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Altitudinal Clinal Variation in Wing Size & Shape in African *Drosophila melanogaster*: One Cline or Many?

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Abstract

Geographical patterns of morphological variation have been useful in addressing hypotheses about environmental adaptation. In particular, latitudinal clines in phenotypes have been studied in a number of *Drosophila* species. Some environmental conditions along latitudinal clines – e.g. temperature – also vary along altitudinal clines, but these have been studied infrequently and it remains unclear whether these environmental factors are similar enough for convergence or parallel evolution. Most clinal studies in *Drosophila* have dealt exclusively with univariate phenotypes, allowing for the detection of clinal relationships, but not for estimating the directions of co-variation between them. We measured variation in wing shape and size in *Drosophila melanogaster* derived from populations at varying altitudes and latitudes across sub-Saharan Africa. Geometric morphometrics allows us to compare shape changes associated with latitude and altitude, and manipulating rearing temperature allows us to quantify the extent to which thermal plasticity recapitulates clinal effects. Comparing effect vectors demonstrates that altitude, latitude and temperature are only partly associated, and that the altitudinal shape effect may differ between Eastern and Western Africa. Our results suggest that selection responsible for these phenotypic clines may be more complex than just thermal adaptation.

Keywords

latitude; allometry; geometric morphometrics; thermal plasticity; adaptation; thermal plasticity

Introduction

How predictable are evolutionary responses to predictable environmental variation? Geographical patterns of variation for ecological variables provide opportunities to address such questions about adaptive evolution, by providing evidence of association between the environmental gradient of the putative agent of selection and the target phenotype. Indeed such studies of clinal variation can often provide an initial indication for candidate adaptive phenotypes. This approach has been taken with a number of *Drosophila* spp., and researchers have characterised geographical variation in many aspects of the *Drosophila* phenotype, including physiological, (e.g. (Gibert & Huey 2001)), biochemical (Eanes 2010), morphological (e.g. (Bubliy & Loeschcke 2005)) and life history (Schmidt et al. 2005; Schmidt et al. 2008; Rako et al. 2009). These patterns of apparent clinal variation track a measured or imputed ecological gradient (Starmer et al. 1997; James & Partridge 1998; Hoffmann et al. 2002; Bubliy & Loeschcke 2005). Where clines are well-studied, co-variation with the gradient has been found for multiple characters; for example the

latitudinal cline along the eastern coast of Australia has been demonstrated to be predictive for several traits in *D. melanogaster* including body size (James et al. 1997), egg size (Azevedo et al. 1996), competitive ability (James & Partridge 1998) and pigmentation (Telonis-Scott et al. 2011). Patterns of ecological variation like these have been exploited by investigators working on the genetic and molecular basis of adaptive changes in these traits: such as the genetics of body size clines (A. Gilchrist & Partridge 1999; Paaby et al. 2010; McKechnie et al. 2010; Lee et al. 2011) and temperature-related variation in the regulation of heat shock protein expression (Sorensen et al. 2001). Furthermore, pairs of potentially parallel clines can be compared in order to address the issues of contingency/repeatability in phenotypic evolution (e.g. (Huey et al. 2000; G. Gilchrist et al. 2001)), and its genetic basis (Turner et al. 2008).

Notably, the majority of work on clinal variation in the *Drosophila* literature addresses latitude, with differentiation of size related traits being particularly well documented (Capy et al. 1993; Imasheva et al. 1994; James & Partridge 1998; Huey et al. 2000; Hallas et al. 2002). Whilst there are a number of climatic and ecological factors that may vary along a latitudinal cline, one of the most predictable of these factors is temperature, with evidence implicating it as a selective agent responsible for latitudinal clines for several traits (Reeve et al. 2000; Norry et al. 2001; Bublly & Loeschcke 2000; Santos et al. 2006). One complication in making inferences from latitudinal variation is that, while temperature may vary predictably over latitude, there are many other factors whose variation may be unpredictable over distances of even a degree of latitude (which is more than 100km). Comparison with altitudinal gradients, which occur over much smaller distances, may thus be informative.

