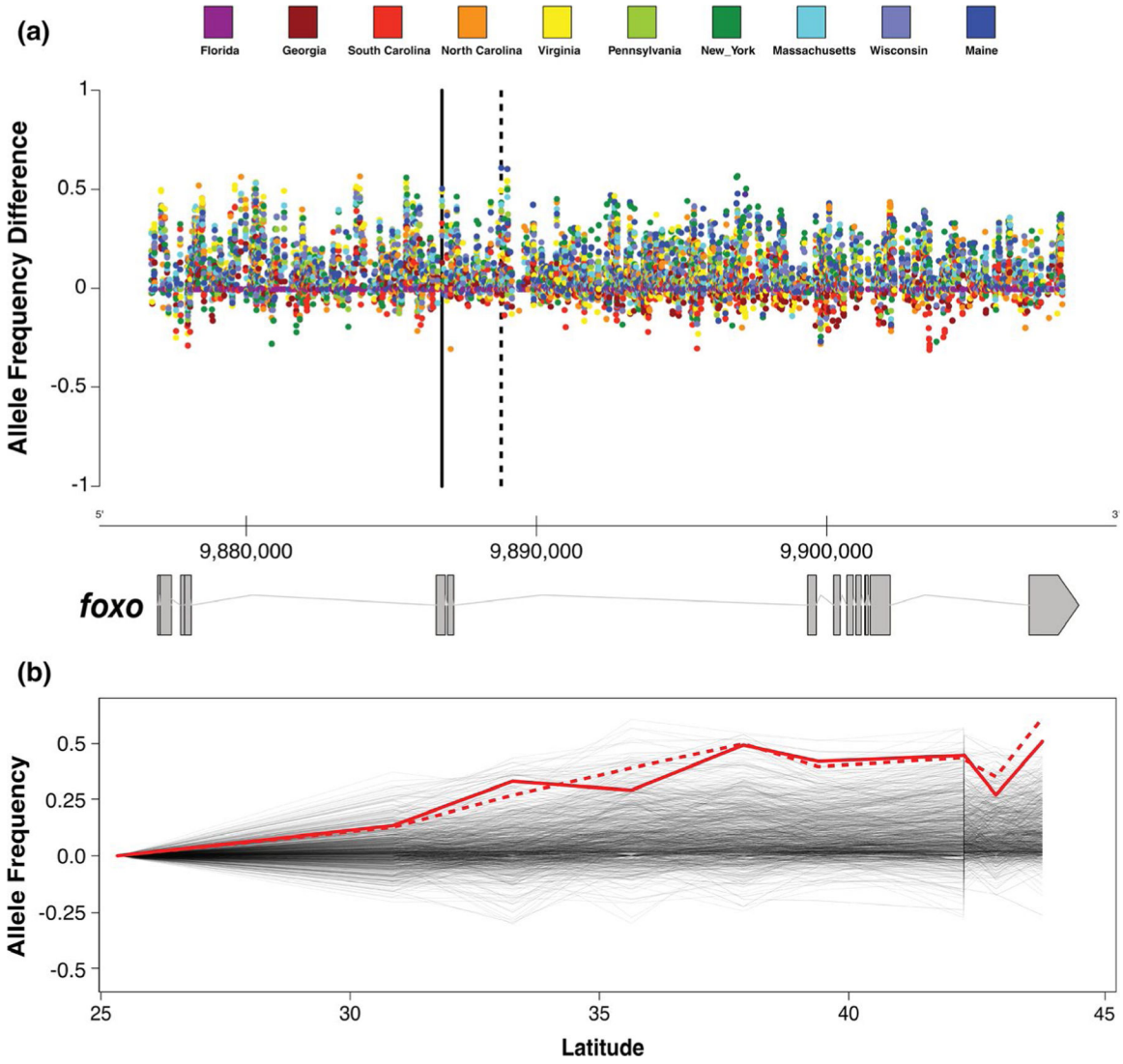

FIGURE 1.

