HHS Public Access

Author manuscript

J Evol Biol. Author manuscript; available in PMC 2017 May 01.

Published in final edited form as:

J Evol Biol. 2016 May ; 29(5): 1059–1072. doi:10.1111/jeb.12847.

Parallel effects of the inversion *In(3R)Payne* on body size across the North American and Australian clines in *Drosophila melanogaster*

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Abstract

Chromosomal inversions are thought to play a major role in climatic adaptation. In *D. melanogaster*, the cosmopolitan inversion *In(3R)Payne* exhibits latitudinal clines on multiple continents. Since many fitness traits show similar clines, it is tempting to hypothesize that *In(3R)P* underlies observed clinal patterns for some of these traits. In support of this idea, previous work in Australian populations has demonstrated that *In(3R)P* affects body size but not development time or cold resistance. However, similar data from other clines of this inversion are largely lacking; finding parallel effects of *In(3R)P* across multiple clines would considerably strengthen the case for clinal selection. Here, we have analyzed the phenotypic effects of *In(3R)P* in populations originating from the endpoints of the latitudinal cline along the North American east coast. We measured development time, egg-to-adult survival, several size-related traits (femur and tibia length, wing area and shape), chill coma recovery, oxidative stress resistance and triglyceride content in homokaryon lines carrying *In(3R)P* or the standard arrangement. Our central finding is that the effects of *In(3R)P* along the North American cline match those observed in Australia: standard arrangement lines were larger than inverted lines, but the inversion did not influence development time or cold resistance. Similarly, *In(3R)P* did not affect egg-to-adult survival, oxidative stress resistance and lipid content. *In(3R)P* thus seems to specifically affect size traits in populations from both continents. This parallelism strongly suggests an adaptive pattern, whereby the inversion has captured alleles associated with growth regulation and clinal selection acts on size across both continents.

Keywords

Inversions; clines; spatially varying selection; adaptation; body size; life history; *D. melanogaster.*

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Data accessibility

Raw data have been deposited at Dryad (doi:10.5061/dryad.8ns67).

Introduction

One of the central goals of evolutionary biology is to understand how organisms adapt to environmental heterogeneity (Hoffmann & Sgrò, 2011; Savolainen *et al.*, 2013). A promising approach towards this end is to investigate systematic, gradual phenotypic and genotypic changes along environmental (e.g., climatic) gradients, so-called "clines", that are thought to be driven by spatially varying selection (Mayr, 1963; Endler, 1977; de Jong & Bochdanovits, 2003; Charlesworth & Charlesworth, 2010).

A classical model system for studying clinality is *Drosophila melanogaster* (de Jong & Bochdanovits, 2003; Hoffmann & Weeks, 2007; Adrion *et al.*, 2015), an ancestrally tropical vinegar (fruit) fly that has migrated out of sub-Saharan Africa about 10,000 to 15,000 years ago and subsequently colonized the rest of the world as a human commensal (David & Capy, 1988; Keller, 2007). As a result of its colonization history, this species had to adapt to a wide range of climatic and ecological conditions, including temperate and seasonal habitats. This is evidenced by patterns of clinal differentiation of numerous life history, morphological, and physiological traits across latitude: clinally varying traits include development time (James & Partridge, 1995), body size (Coyne & Beecham, 1987; Imasheva *et al.*, 1994; James *et al.*, 1995, 1997; Zwaan *et al.*, 2000; Gockel *et al.*, 2001; Gibert *et al.*, 2004; Klepsatel *et al.*, 2014; Fabian *et al.*, 2015), wing loading (Stalker, 1980; Azevedo *et al.*, 1998), pigmentation (Telonis-Scott *et al.*, 2011), ovariole number (Capy *et al.*, 1993; Gibert *et al.*, 2004; Klepsatel *et al.*, 2014), diapause propensity (Schmidt *et al.*, 2005; Schmidt & Paaby, 2008), cold and heat resistance (Hoffmann & Shirriffs, 2002), and desiccation resistance (Hoffmann & Parsons, 2009).

Consistent with spatially varying selection, many of these traits exhibit parallel clinal patterns across latitude on multiple continents, even though demography (e.g., admixture) can also contribute to patterns of clinality (Kao *et al.*, 2015; Bergland *et al.*, 2015; Flatt, 2016). For example, qualitatively identical latitudinal clines have been reported across several continents for body size (Coyne & Beecham, 1987; James *et al.*, 1995; van't Land *et al.*, 1999; Klepsatel *et al.*, 2014; Fabian *et al.*, 2015), pigmentation (David *et al.*, 1985; Munjal *et al.*, 1997; Telonis-Scott *et al.*, 2011), and chill coma recovery time (Gibert *et al.*, 2001; Hoffmann *et al.*, 2002; Ayrinhac *et al.*, 2004).

Despite much work on phenotypic clines in *Drosophila*, and although several single genetic markers are known to covary latitudinally with trait clines (de Jong & Bochdanovits, 2003; Hoffmann & Weeks, 2007; Adrion *et al.*, 2015; and references therein), little is known about the genetics underlying clinal trait variation (for some exceptions see Schmidt *et al.*, 2008; Paaby *et al.*, 2014) and the mechanisms by which clines are formed and maintained. Recent progress comes from genome-wide studies of the Australian and North American clines that have identified hundreds of clinally varying single nucleotide polymorphisms (SNPs) (Kolaczkowski *et al.*, 2011; Fabian *et al.*, 2012; Bergland *et al.*, 2014; Reinhardt *et al.*, 2014; Bergland *et al.*, 2015; Kapun *et al.*, 2016). While some proportion of these clinal variants is expected to causally contribute to clinal trait variation, other variants might be subject to hitchhiking (genetic draft) or admixture (Fabian *et al.*, 2012; Bergland *et al.*, 2015; Kapun *et*

al., 2016). Thus, identifying the true genic targets of clinal selection remains a considerable challenge (Adrion *et al.*, 2015; Flatt, 2016).

Information on potentially functionally relevant genomic sites or regions might be gleaned from the genome-wide distribution of clinal SNPs. Remarkably, even though clinally varying SNPs occur throughout the genome, *the majority* of clinal variants is located on the right arm of the third chromosome (*3R*), especially within the region spanned by a large (~8 Mb), cosmopolitan chromosomal inversion, *In(3R)Payne* (also called *In(3R)P*) (Kolaczkowski *et al.*, 2011; Fabian *et al.*, 2012; Kapun *et al.*, 2016).