Altitudinal gradients have, in common with latitudinal gradients, an associated temperature gradient. If temperature represents an important agent of selection, we would predict that adaptations to altitude would recapitulate adaptations to latitude (Lencioni 2004). Though altitudinal clines have been widely studied in other insects (reviewed by Hodkinson 2005), they have been under-utilized by *Drosophila* researchers. Where such altitudinal clines have been studied, clinal increases in size-related traits – similar to those found along latitudinal clines – have been documented in *D. buzzatii* (Dahlgaard et al. 2001; Sambucetti et al. 2006), *D. mediopunctata* (Bitner-Mathe & Klaczko 1999), *D. birchii* (Bridle et al. 2009), *D. nepalensis* and *D. takahashi* (Parkash et al. 2005) with some evidence for *D. melanogaster* (Collinge et al. 2006). Bublly & Loeschcke (2005) also collected data from a potential altitudinal cline in *D. simulans*, but found neither life-history nor morphological traits to be co-varying with altitude. Whilst high altitude and high latitude share low temperatures – which is likely to be important in small ectotherms like insects – there are other potential agents of selection that may be important at high altitude but are unrelated to latitude. In particular, air density and therefore the partial pressure of oxygen decreases with altitude, and both have the potential to influence insect physiology (Dillon et al. 2006). In addition, with increased altitude come steeper local temperature gradients (e.g. between shade and sunlight), increased incident UV radiation and increased rates of evaporation due to decreased water vapour pressure (Somme 1989). The widely touted ‘Bergmann’s Rule’ of increased body size at lower temperatures has support from some studies of altitudinal variation in *Drosophila* (e.g. Bitner-Mathe & Klaczko 1999; Dahlgaard et al. 2001) but not others (e.g. Norry et al. 2001; Bublly & Loeschcke 2005), and no pattern has been found in wild-caught flies (reviewed by Dillon et al. 2006). Since reduced temperature alone may select for increased body size (Reeve et al. 2000), the lack of this pattern may be due to other altitudinal factors or their interaction with temperature (Dillon & Frazier 2006). In *D. melanogaster*, locomotor performance at high altitude appears to be affected by just such an interaction between temperature and air pressure, with flight performance being more

strongly reduced than walking speed (Dillon & Frazier 2006). Wing morphology, therefore, might experience selection for clinal variation even if body size did not.

An obstacle in assessing whether altitudinal and latitudinal clines demonstrate similar associations with a phenotype (thus implying a common agent of selection such as temperature) is the “low dimensionality” of the measured phenotypes. For instance, if we observe an increase of body size with both altitude and latitude it may well be due to a common agent of selection (such as temperature) acting on size. However, since body size can only vary along a single axis (smaller – larger), there can be – with sufficiently large sample sizes, and therefore high power – a 50:50 chance of both of the environmental gradients showing a “significant” effect in the same direction. Working in this simple phenotypic space therefore reduces our confidence that biologically important agents of selection act similarly across altitude and altitude. However, as the dimensionality of the “trait” increases, the chance of spurious changes in the same direction (i.e. that independent vectors point in the same direction) decreases. In such cases it follows that an integrated multivariate approach (working in a high dimensional phenotypic space) is advantageous, since we can ask whether the vector of changes associated with both altitude and latitude are similar. Thus a clear multivariate approach may aid substantially in our understanding of evolutionary trajectories, convergence and parallelism (Schluter 1996).

In this study, we ask how variation for multivariate wing morphology in *D. melanogaster* is associated with altitude, latitude and rearing temperature, and how similar the associated shape effects are among these different influences. Using iso-female lines derived from populations of *D. melanogaster* collected at a range of altitudes and latitudes from across sub-Saharan Africa, we model the influence of these factors and look at the vector correlations between modelled effects in a 58 dimensional representation of wing shape. Our results are consistent with a small but significant influence of altitude on wing shape and size. Interestingly the vector of effects for altitude and latitude only show weak to moderate correlations with one another. We also used a manipulation of rearing temperatures for a subset of lines in order to assess the extent to which altitudinal variation might be related to thermal plasticity for size and shape. Variation in wing morphology associated with rearing temperature was more similar to static allometric variation and latitude than to the altitudinal effects, and was consistent over different altitudinal ranges. Finally, we find that the modelled directions of altitude-associated shape variation from Eastern and Western sub-Saharan Africa are poorly aligned, while allometric and sexual dimorphic effects across East and West remain strongly correlated. This suggests that local altitudinal clines may be somewhat independent from one another. We discuss these results within the context of the potentially related effects of altitude and latitude, and the underlying agents of selection.