Allele frequency changes for *foxo*-associated SNPs in 10 populations sampled from the eastern U.S (population specifics given in Table S1). Both plots show allele frequency differences conditioned to increase from south to north, with frequencies in Florida being set to zero. (a) Allele frequency differences for a given population (colour coded) compared to the reference (Florida) for all SNPs according to their genomic position. The *foxo* candidate SNPs are denoted by two vertical black lines (solid: 3R: 9,892,517; dashed: 3R: 9,894,449; *D. melanogaster* reference genome v6). (b) Shows how allele frequencies change with latitude. The two *foxo* candidate SNPs are shown in red (solid: 3R: 9,892,517; dashed: 3R: 9,894,449) [Colour figure can be viewed at wileyonlinelibrary.com]

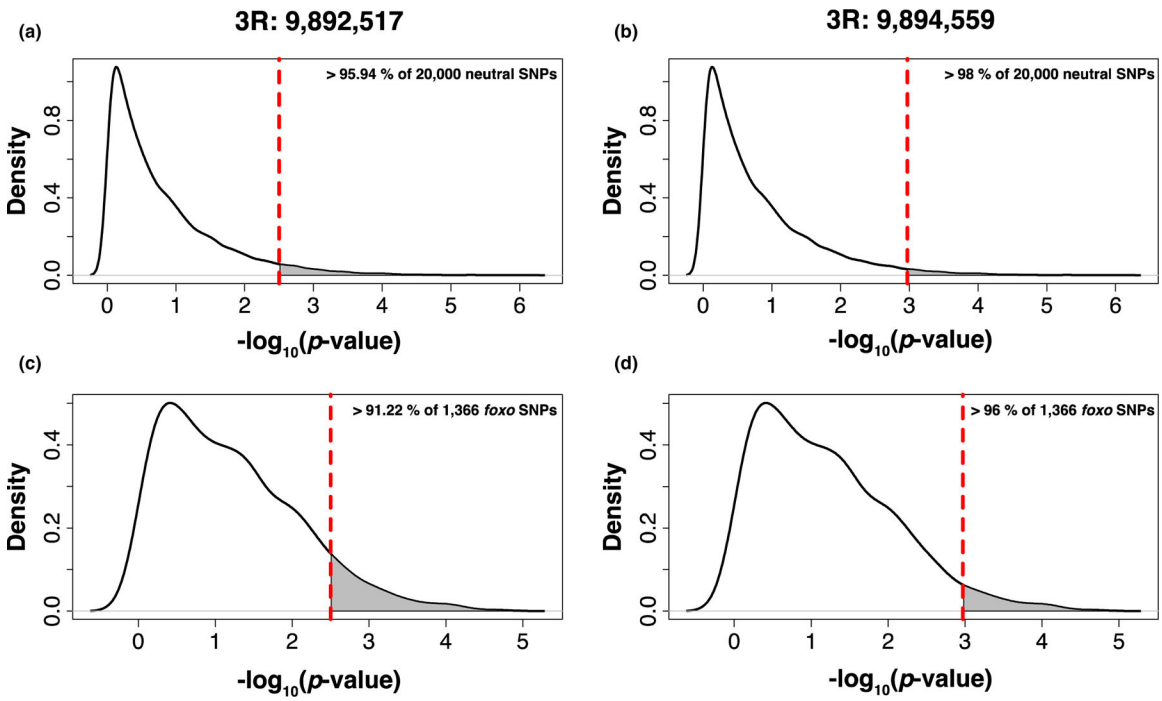

FIGURE 2.

Empirical cumulative density functions (ECDF; total area = 1) calculated from the distribution of $-\log_{10}$ (*p*-values) for generalized linear models that test for associations between allele frequencies and latitude in 20,000 neutrally evolving SNPs (a,b) and 1372 noncandidate SNPs located inside or within 2 kbp distance to *foxo*. The vertical dashed lines indicate the significance values of the two candidate SNPs 3R: 9,892,517 (a,c) and 3R: 9,894,449 (b,d). The grey areas limited by the dashed line indicate the percentiles of neutral or noncandidate *foxo* SNPs with significance values larger than the candidates [Colour figure can be viewed at wileyonlinelibrary.com]