The *In(3R)P* inversion is of particular interest for four reasons. First, in several geographic areas (e.g., North American east coast, Australian east coast, India, Japan) this inversion exhibits steep, parallel latitudinal clines: the inverted karyotype reaches intermediate frequencies at low latitudes but is rare or absent at high latitudes (Mettler *et al.*, 1977; Inoue & Watanabe, 1979; Stalker, 1980; Knibb *et al.*, 1981; Knibb, 1982; Das & Singh, 1991; Matzkin *et al.*, 2005; Fabian *et al.*, 2012; Kapun *et al.*, 2014; Rane *et al.*, 2015; Kapun *et al.*, 2016). For example, along the North American cline this arrangement reaches a frequency of ~50% in southern Florida but is absent in Maine (Mettler *et al.*, 1977; Knibb, 1982; Fabian *et al.*, 2012; Kapun *et al.*, 2014, 2016); thus, flies from high-latitude populations are fixed or nearly fixed for the standard arrangement. Second, in Australia and North America, the latitudinal slopes of the *In(3R)P* clines have remained stable across >40 years of observation, consistent with the clines being maintained by spatially varying selection (Anderson *et al.*, 2005; Umina *et al.*, 2005; Kapun *et al.*, 2014, 2016); in Australia, the intercept of the clinal slope has recently shifted – possibly as a consequence of climate change (Anderson *et al.*, 2005; Umina *et al.*, 2005). Third, recent evidence suggests that the North American cline of *In(3R)P* is maintained non-neutrally and independent of population structure or admixture (Kapun *et al.*, 2016). Fourth, several inversions in *Drosophila* have previously been found to be associated with development time, egg-to-adult survival, size-related traits, fecundity and fertility, stress resistance (to cold, heat, starvation), and lifespan (Sperlich & Pfriem, 1986; Hoffmann *et al.*, 2004; Hoffmann & Weeks, 2007; Hoffmann & Rieseberg, 2008; and references therein). Thus, although many alleles within *In(3R)P* might be in linkage disequilibrium (LD) and thus subject to hitchhiking, the observation that the majority of clinal SNPs resides in the genomic region spanned by this inversion suggests that clinal trait variation might at least partly be driven by *In(3R)P* (de Jong & Bochdanovits, 2003; Fabian *et al.*, 2012; Kapun *et al.*, 2016).

Indeed, several association mapping studies have linked *In(3R)P* to clinal size variation among Australian populations (Weeks *et al.*, 2002; Rako *et al.*, 2006; Kennington *et al.*, 2007). Similarly, using quantitative trait locus (QTL) mapping, Calboli *et al.* (2003) found that the largest QTL peak for body size for the endpoints of the Australian and South American clines overlaps the region of *In(3R)P*. However, little is known about associations between *In(3R)P* and clinal phenotypes (including size) for other continents; finding parallel phenotypic effects of *In(3R)P* across multiple clines would considerably strengthen the case for spatially varying (clinal) selection. Moreover, effects of this inversion polymorphism on clinal fitness-related traits other than size remain largely unknown (cf. Rako *et al.*, 2006).

Here, we investigate – for the first time – the phenotypic effects of *In(3R)P* in populations that approximate the endpoints of the North American east coastal cline (southern Florida versus Maine). We measured several fitness-related traits thought to be clinal (development time, egg-to-adult survival, proxies of body size [femur length, tibia length, wing area, wing shape], chill coma recovery time, oxidative stress resistance, triglyceride content [a correlate of starvation resistance]) in isochromosomal homokaryon lines carrying *In(3R)P* or the standard chromosomal arrangement.

Our results for the effects of *In(3R)P* on several measures of body size mirror those previously observed in populations from the Australian cline (Weeks *et al.*, 2002; Rako *et al.*, 2006; Kennington *et al.*, 2007) – this strongly suggests the existence of parallel adaptive effects of *In(3R)P* on clinal size variation across both continents that are driven by spatially varying selection.

Materials and Methods

Fly stocks and maintenance

We used isofemale lines collected from populations that approximate the endpoints of the clinal gradient running along the North American east coast: a set of lines from subtropical southern Florida (Homestead and Jacksonville) and one from a temperate population in Maine (Bowdoin) (see Table 1; also see Schmidt *et al.*, 2005; Schmidt & Paaby, 2008; Fabian *et al.*, 2015 for further details on these populations). Since we failed to detect phenotypic differences between the two Florida populations (not shown), we combined lines from both populations for statistical analysis. Isofemale lines were kept for long-term maintenance under constant conditions at 18°C and 60% relative air humidity, at a photoperiod of 12h:12h light:dark.

All isofemale lines were screened for the presence of six cosmopolitan inversions (*In(2L)t, In(2R)NS, In(3L)P, In(3R)K, In(3R)Mo, In(3R)P*; see Lemeunier & Aulard, 1992) by extracting DNA from pools of 5–10 individuals from each line with a salt-chloroform extraction protocol and using PCR markers described in Matzkin *et al.* (2005) and Corbett-Detig *et al.* (2012). Consistent with previous data (Mettler *et al.*, 1977; Knibb, 1982; Kapun *et al.*, 2016), *In(3L)P* and *In(3R)P* segregated at intermediate frequencies in the subtropical samples from Florida but were absent in Maine. *In(3R)Mo*, in contrast, showed the opposite trend: it segregated at 11% frequency in Maine but was absent in Florida. None of the other inversions showed clinality (Table 1; also see below).

Generation of isochromosomal lines

To isolate wild-type chromosomes either carrying the inverted *In(3R)P* arrangement or the standard arrangement from isofemale lines (see above), we used a compound (second and third chromosome) balancer (*SMB6; TM6B*; Bloomington *Drosophila* Stock Center [BDSC], stock #5687) in an *ebony* (e^1) mutant background (Fig. S1). For a given isofemale line, we crossed a wild-type male from that line to a female carrying the balancer. F1 pupae heterozygous for the balancer were selected visually based on the dominant *tubby* (Tb^1) mutant phenotype. Upon eclosion, F1 adults were backcrossed to the balancer line to

amplify the isolated wild-type chromosome. After four days of egg laying, F2 adults were screened for the presence or absence of *In(3R)P* using PCR markers described in Matzkin *et al.* (2005). Isochromosomal homokaryon lines were generated by selecting against balancer phenotypes in F3 crosses.