Methods

Experimental Animals

We studied wild-derived isofemale *D. melanogaster* strains from nine African population samples (Table 1 & Figure 1(a)) that varied across altitude and latitude. While flies were sampled from the wild at a number of points between 2001–2009, this did not appear to have a confounding effect (see *Analysis* below). Within a population sample, each strain (line) was founded by a separate wild-caught female. For the primary experiment (experiment one), all flies were reared in vials in a single incubator at 24°C under a 12:12hr light:dark cycle and with humidity controlled at 60%. Each line was maintained for 2 generations under these constant conditions before adults were sampled for measurement. All flies were reared on a standard cornmeal-molasses *Drosophila* media. Once adults eclosed, they were phenotyped using the “wing grabber” as described by Houle et al. (2003).

For the temperature manipulation experiment (experiment two), we reared flies under moderately density-controlled conditions – since larval crowding is known to increase phenotypic variance in morphological traits (Imasheva & Bublly 2003). 20 females & 20 males were selected at random and placed into a fresh bottle containing standard food and allowed to oviposit for 3 days only. This rearing regime was replicated for a subset of lines (Table 1.) at both 24°C and 18°C, but with the same humidity and photoperiod as described above. Once these flies eclosed, they were stored in ethanol, a single wing was dissected and imaged from each individual (For sample sizes by line see Supplementary Table).

Measurement

For experiment one flies were imaged live – the right wing of each fly was immobilised using the wing grabber apparatus, as described in (Houle et al. 2003), and photographed using a Leica DFC400 camera mounted on a M125 microscope (with Leica LAS software v3.4.0). Images were saved in greyscale as TIFF files. To capture landmark and semi-landmark data we followed a modified protocol (Houle et al. 2003) for the use of the ‘WINGMACHINE’ software. First, we used ‘tpsDig2’ (Rohlf 2010) software to record the coordinates of the two starting landmarks needed by WINGMACHINE; the humeral break on the leading edge of the wing and the alula notch on the trailing edge, which lie at either end of the wing hinge. After data formatting, WINGMACHINE measures images by fitting nine B-splines to the veins and margins of the wing in the image. Initially, we set WINGMACHINE to measure 10% of our images, then visually checked the fit of the splines to the images and adjusted the splining parameters accordingly before setting WINGMACHINE to measure all the images. After measuring the full image set we reviewed each splined image; and adjusting spline control points (Figure 1(b)) manually as necessary. We extracted landmark and semi-landmark positions, and performed the Procrustes superimposition in order to separate shape information from size, position and rotation information (Zelditch et al. 2004). After superimposition, the positions of the semi-landmarks were allowed to slide along each segment of the wing margin/veins; this process is iterated to achieve the minimum Procrustes distance from the reference configuration for each wing, after which the mean semi-landmark positions are evenly spaced between the landmarks at either end of their segment. In the case of vein 4 there is no true landmark proximal of the anterior cross-vein that can serve this purpose, and so a semi-landmark is positioned at the point where vein 4 meets the wing hinge in order to give the vein 4 B-spline a defined end-point. After superimposition and semi-landmark sliding this poorly defined semi-landmark is removed from the dataset. Superimposition and semi-landmark sliding was performed using custom MatLab code (available from E. J. Marquez – see acknowledgements).