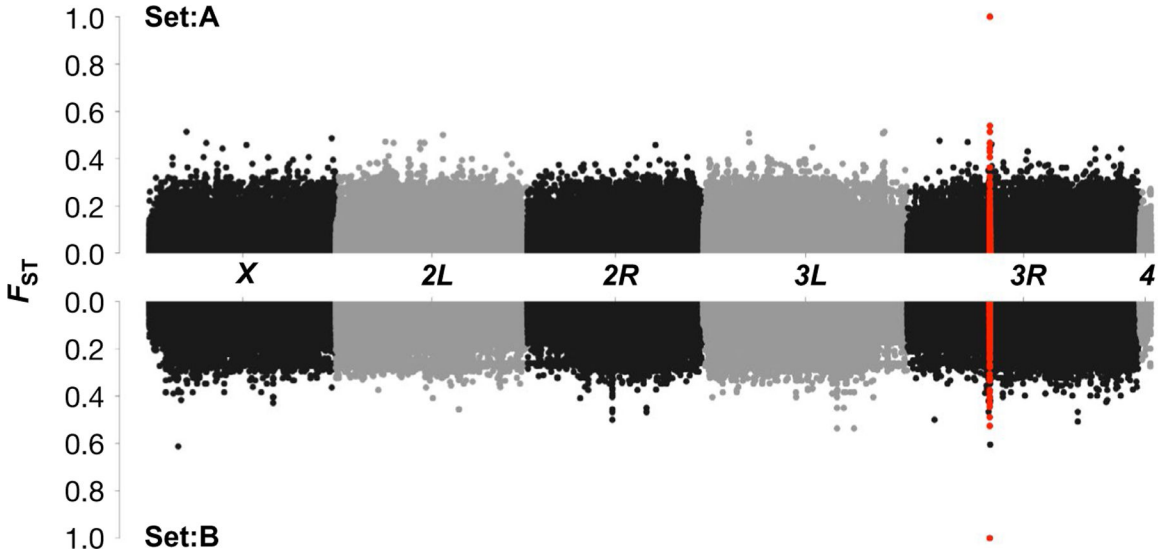

FIGURE 3.

F_{ST} Manhattan plots for the biological replicates that were constructed from independent sets of inbred lines from the DGRP panel. F_{ST} values for all SNPs at the foxo locus are highlighted in red. The analyses show that only the two focal *foxo* SNPs are fixed (F_{ST} =1) for alternative alleles in the low- and high-latitude population cages, and that this is consistent for both of the biological replicates (Set A, top; Set B, bottom); all other *foxo* SNPs shown in red do not reach fixation at F_{ST} = 1. Note that the two focal, fixed *foxo* SNPs are so close to each other that they appear as a single red dot at F_{ST} = 1, both in Set A and Set B. The construction of the recombinant outbred population cages, using 18 inbred lines that are fixed for the SNPs of interest, is thus not confounded by fixed differences between experimental populations at other positions in the genome [Colour figure can be viewed at wileyonlinelibrary.com]