We isolated 41 *3R* chromosomes carrying *In(3R)P* ("Florida inverted", FI) and 30 carrying the standard arrangement ("Florida standard", FS) from the two Florida populations, and 20 chromosomes carrying the standard arrangement from Maine ("Maine standard", MS). In total, we were able to generate 14 FI (34.1% of all FI isolates), 13 FS (43.3% of FS isolates) and 6 MS (30% of MS isolates) isochromosomal homokaryon lines for phenotyping (see below). For the remaining isolates we failed to obtain homokaryons, possibly due to recessive deleterious or lethal variants in the wild-type chromosomes; we maintained these lines as heterozygotes over a balancer chromosome but excluded them from the phenotypic assays reported here. We verified *3R* karyotype by using PCR on 3–5 single individuals per isolated chromosome, as described above.

During the isolation process we did not control for inversions on chromosomal arms other than *3R*: apart from *In(2L)t*, which segregated in ~30% of isolated lines, other inversions were either absent or present at only very low frequencies. Given that *In(2L)t* segregated at approximately equal proportions among the three sets of isochromosomal lines, we did not control for its effects in our analyses.

Phenotypic assays

General methods—Isochromosomal lines were used to measure several pre-adult life history traits (development time, egg-to-adult survival), stress-related and physiological traits (chill coma recovery time, oxidative stress resistance, triglyceride content), and proxies of body size (femur length, tibia length, wing area, wing shape) (see below). Isochromosomal lines were assigned randomized identifiers; assays were performed blind with respect to identifiers to eliminate potential bias. Vials or bottles were maintained and experiments performed at 25°C and 60% relative humidity, under a photoperiod of 12h:12h light:dark.

To avoid non-genetic parental and environmental effects assays were performed on flies from the F2 generation. Prior to the assays, we let 100 flies from each line oviposit for 2 days on standard (cornmeal-agar-yeast) medium. Eclosing F1 individuals were distributed into three replicate bottles (~200 flies per bottle) and aged for 5 days; flies were then transferred to new bottles and allowed to lay eggs for 3 hours. For each line, we collected 200 eggs and placed them into bottles containing 25 mL of standard medium. The positions of experimental bottles were randomized once per day to avoid potential effects caused by environmental heterogeneity inside the incubator. Eclosing F2 adults were collected every 6 hours during the day and every 12 hours overnight and aged for 3 days before being used for phenotypic assays (see below).

Pre-adult life history (development time and egg-to-adult survival)—To assess egg-to-adult development time and egg-to-adult survival (proportion viability) we recorded

eclosion times for each individual and estimated developmental time in hours relative to the time point of egg laying.

Chill coma recovery—Adults were aged for two days after eclosion prior to the chill coma recovery assay. 24 hours before the start of the assay, we anesthetized flies with CO_2 and created new subsets of up to 20 flies per sex and line in new vials with standard medium. To induce chill coma, flies were transferred to empty vials without anesthesia and vials placed on ice at 0°C for 3 hours. Flies were subsequently transferred to petri dishes at room temperature and visually monitored until they woke up. For each individual, the time elapsed between removal from ice and waking was recorded; a fly was deemed "awake" as soon as it was able to stand on all its legs. Flies from this assay were stored for triglyceride measurements at −20°C (see below).

Oxidative stress resistance—Adults were aged for two days after eclosion and split in two replicate subsets of 10 flies per sex and line 24h before the start of the assay. To induce oxidative stress, flies were transferred to media-free vials containing filter paper saturated with 5 mL of 30 mM methyl viologen (paraquat) (Sigma-Aldrich) in 5% sucrose solution (Paaby & Schmidt, 2008). To prevent evaporation, each vial was sealed with parafilm. We monitored mortality every two hours until ~ 90% of all flies had died. We continued monitoring flies in 8 hr intervals until all flies were dead. Corpses were preserved for morphometric measurements in ethanol (see below).

Triglyceride content—Since starvation resistance is often correlated with lipid content (Hoffmann and Harshman, 1999; Schmidt *et al.*, 2005; Goenaga *et al.*, 2013), we measured whole-body triglyceride (triacylglyceride [TAG]) content as a proxy. For each sample, we generated homogenates using 2 pooled flies and estimated serum TAG levels in micrograms per fly from blanks and standards run with each plate, using an enzymatic assay kit (Serum Triglyceride Determination Kit; Sigma-Aldrich) (also see McGowan *et al.*, 1983; Tennessen *et al.*, 2014).

Size-related traits and morphometric analysis—For morphometric measurements we removed the first right leg and right wing of each fly. Both body parts were mounted on slides with CC/Mount™ tissue mounting medium (Sigma-Aldrich) and sealed with cover slips. Images of legs and wings were taken with a digital camera (Leica DFC 290, Leica Microsystems GmbH, Wetzlar, Germany) attached to a stereo dissecting microscope (Leica MZ125). Femur and tibia length were measured as the distance between two sets of landmarks with ImageJ (v.1.47d), following the approach described in Debat *et al.* (2011).

To minimize measurement error, we repeated all measurements three times and used the average lengths for statistical analysis. For wing measurements, we used ImageJ (v.1.47d) to define two orientation landmarks at the distal side of the humeral break at the posterior end of the costal cell (C) and the notch at the sinus between the alula (Al) and the axillary cell (Ax) of the wing (Fig. S2). These landmarks were used to infer semi-landmarks and to fit B-splines along the outline of the wing and along wing veins with Wings4 and CPR software (van der Linde & Houle, 2009; http://bio.fsu.edu/dhoule/wings.html). Males and females were analyzed separately, and landmark data for every image were processed manually. We

applied multivariate outlier detection based on principal components analysis (PCA) of landmark coordinates using CPR and excluded extreme outliers caused by broken wings or images of insufficient quality. As a proxy for wing size we used total wing area, based on spline functions along the wing outline. Wing shape variation was analyzed using LORY software (http://bio.fsu.edu/dhoule/lory.html), following the methods described by Márquez *et al.* (2012). We obtained point estimates of shape deformation by locally evaluating Jacobian matrices of interpolation functions at pseudo-landmarks using LORY. Log ($-log_2$) -transformed determinants of Jacobian matrices contain information about local space contractions or expansions relative to a reference configuration and can be used as discrete summary variables that describe shape variation.