The data were checked for visual outliers on scatterplots, and putative outliers were examined, and fixed by hand if necessary. The 48 pairs of Procrustes coordinates (14 two-dimensional landmarks and 34 semi-landmarks) and centroid size were passed to ‘R’ for analysis. Procrustes coordinates were first subjected to principal components analysis, since Procrustes configurations – particularly when using semi-landmarks – by definition will include dimensions without variance. In the case of our data we have 96 Procrustes coordinates. The Procrustes superimposition results in a deficiency of 4 ranks (1 each for removed size and rotation information, and 2 for position), and each semi-landmark contributes approximately 1 dimension of information (Zelditch et al. 2004; Adams et al. 2004), so we retained the first $96 - (4 + 34) = 58$ principal components whose eigenvalues were non-zero. Upon empirical examination, all shape variation in our data is represented in these 58 dimensions: i.e. the total variation in these 58 principal components (sum of the eigenvalues) is equal to the total variance present in the procrustes coordinates (the trace of their covariance matrix).

Analysis

All analyses were run in **R** V2.13 (R Development Core Team 2011), on a node at the High Performance Computing Center in the Institute for Cyber-Enabled Research (running 64-bit Red Hat Enterprise Linux, V6.1), and mixed models were run using the MCMCglmm V2.15 package (Hadfield 2010). All analyses can be found in the scripts (DRYAD DOI:XXX), including custom functions written for this study.

Modelling Size variation—For Experiment one, we fitted a generalized linear mixed model (GLMM) to our wing centroid size data. Formally this model was:

$$y \sim \beta_{0ij} + \beta_1 A + \beta_2 S + \beta_3 La + \beta_4 Lo + \varepsilon$$

$$\beta_{0ij} \sim N(\beta_{0j}, \sigma_{\beta_0}^2)$$

...where y is the vector of wing size measures, β_1 is the regression coefficient for altitude, β_2 is that for sex, β_3 that for latitude and β_4 is that for longitude. We fitted latitude in order to compare the latitudinal effect with the altitudinal. Additionally fitting longitude has the effect of modelling the population structure without specifying a population term, but avoids the collinearity of population with altitude. We treated 'line' as a random effect (β_j) and used the relatively uninformative prior $\sim IW(V=I_1, \nu=0.05)$ for both the random and residual (**G** & **R**) co-variance matrices. The MCMC chain was run for 200,000 updates with a burn-in of 2000 and thinning at intervals of 20 to minimize any autocorrelation in the chain – we therefore had effective sampling of 19800 updates. To quantify variance accounted for, we also fitted the model as a least squares ANCOVA, expressing the data as within-sex within-line means. We calculated an R^2 value for the full model from the ANCOVA, and we also estimated the influence of each factor considered separately in two ways. Firstly we report the R^2 values for single-factor models, and secondly we calculated the partial R^2 (coefficient of partial determination) for each factor, which measures the marginal variance accounted for in the observed response, given all other predictors are already included for in the model (Kutner et al. 2004). These were estimated using a custom function ('PRsq') in R.

For the analysis of the rearing temperature-manipulation experiment (experiment two) we modelled variation as described above, but also included the influence of temperature. For both analyses we examined models including interaction terms and assessed model fit using the deviance information criterion (DIC). For the experiment one, the additive model was the best fit to the data ($\Delta \text{DIC} \sim 5$), but for experiment two the best model included interaction effects of rearing temperature, sex and altitude ($\Delta \text{DIC} \sim 10$).

Modelling Shape variation—We took an analogous approach to modelling wing shape, using a multivariate GLMM to and expressing wing shape as the 58 non-zero principal components (which contain all of the variation for shape). This model was:

$$\mathbf{Y} \sim \beta_{0ij} + \beta_1 A + \beta_2 S + \beta_3 Cs + \beta_4 La + \beta_5 Lo + \varepsilon$$

$$\beta_{0ij} \sim N(\beta_{0j}, \sigma_{\beta_0}^2)$$

...where $\mathbf{Y}$ is the matrix of shape variables, β_1 is the vector of regression coefficients for altitude, β_2 for sex, β_3 for wing centroid size and β_4 and β_5 are those for latitude and longitude respectively. For these multivariate GLMMs we fitted a purely additive model in both cases since 58 parameters are estimated per 'fixed' predictor variable, leading to concerns of being able to estimate all parameters without requiring a highly informative prior. The only difference for the analysis of the temperature-manipulation dataset was the