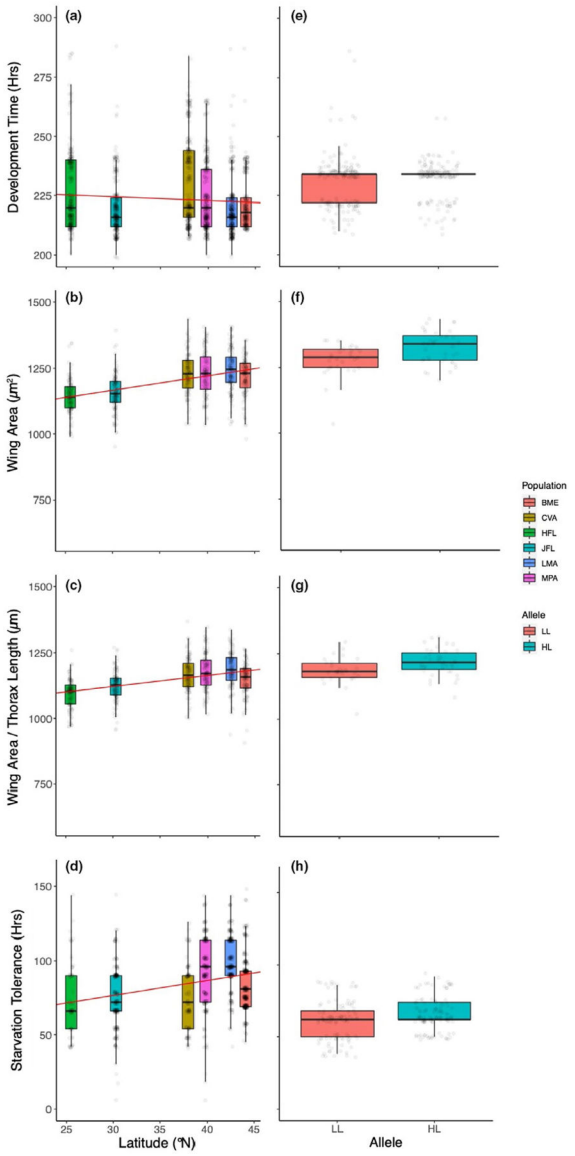

FIGURE 4.

Phenotypic variation for females. Trait variation among natural populations collected across the latitudinal gradient in the eastern U.S. is plotted on the left (a–d) and traits exhibited by the homozygous high- and low-latitude *foxo* genotypes are depicted on the right (e–h). Box plots show the median (50th percentile; bold horizontal line in the box) and the 25th and 75th percentile (upper and lower horizontal edges of the box); the upper whisker represents the maximum value of the data that falls within 1.5 times the interquartile range over the 75th percentile; the lower whisker is the minimum value of the data that is within 1.5 times the interquartile range under the 25th percentile. In (a–d), regression lines (regression of means to latitude) are in red (Table S3) and indicate the extent of clinality. Development time did not vary predictably with latitude (a), and was also equivalent between *foxo* alleles (e). Wing area (c) and the ratio of wing area to thorax length (d) exhibit a positive latitudinal cline; these patterns of size variation in the natural populations were mirrored in both

magnitude and direction by the observed differences in size parameters between the low and high-latitude *foxo* alleles (g, h). Starvation tolerance increased with increasing latitude (d); similarly, the high-latitude *foxo* allele was associated with increased starvation resistance (h) [Colour figure can be viewed at wileyonlinelibrary.com]

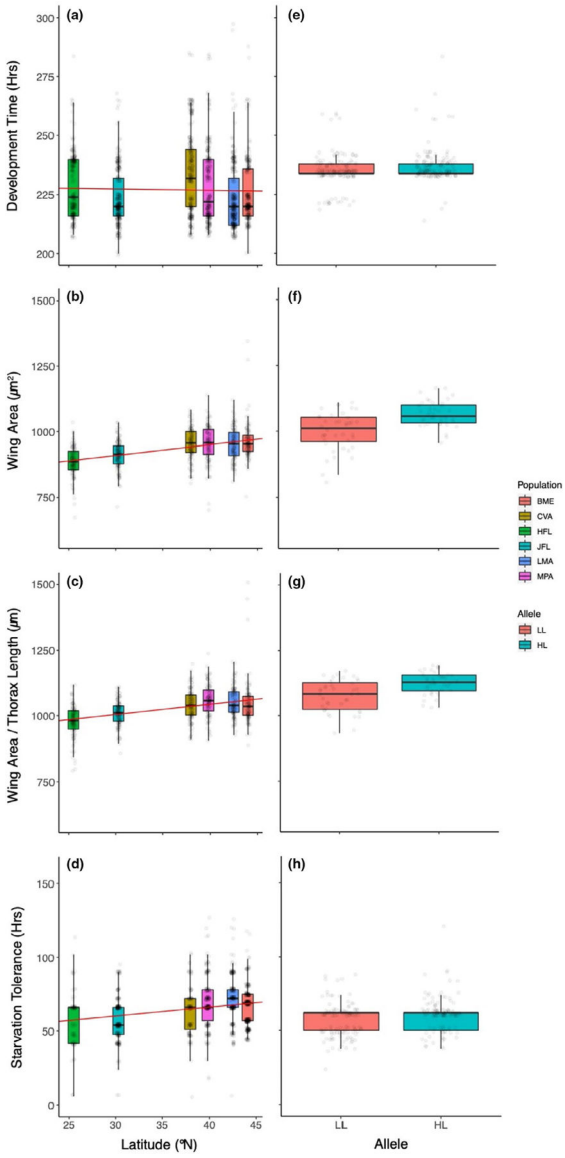

FIGURE 5.