Deformations of individual configurations were analyzed relative to Procrustes-transformed landmark coordinates, averaged across all individuals for each sex. We fitted elastic body splines (EBS) as interpolation functions at 122 (females) and 124 (males) evenly distributed pseudo-landmarks and calculated log-transformed Jacobian determinants for each individual. To visualize shape differences, we averaged Jacobian determinants across all individuals for each pseudo-landmark, group (FI, FS, MS) and sex. To interpolate shape values between landmarks we performed “kriging” (Gaussian process regression) using the *R* package *kriging* and plotted wings by showing interpolated Jacobian determinants for each group and sex using custom software (available upon request from M.K). Finally, to examine variation in allometry between body parts among the three karyotypic groups (FI, FS, MS) we calculated the ratios of (1) femur length to tibia length, (2) femur length versus wing area, and (3) tibia length versus wing area.

Statistical analysis

Statistical analyses were performed using JMP (v.11.1.1) and *R* (v.3.2.1) software. Given that the *In(3R)P* is absent in Maine, we could not analyze data with a fully factorial (orthogonal) model, testing the effects of karyotype (standard versus inverted), geography (Florida versus Maine), and the karyotype by geography interaction. We thus created a compound grouping factor g with three levels (“Florida inverted”, FI; “Florida standard”, FS; “Maine standard”, MS) (also see below).

We first performed multivariate analysis of variance (MANOVA) to test the effects of karyotype and geography on multivariate phenotype (i.e., a linear combination of all measured traits, except wing shape [due to its high dimensionality] and size ratios), using the following model: $Y_i = g + s + g \times s$, where Y_i denotes the matrix of measured individual traits averaged by line and sex for the i^{th} line, g is the nominal fixed grouping factor (with levels FI, FS, MS), s denotes the fixed effect of sex, and $g \times s$ denotes the interaction term. We also used MANOVA to analyze multivariate wing shape based on multiple Jacobian determinants, separately for each sex, by using the following model: $Y_i = g + l_{(g)}$, where $l_{(g)}$ represents the effect of line nested within the grouping factor g (also see below).

Next, we analyzed each trait (including size ratios; see above) separately using a nested mixed-effects analysis of variance (ANOVA) model of the following form: $y_i = g + s + g \times s + l_{(g)}$, where y_i is the measured phenotype for the i^{th} individual, g denotes the grouping factor, s denotes sex, and $l_{(g)}$ is the random effect of line nested in g, estimated using

restricted maximum likelihood (REML). The random line effect was included to account for variation among lines, but we were not primarily interested in the variance component estimates of this effect; we therefore do not report these estimates.

To analyze egg-to-adult survival (proportion viability) we used the following ANOVA model: $arcsine\ squareroot\ (y_i) = g + s + g \times s$, where y_i is the proportion of egg-to adult survival of the i^{th} line and g and s denote the grouping factor and sex, respectively; note that in this analysis "line" was the lowest level of replication.

To tease apart the effects of karyotype and geography we performed post-hoc tests using Tukey's Honest Significant Difference (HSD) tests implemented in JMP, whenever the effect of the grouping factor g was significant; Tukey's HSD method corrects for multiple testing (i.e., the family-wise error rate). (For MANOVAs, we used planned contrasts instead since post-hoc tests were not available in JMP.) We were specifically interested in using these tests to determine the effects of *In(3R)P* karyotype; the effects of geography were only of secondary interest. Significant differences between FI and FS and between FI and MS, with the comparison FS versus MS being non-significant, imply a clear-cut effect of karyotype, and that the standard homokaryons from Florida and Maine have qualitatively identical effects. A pattern where FI versus FS, FI versus MS, and FS versus MS are all significantly different implies that inverted versus standard karyotypes differ in their effect, but that the two standard arrangement genotypes from Florida and Maine differ as well. In this situation, the effects of karyotype and geography can not be completely separated; nonetheless, the significant difference between FI and FS indicates an effect of *In(3R)P* karyotype. Under either scenario it thus seems safe to conclude that *In(3R)P* karyotype affects the phenotype of interest.

To compare our results for the differential effects of *In(3R)P* karyotype on wing area in North America to those from Australia (Queensland; Rako *et al.* 2006) we calculated Cohen's standardized effect sizes d (Cohen, 1988) (1) from lines means and standard deviations for the FI and FS lines from Florida (this study) and (2) from approximate values of line means and standard deviations of inverted and standard lines obtained from Fig. 1 in Rako *et al.* (2006), using the online tool WebPlotDigitizer (Rohatgi, 2015).

In contrast to size data, the assumptions of normality and homoscedasticity underlying ANOVA were not always fulfilled for other traits. Since data for development time, egg-to-adult survival, chill coma recovery and oxidative stress resistance represent failure time or time-to-event data that can violate ANOVA assumptions, we additionally analyzed these traits using mixed-effects Cox (proportional hazards) regression implemented in the *R* package *coxme* (Therneau, 2012), following the same model structure as defined above. These analyses yielded outcomes that were qualitatively identical to those based on ANOVA (not shown).

Results

Effects on multivariate phenotype

To account for potential phenotypic correlations among traits we performed MANOVA analysis of the multivariate phenotype, i.e. a linear combination of all measured traits (except wing shape; see below). Examination of contrasts for the grouping factor *g* (FI versus FS, FI versus MS, FS versus MS) indicated that inverted *In(3R)P* and standard arrangement differ in their effects on multivariate phenotype (Table S1; also see below and Table S3). The karyotypic effect of *In(3R)P* was most clearly revealed by the significant difference between the FI and FS groups. Inspection of contrasts also suggested that geographical origin (Florida versus Maine) might affect multivariate phenotype (Table S1). In particular, the significant difference between FS and MS might be consistent with an effect of geography; however, a non-mutually exclusive alternative is that standard arrangements from Florida and Maine differ genotypically in their effects upon phenotype.

Effects on pre-adult life history and stress resistance

Pre-adult life history traits (development time, egg-to-adult survival) were neither affected by *In(3R)P* karyotype nor by geography (Table 2). Similarly, karyotype and geography had no measurable effect on any of the stress resistance or physiological traits (chill coma recovery time, oxidative stress resistance, triglyceride content) (Table 2).

Effects on size, shape and allometry

In contrast to life history and stress resistance, inverted and standard chromosomal arrangements differed in their effects on size-related traits. Inverted and standard lines from Florida differed significantly for both femur and tibia length, suggesting an effect of *In(3R)P* on body size (Table 2). The tibiae of inverted homokaryons were significantly shorter than those of non-inverted lines for both sexes; the same effect was seen for femur length but only in males (Fig. 1, Table 2). Although for both traits standard arrangement lines from Maine did not differ from the two Florida karyotypes (Fig. 1, Table 2), we failed to identify a clear effect of geography when comparing lines from Florida and Maine without accounting for karyotype (not shown). These observations indicate that *In(3R)P* karyotype affects size, even though geographic differences independent of karyotype might also make a contribution.