addition of a temperature term to this model. We treated ‘line’ as a random effect (γ) and used the relatively uninformative prior $\sim \text{IW}(V=\mathbf{I}_{58}, \nu=0.1)$ for both the random and residual ($\mathbf{G}$ & $\mathbf{R}$) co-variance matrices. We avoided fitting a completely unstructured co-variance matrix for either the random effect of line or for residual variation since this would require the estimation of additional 1711 (co)-variances for each matrix; which would be difficult to estimate (and would require a highly informative prior to behave computationally). Therefore we utilized the ‘idh’ function in MCMCglmm to fit matrices where all off-diagonal (co)-variances elements are fixed to zero. Because we express shape as principal components, this is true by definition for the phenotypic co-variance matrix. However, this same structure must be assumed to also hold in the decomposition of the genetic and residual co-variance matrices. The posterior distribution was generated from 200,000 updates with a burn in of 2000 and thinning of 20, thus our effective sampling was 19800 updates of the Markov chain posterior. (NB: Some of the stocks used here were collected at different times (Table 1). In order to check that lab adaptation/drift was not a confounding factor in any differences between populations, we ran a model that included a term for the ‘age’ of the lineages (time since the progenitor females were collected and brought into the lab). This model was a poorer fit to the data (DIC~4000), and the ‘age’ effect accounted for >0.5% of the variance and was not highly correlated (0.36) with the fitted altitude effect.)

As with our analyses of size, we also fitted a least square MANCOVA to within-sex within-line means from the shape data to quantify variance accounted for. We computed a multivariate extension of the coefficient of determination {Claude 2008} as:

$$\frac{\text{Tr}(\mathbf{V}_{\hat{\mathbf{Y}}})}{\text{Tr}(\mathbf{V}_{\mathbf{Y}})}$$

...where $\text{Tr}(\mathbf{V}_{\hat{\mathbf{Y}}})$ and $\text{Tr}(\mathbf{V}_{\mathbf{Y}})$ represent the trace of the estimated covariance matrices for the model fitted values, and observed values (for shape variables) respectively. This is equivalent to the established ‘Procrustes variance’ metric (e.g. Breuker et al. 2006). Identical results are calculated with Procrustes residuals or with PCs (data not shown). We report these multivariate R^2 values from single-factorial MANOVAs for each predictor. In addition to these measures, we wrote a function (‘shapePRsq’) to calculate the multivariate extension for the coefficients of partial determination (partial R^2) extending the derivation of Kutner et al. (2004).

To illustrate the shape cline as a measure of shape change per metre of altitude we calculated a shape score for altitude (Drake & Klingenberg 2008). Briefly, we projected the shape data onto a line in the direction defined by the vector of regression coefficients (β) from the above model. For altitude, the shape score s is:

$$s = \mathbf{y}\beta^T(\beta\beta^T)^{-0.5}$$

...which can be plotted against altitude (Figure 2(a)) as a graphical means to assess the strength and linearity of the clinal relationship (Drake & Klingenberg 2008). In addition to using the vectors of regression coefficients to calculate shape scores, we calculated vector correlations between them to quantify the level of similarity between the different modelled effects on shape. Vector correlations were calculated as:

$$r_{vc} = \frac{|\mathbf{a} \cdot \mathbf{b}|}{\|\mathbf{a}\| \times \|\mathbf{b}\|}$$

... where $|\mathbf{a} \cdot \mathbf{b}|$ is the absolute value of the scalar product between vectors $\mathbf{a}$ and $\mathbf{b}$, while $\|\mathbf{a}\|$, and $\|\mathbf{b}\|$ are the magnitudes (norms) of each vector. We computed the credible intervals as the 95% span of highest posterior density for the value of this vector correlation.