Patterns of phenotypic variation for males largely mirror those observed for females. Natural populations are depicted in (a–d) and are arranged by increasing latitude of origin; *foxo* genotypes are given in (e–h). Clines and concordant differences between *foxo* genotypes were observed for size-related traits (b,c and f,g) and starvation tolerance (d,h); development time was not clinal but was distinct between the assayed *foxo* alleles. Box plots show the median (50th percentile; bold horizontal line in the box) and the 25th and 75th percentile (upper and lower horizontal edges of the box); the upper whisker represents the maximum value of the data that falls within 1.5 times the interquartile range over the 75th percentile; the lower whisker is the minimum value of the data that is within 1.5 times the interquartile range under the 25th percentile. In (a–d), regression lines (regression of means to latitude) are in red (Table S3) and indicate the extent of clinality. [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1

Analyses of variance (ANOVA) for the assayed phenotypic traits among natural populations from across the latitudinal cline. The models shown below account for the random effect of line nested in population (estimated with restricted maximum likelihood, REML), but we do not report variance component estimates here since they are of little biological interest. Note that we did not use experimental blocks for measuring starvation tolerance. See Materials and Methods for further details

	Females				Males			
	F	**df_{num}**	**df_{den}**	**p**	**F**	**df_{num}**	**df_{den}**	**p**
Developmental time								
Population	6.75	5	133.3	<.0001	6.42	5	132.1	<.0001
Block	69.69	2	649.8	<.0001	75.51	2	539.6	<.0001
Thorax length								
Population	4.71	5	135.4	.0005	3.28	5	140.9	.0078
Block	0.05	2	363.6	.96	1.96	2	365.8	.14
Wing area								
Population	14.15	5	136.8	<.0001	11.54	5	136.4	<.0001
Block	2.72	2	386.6	.067	4.71	2	394.9	.0095
Wing area/thorax length								
Population	13.91	5	135.6	<.0001	11.45	5	137.7	<.0001
Block	3.69	2	365.3	0.026	3.27	2	372.1	0.039
Starvation tolerance								
Population	10.41	5	116.5	<.0001	5.53	5	113.7	.0001

TABLE 2

Analyses of variance (ANOVA) for the phenotypic effects of the low-latitude (LL) and high-latitude (HL) alleles at the *foxo* locus. The models show the effects of allelic state, set nested in allele, and of replicate cage nested in the combination of allele and set. See Materials and Methods for further details

	Females			Males		
	F	**df**	**p**	**F**	**df**	**p**
Developmental time						
Allele	0.67	1	.41	8.73	1	.0034
Set (A)	2.44	2	.09	8.48	2	.0003
Cage (A,S)	7.04	4	<.0001	3.74	4	.0055
Thorax length						
Allele	2.85	1	.096	9.97	1	.0023
Set (A)	1.70	2	.19	4.95	2	.0097
Cage (A,S)	3.85	3	<.014	3.99	4	.0055
Wing area						
Allele	8.72	1	.0044	41.35	1	<.0001
Set (A)	2.05	2	.14	10.23	2	.0001
Cage (A,S)	0.78	3	.51	11.99	4	<.0001
Wing area/thorax length						
Allele	4.66	1	.035	34.19	1	<.0001
Set (A)	1.92	2	.16	6.36	2	.0029
Cage (A,S)	0.52	3	.67	13.95	4	<.0001
Starvation tolerance						
Allele	8.38	1	.0042	8.14	1	.0047
Set (A)	9.41	2	.0001	7.79	2	.0005
Cage (A,S)	4.63	4	.0013	2.36	4	.055