The notion that *In(3R)P* inverted versus standard arrangements have differential effects on size was clearly confirmed by an analysis of variation in wing size: for both sexes, Florida inverted lines had significantly smaller wings than Florida standard and Maine standard lines, whereas standard arrangement lines from Florida and Maine did not differ from each other (Fig. 1, Table 2). Despite different measurement methods and sample sizes, we found that the effect sizes for wing size differences between inverted and standard karyotypes from low-latitude populations in North America (Florida; our data) and Australia (Queensland; Rako *et al.*, 2006) were large (i. e., Cohen's $d > 1.4$) and qualitatively very similar (Florida: $d=1.74$; Queensland, Australia: $d=1.64$) across both continents (Table S2).

MANOVA applied to a linear combination of femur length, tibia length and wing area, thus accounting for potential intercorrelations among size-related traits, also revealed significant

among-group contrasts consistent with effects of karyotype and geography on size (Table S3).

We next analyzed among-group variation in wing shape. Contrasts from MANOVA performed on Jacobian determinants of pseudo-landmarks showed significant effects of karyotype and geography on wing shape for both sexes (Table S4). Florida inverted and Maine standard lines differed most strongly in their effects on wing shape, with Florida standard lines being intermediate. In both sexes, areas that showed largest variation for wing shape were located at the proximal part of the wing around the humeral break, around the terminal end of the distal (L5) wing vein, and at the distal end of the 1st posterior (1P) wing cell (Fig. 1, Fig. S2).

We also examined whether the three groups differ in allometry by analyzing among-group variation in the size ratios of leg parts (femur length versus tibia length) and different body parts (femur length versus wing area, tibia length versus wing area). While we failed to detect effects for the ratio of femur:tibia length, both group and sex affected the ratios of leg parts to wing area, with the ratios being larger for males than females (Table 2, Fig. S3). This suggests that in males wing size is smaller relative to leg size. For both measures of leg:wing size, Florida inverted lines exhibited larger ratios than Maine standard lines, irrespective of sex. The effect of *In(3R)P* karyotype was most clear-cut for the femur length:wing area ratio in males: Florida inverted lines had a greater ratio than both Florida and Maine standard lines, while standard lines from Florida and Maine did not differ from each other (Table 2, Fig. S3).

Together, our results indicate that *In(3R)P* affects multiple aspects of body size, shape and allometry but does not seem to have detectable effects upon pre-adult life history, stress resistance (e.g, chill coma recovery, oxidative stress resistance), and fat content.

Discussion

Chromosomal inversion polymorphisms are commonly found in *D. melanogaster* populations (Lemeunier & Aulard, 1992) but evidence for selection acting on them is surprisingly scarce (Kapun *et al.*, 2016). In support of a role for selection, *In(3R)Payne*, a cosmopolitan inversion that is clinally distributed along latitudinal gradients in Australia and North America, has been associated with body size clines in Australian populations (Weeks *et al.*, 2002; Rako *et al.*, 2006; Kennington *et al.*, 2007). However, comparable phenotypic data from other continents are not available, and whether the observations from the Australian cline represent a local phenomenon or a general pattern remains unclear. Moreover, effects of this inversion on traits other than size remain largely unknown (cf. Rako *et al.*, 2006). Here we have investigated the phenotypic effects of *In(3R)P* in populations originating from the endpoints of the latitudinal cline running along the North American east coast.

In(3R)P has parallel effects on size across the North American and Australian clines

Our study provides the first evidence for an association between *In(3R)P* and the body size cline (cf. Coyne & Beecham, 1987) in North America. For the endpoints of the Australian

cline, Rako *et al.* (2006) reported that flies carrying *In(3R)P* had smaller wings than standard arrangement flies. Similarly, for several proxies of body size, we found that inverted flies from the North American cline are smaller than flies carrying the standard chromosomal arrangement. Our findings thus mirror previous observations from the Australian cline (Weeks *et al.*, 2002; Rako *et al.*, 2006; Kennington *et al.*, 2007) and suggest that *In(3R)P* has parallel – very likely adaptive – effects on body size along both clinal gradients (cf. Kapun *et al.*, 2016).

Another size trait known to exhibit clinal variation on multiple continents – and thus likely to be subject to spatially varying selection – is wing "loading" (the intercept of the relationship between body and wing size) (Azevedo *et al.*, 1998; Gilchrist *et al.*, 2000). Stalker (1980), for example, reasoned that larger wings relative to body size (i.e., low wing loading) might result in increased lift and would thus compensate for lower beat frequencies at lower temperatures experienced at higher latitudes. Perhaps consistent with this prediction, we observed lowest wing loading for standard arrangement lines from Maine, intermediate loading in standard arrangement lines from Florida, and highest loading in inverted lines from Florida. It is noteworthy in this context that QTL mapping has identified a major peak for male flight duration within the region spanned by *In(3R)P* (Luckinbill *et al.*, 2005; see discussion in Rako *et al.*, 2006).

We also found karyotypic and geographic variation in wing shape. Inverted lines from Florida and standard arrangement lines from Maine differed most strongly in wing shape, while standard lines from Florida showed an intermediate pattern. Consistent with observations by Gilchrist *et al.* (2000), who investigated wing shape variation along size clines from three continents (albeit without examining *In(3R)P*), we observed large shape deformations in the anterior distal region between the medial and cubital vein. Moreover, we identified large shape differences at the discal cell and the 3rd posterior cell along the distal vein (L5), indicating shape expansion in Florida inverted lines but shape contraction in Maine standard lines. In contrast, shape differentiation was minimal along the leading edge of the wing. This is in good agreement with kinetic analyses of wing aerodynamics: the anterior-posterior wing region might potentially be functionally constrained since it maintains the rotation axis close to the leading edge (Dickinson *et al.*, 1999; Gilchrist *et al.*, 2000). However, the evolutionary mechanisms that maintain variation in wing shape remain poorly understood; while wing size is subject to directional selection, wing shape seems to be the result of optimizing (stabilizing) selection (potentially due to selection for "canalization" [Flatt, 2005]) rather than directional selection (Gilchrist and Partridge, 2001). Additional data will be required to unravel the potentially adaptive effects of *In(3R)P* on variation in wing shape.