In addition to applying this analysis framework to the data from the main experiment and to the temperature-manipulation dataset, we also partitioned the main dataset in two ways. Firstly, we separated the dataset into a western and an eastern group of populations: the NG, CO, CN & GA populations to the west and the ED, EZ, KO, RG & MW populations to the east (Figure 1(a) & Table 1). It is possible that this may be representative of an underlying phylogenetic division between semi-independent eastern and western radiations, since our choice of groups was guided by the unrooted population distances assigned from F_{ST} values by Pool & Aquadro (2006), however it should be noted that F_{ST} values are low throughout most of this region. Secondly, in order to assess the geographical scale over which the shape cline might vary, we reanalysed two subsets of the main dataset relating to populations CO & CN (both from Cameroon) and to populations ED & EZ (both from Ethiopia) that varied altitudinally (Table 1). In each case the same analysis framework was applied to each subset separately, and vector correlations were computed as described above.

Results

Previous work has demonstrated the influence of both latitude (Gilchrist et al. 2001) and rearing temperature (Debat et al. 2009) on wing size and shape in *Drosophila melanogaster*. To investigate how much influence altitude has on wing shape, we collected, reared and measured (see methods) flies from multiple populations of Sub-Saharan Africa that vary greatly in altitude (Figure 1(a) & Table 1). Size in particular is well known to increase with latitude, across a number of continents (Capy et al. 1993), with both strong genetic and temperature mediated plasticity influencing these effects (Debat et al. 2009; McKechnie et al. 2010). Consistent with these previous observations, we see a general increase in wing size with altitude (Figure 2(b)), although this effect is not large – estimated at $0.07\text{mm}^2/\text{km}$ (95% credible interval $0.05 - 0.09\text{mm}^2/\text{km}$) – it is more important than the effect of latitude in our sample, which we estimated at $0.014\text{mm}^2/\text{degree}$ (95% CI $0.012 - 0.016\text{mm}^2/\text{degree}$). These effects are obviously operating along different scales (1 degree of latitude is $\sim 110\text{km}$ at the equator), but equates to ~ 5 orders of magnitude more change per km, presumably reflecting the steepness of the altitudinal climatic gradient. This effect of altitude is similar between the sexes (no evidence of a sex by altitude interaction), as has been found previously in several studies of latitudinal variation on wing size (Zwaan et al. 2000; Morin et al. 1999). Our analysis also revealed a comparatively strong sexual size dimorphism – males' wings were 0.56mm^2 smaller (95% CI $0.55 - 0.58\text{mm}^2$) than females – and a subtle trend of decreasing size with longitude estimated at $-0.002\text{mm}^2/\text{degree}$ (95% CI $-0.001 - -0.004\text{mm}^2$). Sexual dimorphism (Figure 1(c)), and variation between lines (Figure 1(b)) accounted for the majority of size variance (Table 2).

The principal focus of our study was on influence of altitude on the shape of the wing. We fitted a fully multivariate GLMM of shape modelled as a function of altitude, sex, wing size, latitude and longitude (see methods section). Our 58 dimensional geometric representation of wing shape includes 14 two-dimensional landmarks representing positions where veins intersect veins and the wing margin and 34 semi-landmarks along the margin and veins. As shown in Figure 2(c), the altitudinal shape change is particularly interesting, with the size

corrected shape effects showing a proximal repositioning of the posterior crossvein, a distal repositioning of the anterior crossvein and a deepening of the anal lobe, with only minor influences elsewhere. This is quite different from what has been observed for shape/size relationships previously (Debat et al. 2003), but whether this is due to a more complete representation of wing shape or the underlying relationship to altitude is unclear.

Shape is clearly changing with respect to altitude, with a similar magnitude for both males and females (Figure 2(a)). However while the shape score is informative about the magnitude and rate of shape change, it does not tell us about the direction of that change. To compare the directions of altitudinal shape-change between the sexes, we calculated the vector correlation between the vectors of model coefficients for altitude from sex-specific models. The model-adjusted effects of altitude and size on shape calculated from our model were weakly correlated (vector correlation 0.11 – 95% credible interval between 0.01 & 0.50, Figure 4), suggesting that the allometric pattern of shape change in the wing shares only a small degree of similarity with that due to variation along the altitudinal cline. The altitude effects on males and females are quite similar – their vector correlation between altitude effects when the sexes were modelled separately was 0.89 (95% credible interval 0.29 – 0.90). Altitude is associated with similar increases in wing size in both sexes (above) and the size (allometry) and sex (dimorphism) effects were somewhat correlated (vector correlation 0.14 – 95% credible interval 0.04 – 0.23). Taken together, these statistics indicate that the altitudinal variation and sexual dimorphism in wing shape are, in part, likely to be mediated by allometry.