In(3R)P and the genetic basis of size and shape

Further support for potentially causal links between *In(3R)P* and size-related traits comes from studies of the genetic basis of size and shape variation in *Drosophila* (see de Jong & Bochdanovits, 2003; Mirth & Shingleton, 2012; and references therein). Gockel *et al.* (2002) and Calboli *et al.* (2003), for example, used QTL analysis to map genetic variation associated with thorax length and wing size and found that the third chromosome accounts

for a major proportion of size variation between the endpoints of the Australian and South American clines. Weeks *et al.* (2002) identified three indel (insertion deletion) and microsatellite polymorphisms within the region spanned by *In(3R)P* that are strongly associated with body size variation among Australian populations. Similarly, Kennington *et al.* (2007) found that microsatellite alleles associated with decreased wing size are in strong LD with *In(3R)P*. Moreover, the gene *Dca* (*Drosophila cold acclimation*; also known as *smp-30*), which is located close to the proximal breakpoint of *In(3R)P* and likely associated with this inversion through hitchhiking, accounts for approximately 5–10% of natural wing size variation in Australian populations (McKechnie *et al.*, 2010), and a clinal promoter polymorphism in this gene has been shown to decrease wing size (McKechnie *et al.*, 2010; Lee *et al.*, 2011).

In agreement with these findings, the region spanned by *In(3R)P* harbors several genes known to be important for growth regulation and the determination of body size (de Jong & Bochdanovits, 2003; Fabian *et al.*, 2012; Kapun *et al.*, 2016; see flybase.org for details of gene function and original source references). For example, *In(3R)P* contains multiple loci involved in insulin/insulin-like growth factor signaling (IIS), a pathway that plays a major role in regulating growth, size and shape, including *InR* (*insulin-like receptor*), *Tsc1* (*tuberous sclerosis complex 1*), and *Pi3K* (*Pi3K92E*, *phosphoinositide 3-kinase at 92E*; also known as *Dp110*) (Brogiolo *et al.*, 2001; de Jong & Bochdanovits, 2003; Oldham & Hafen, 2003; Edgar, 2006; Shingleton *et al.*, 2007; Mirth & Shingleton, 2012; Nässel *et al.*, 2015; also see below). Importantly, *InR* harbors many alleles that are strongly clinal along the North American east coast (Fabian *et al.*, 2012; Paaby *et al.*, 2014); indeed, a naturally occurring, clinal indel polymorphism in *InR* (albeit apparently not in LD with *In(3R)P*) affects body size in North American populations (Paaby *et al.*, 2014).

Whole-genome analyses of clinal variation associated with *In(3R)P* have also uncovered candidates with known effects on growth, including clinally varying alleles in *InR* (see above), *Tsc1* (see above), *Hmgcr* (*hydroxymethlyglutaryl coenzyme A reductase*, known to interact with IIS), *Orct2* (*organic cation transporter 2* or *calderón*, involved in IIS as well) and *Stat92E* (*signal-transducer and activator of transcription protein at 92E*, a transcription factor involved in JAK/STAT signaling) (Fabian *et al.*, 2012; Kapun *et al.*, 2016). Several of these genes, including *InR*, *Orct2* and *Stat92E*, also vary clinally along the Australian cline (Kolaczkowski *et al.*, 2011).

Two other interesting candidates are *hh* (*hedgehog*) and *Dad* (*Daughters against DPP*), both of which harbor clinal alleles associated with *In(3R)P* in North America (Fabian *et al.*, 2012; Kapun *et al.*, 2016). The *hh* locus encodes a signaling protein, which forms gradients in the developing wing and controls the placement and spacing of the longitudinal wing veins L3 and L4 (Blair, 2007; Matamoro-Vidal *et al.*, 2015). Perhaps consistent with the involvement of this gene, we identified strong variation in the spacing of these veins among karyotypes (see Fig. 1). *Dad* encodes a negative regulator of *Dpp* (*Decapentaplegic*), a morphogen that modulates the placement of the L2 and L5 wing veins (Tsuneizumi *et al.*, 1997; Matamoro-Vidal *et al.*, 2015); notably, we observed strong shape variation among karyotypes within the 3rd posterior cell along the L5 vein.

Thus, multiple lines of evidence suggest that *In(3R)P* harbors clinal variants in several major genes known to affect growth, size and shape. Although the causative effects of *In(3R)P*-linked alleles at these loci on size and shape remain unknown, these variants represent promising candidates for functional testing (cf. Kapun *et al.*, 2016).

In(3R)P has no measurable effects on pre-adult life history or stress resistance

Little is known about whether *In(3R)P* affects traits other than size. For example, with regard to Australian populations, a study by Anderson *et al.* (2003) reported an association between cold resistance and *In(3R)P*, and McColl *et al.* (1996) found an association between the response to thermal selection and the *hsr-omega* and *hsp68* genes, both located in the region spanned by *In(3R)P* (Anderson *et al.*, 2003). However, Rako *et al.* (2006), using a more direct genetic association approach based on *In(3R)P* homokaryon lines, failed to find an effect of *In(3R)P* on cold resistance. These findings are in good agreement with ours: we also did not detect any measurable effects of *In(3R)P* on cold resistance. Although several genes known to be involved in cold resistance are located within the region of *In(3R)P* (Anderson *et al.*, 2003), it is unknown whether alleles at these loci are in LD with this inversion (cf. Weeks *et al.* 2002; Rako *et al.*, 2006).

Rako *et al.* (2006) also found no effects of *In(3R)P* on development time for the Australian cline, an observation that is again consistent with ours. Given the usually tight physiological and genetic correlations between development time and body size (e.g., in artificial selection or experimental evolution experiments; see de Jong & Bochdanovits, 2003; and references therein), it is perhaps surprising that *In(3R)P* does not affect development time. However, clinal patterns for this trait often seem to be weak (James & Partridge, 1995) or absent (Fabian *et al.*, 2015); in line with this, development time and body size do not seem to be associated among populations along the Australian cline (James *et al.*, 1995). This raises the interesting but unresolved question of how, in terms of physiological mechanisms, *In(3R)P* affects size.

We also measured several traits that were not assayed by Rako *et al.* (2006), including egg-to-adult survival, oxidative stress resistance and triglyceride content; however, again, we could not find any measurable effects of *In(3R)P* on these traits. For the South American cline, Robinson *et al.* (2000) also failed to find a cline for fat content (and starvation resistance), albeit without examining *In(3R)P*. Together with the previous findings from Australia, our results therefore suggest that *In(3R)P* might have quite specific effects on size-related – but not necessarily other fitness-related – traits; yet, two important caveats remain. First, this inversion might have subtle effects on the non-significant traits we have measured but our statistical power for finding these effects was perhaps insufficient. Secondly, there are other major fitness-related traits known to be clinal (e.g., ovariole number, fecundity, lifespan, reproductive diapause) that we have not measured as a function of *In(3R)P* karyotype.

The adaptive significance of *In(3R)P*

The *In(3R)P* polymorphism exhibits steep, persistent latitudinal frequency clines between subtropical/tropical and temperate, seasonal environments on multiple continents (e.g.,

North America, Australia, Indian subcontinent, Japan), but – intriguingly – does not seem to be clinal within the tropics proper (e.g. sub-Saharan Africa, Southeast Asia) (Aulard *et al.*, 2002; Glinka *et al.*, 2005). This strongly suggests that the inverted arrangement is selectively favored in warm, low-latitude habitats, whereas the standard arrangement is favored in temperate, seasonal, high-latitude habitats.

Recent findings indeed support the notion that latitudinal clines of *In(3R)P* are maintained by spatially varying selection: in North America the latitudinal cline of *In(3R)P* has remained stable for >40 years, deviates from neutral expectation, and is maintained independent of isolation by distance and admixture (Kapun *et al.*, 2016). Moreover, the majority (>90%) of the most strongly clinally varying single nucleotide polymorphisms (SNPs) contained in *In(3R)P* are shared between the North American and Australian clines, consistent with parallel effects of spatially varying selection across both continents (Kapun *et al.*, 2016).

Interestingly, in areas where *In(3R)P* is known to be clinal (e.g., North America, Australia, India, Japan), body size also exhibits latitudinal clines (see Introduction). Together with the observation that *In(3R)P* is associated with body size in both Australia and North America, this suggests that *In(3R)P* clines might be driven by selection on body size. While the selective forces shaping body size clines still remain largely unknown (Partridge & Coyne, 1997), thermal experimental evolution experiments in *Drosophila* have shown that adaptation to warm versus cool conditions favors small versus large size (Partridge *et al.*, 1994). Thus, temperature might represent the most parsimonious selective agent underlying latitudinal size clines. As hypothesized by James & Partridge (1995), a possible reason for the existence of a temperature-latitude-size correlation in *Drosophila* could be that larval food resources might be more ephemeral in the tropical climates due to increased competition, and that this would cause selection to favor rapid development and thus smaller adult size. In temperate habitats, in contrast, resources might be more stable and selection might thus favor longer development time and larger adult size (James & Partridge, 1995). Even though we did not find an effect of *In(3R)P* on development time, the fact that *In(3R)P* causes smaller size (through as of yet unknown developmental effects) and that its frequency is much more prevalent in warmer areas might be consistent with such a scenario.

The idea that inversions such as *In(3R)P* might be shaped by climatic adaptation is underscored by several observations. First, in North America *In(3R)P* frequency is strongly positively associated with multiple measures of temperature and precipitation, whereas temperature dispersion (range) and seasonality seem to favor higher frequencies of the standard chromosomal arrangement (Kapun *et al.*, 2016; also see Knibb, 1982). Second, along the Australian east coast, the latitudinal cline of *In(3R)P* has shifted in position (intercept) across a time span of 20 years in response to recent climate change; since no single climatic factor could fully account for this pattern, it is likely that a combination of climatic variables, not temperature alone, has driven this shift (Umina *et al.*, 2005). Third, in support of climatic selection, we have previously found in an experimental evolution experiment that *In(3R)Mo* and *In(3R)C*, two inversions that partly overlap with *In(3R)P*, were selectively favored in replicate populations exposed to cold versus warm temperatures, respectively (Kapun *et al.*, 2014). However, an important caveat is that in the same

experiment *In(3R)P* itself was rapidly lost, from an initial frequency of ~20%, in both cold and warm environments. Thus, together with the findings mentioned above, unknown selective factors other than – or in addition to –temperature must play a major role in maintaining this inversion. It will clearly be of great interest – as well as a major challenge – to determine the selective factors affecting *In(3R)P* in future work.

Conclusions

Here, we have demonstrated that the chromosomal inversion *In(3R)P* affects several size-related traits in North American populations of *D. melanogaster*. Remarkably, these effects go in the same direction – and are of similar magnitude (e.g., see Table S2) – as those that have been previously reported for the Australian cline (Rako *et al.*, 2006). In conjunction with the Australian data, our results thus suggest a major role of *In(3R)P* in shaping clinal size variation across *both* continents, thereby considerably strengthening the case for spatially varying selection acting on body size via genetic variants contained within this inversion. However, the effects we have identified here remain correlational; future efforts will be required to dissect the functional links between size and the causative genetic variants harbored by this inversion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank two anonymous reviewers and Kathrin Garschall for helpful comments; David Houle, Brian Hollis and Fiona Hollis for advice on morphometrics; Tad Kawecki, Jérôme Goudet and Tanja Schwander for statistical advice; and Daria Martynow for help in the laboratory. Our research was supported by the Swiss National Science Foundation (SNSF) (grant PP00P3_133641 to T.F.), the National Institutes of Health (NIH) (grant 5R01GM100366 to P.S.S.), the National Science Foundation (NSF) (grant DEB 0921307 to P.S.S.), and the Department of Ecology and Evolution at the University of Lausanne.

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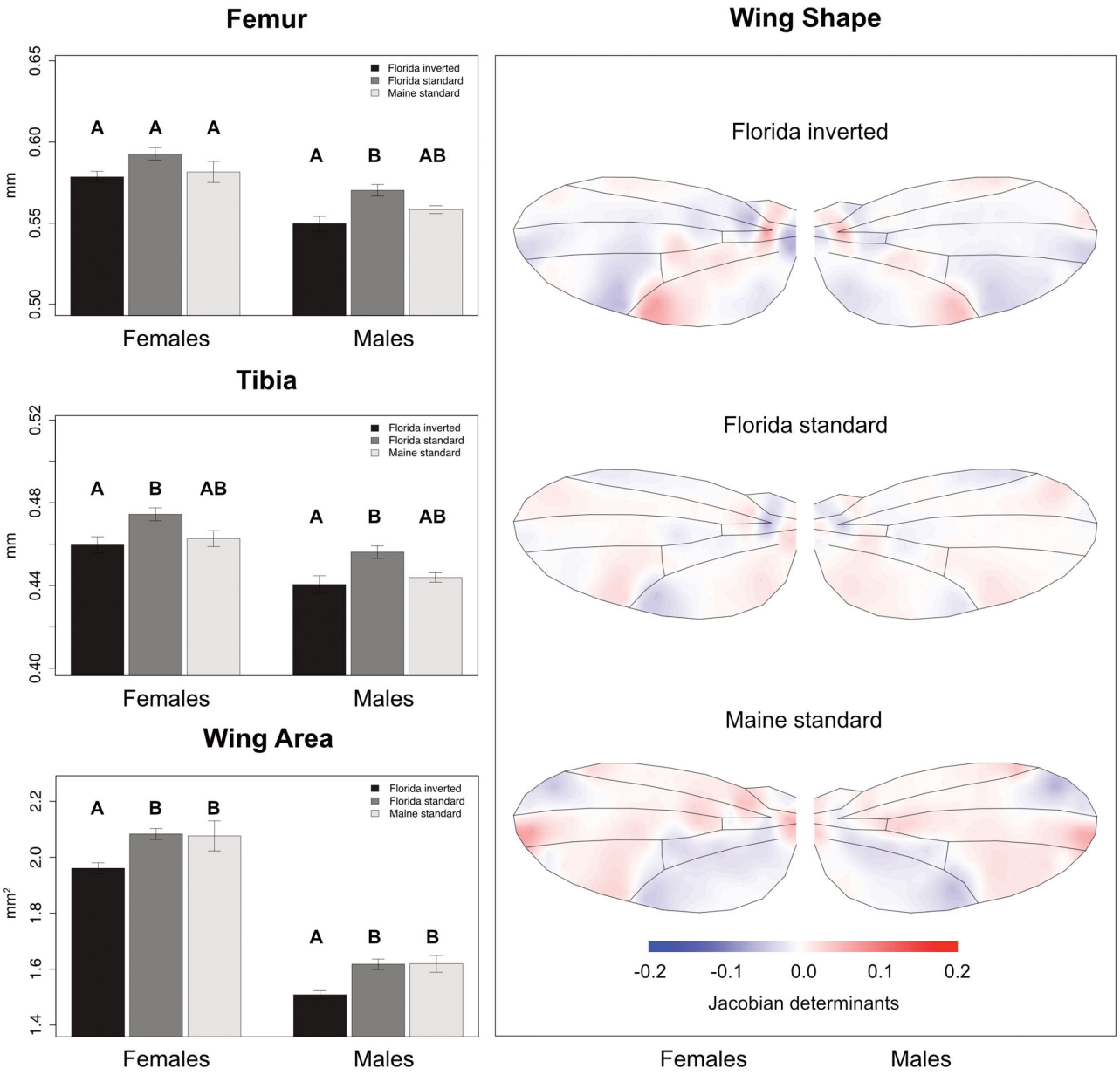

Figure 1.

The effects of *In(3R)P* on size-related traits. The left panel shows trait values averaged across line means for the three different groups differing in *In(3R)P* karyotype ("Florida inverted", FI; "Florida standard", FS; "Maine standard", MS). Error bars show standard errors. Letters above bars show the outcomes of Tukey's HSD post-hoc tests, carried out for each sex separately: groups that not containing the same letter are significantly different ($p<0.05$). The right panel shows average wing outlines and Jacobian determinants for each of the three groups (FI, FS, MS). Jacobian determinants, interpolated with kriging, represent local expansion (positive values; red) or contractions (negative values; blue) relative to the grand mean.

Table 1

Summary of samples used in this study and estimates of inversion frequencies. *N* = number of isofemale lines screened to isolate *3R* homokaryons.

Location	State	*N*	Latitude	Longitude	Date	Collector	Inversion frequencies					
							In(2L)t	*In(2R)NS*	*In(3L)P*	*In(3R)K*	*In(3R)Mo*	*In(3R)P*
Homestead	Florida	51	25.5°N	−71.06 °E	5/2011	P. Schmidt	0.38	0.06	0.42	0.09	0.00	0.63
Jacksonville	Florida	32	30.3°N	−81.6°E	8/2011	R. Cogni	0.63	0.05	0.20	0.42	0.09	0.31
Bowdoin	Maine	35	42.3°N	−80.5°E	10/2012	P. Schmidt	0.43	0.03	0.00	0.03	0.11	0.00

Table 2

Mixed-effects ANOVA tables for phenotypic analyses.

Trait	Factors		
	group (*g*)	**sex (*s*)**	***g* × *s***
Development time (h)	$F_{2,31}$= 1.07	$F_{1.3554}$= 402.52***	$F_{2,3554}$= 0.06
Egg-to-adult survival (%)	$F_{2,62}$= 2.88	$F_{1,62}$= 3.12	$F_{2,62}$= 0.577
Wing area (mm^2)	$F_{2,29}$= 10.24**	$F_{1,1075}$= 3551.66***	$F_{2,1075}$= 0.89
Femur length (mm)	$F_{1,29}$= 6.3**	$F_{1,1053}$= 525.04***	$F_{2,1053}$= 5.1**
Tibia length (mm)	$F_{1,29}$= 6.39**	$F_{1,1053}$= 318.66***	$F_{2,1053}$= 0.23
Femur-to-tibia ratio	$F_{1,28}$= 0.9	$F_{1,1059}$= 0.9	$F_{2,1059}$= 0.9
Femur-to-wing area ratio	$F_{1,29}$= 7.72**	$F_{1,1056}$= 2268***	$F_{2,1056}$= 2.58
Tibia-to-wing area ratio	$F_{1,29}$= 5.77**	$F_{1,1055}$= 2119***	$F_{2,1055}$= 3.4*
Chill coma recovery (time to recovery, h)	$F_{1,28}$= 1.29	$F_{1,1041}$= 20.3***	$F_{2,1040}$= 9.09**
Oxidative stress resistance (age at death, h)	$F_{1,29}$= 0.56	$F_{1,1183}$=0.65	$F_{2,1183}$= 0.03
Triglyceride content (μg)	$F_{1,29}$= 0.61	$F_{1,488}$=264.76***	$F_{2,488}$= 2.68

* p <0.05;

** p < 0.01;

*** p < 0.001.

Significant among-group effects for the grouping factor *g* were analyzed using Tukey's HSD post-hoc tests; results of these tests are shown in Fig. 1. See Materials and Methods and Results sections for further details.