Altitudinal effects vs. Thermal plasticity for size and shape of the wing

Flies at high altitude experience generally lower temperatures, and thermal plasticity has been reported for wing morphology (Debat et al. 2009; Bubliy & Loeschcke 2002; Bubliy & Loeschcke 2000; Santos et al. 2004; Santos et al. 2006). Altitudinal adaptation might therefore be predicted to proceed in the direction of the plastic developmental response to temperature, as has been found for latitudinal variation (Bitner-Mathe & Klaczko 1999; Dahlgaard et al. 2001). To determine the extent to which (evolved) altitude effects are recapitulated by (plastic) rearing temperature, we reared a subset of the lines (Table 1) at controlled density at both 18°C and 24°C, and examined the joint influence of altitude, site of origin (latitude and longitude) and rearing temperature and sex on both wing size (Table 4) and shape (Table 5).

Consistent with our earlier observations, there was a strong sexual size dimorphism effect at both temperatures – with females' wings 0.47mm^2 (CI 0.39 – 0.56mm^2) larger than males – and flies of both sexes reared at 18°C were an average 0.37mm^2 (CI 0.29 – 0.45mm^2) larger than those reared at 24°C. We also measured a wing size increase with altitude of $0.18\text{mm}^2/\text{km}$ (CI 0.09 – $0.27\text{mm}^2/\text{km}$), consistent with the effect observed previously (Table 4). Our best model, as selected by DIC, revealed interaction effects of altitude with sex and temperature. Males' wings showed a $0.07\text{mm}^2/\text{km}$ less steep relationship with temperature than females (CI 0.01 – 0.13), and flies reared at 18°C showed a $0.11\text{mm}^2/\text{km}$ steeper wing size–altitude effect (CI 0.06 – 0.17, see Figure 3(b)). We did not detect latitude or longitude effects in the temperature manipulation data, which we assume to be due to the much smaller size of this dataset, with commensurately reduced power.

Shape change associated with altitude also seems to be partially recapitulated by the rearing temperature treatment. The model-adjusted temperature and altitude effects were weakly correlated, with a vector correlation of 0.20 (credible interval 0.00 – 0.49). Moreover, when we re-ran model the full model separately for high altitude (populations CN, CO & KO) and low altitude populations (GA, MW & NG – see Table 1), the effects of temperature were very closely aligned, with a vector correlation of 0.98 (CI 0.92 – 0.99). This suggests that

despite the overall influence of thermal plasticity on wing size and shape, the influence of altitude appears to be largely consistent. We found that the influence of size (allometry) on shape was moderately correlated with that for sex (dimorphism) in both experiments (CI's 0.04 – 0.23 and 0.27 – 0.54 respectively), and that allometry also correlated strongly with the temperature effect in the experiment two (0.75 CI 0.68 – 0.80). The rearing temperature effect was moderately correlated with both the altitude effect (0.20 CI 0.00 – 0.49) and with dimorphism (0.20 CI 0.05 – 0.35). The effects of altitude and sex also showed a consistent moderate correlation in both experiments: 0.26 in the main dataset and 0.24 in the temperature manipulation dataset (CI's 0.00 – 0.53 and 0.00 – 0.56 respectively). The comparison of allometry and altitude found a somewhat less consistent correlation – it was estimated at 0.11 (CI 0.00 – 0.50) from the main dataset, and at 0.35 (CI 0.00 – 0.65) from the temperature manipulation experiment (Figure 4.). The latitudinal and altitudinal shape clines were moderately correlated in both datasets – 0.32 and 0.24 respectively (CI's 0.00 – 0.50 and 0.00 – 0.60) – but the latitude effect was much more strongly correlated with the effect of temperature (0.66 CI 0.19 – 0.80). These results indicate that thermal plasticity mostly involves variation in the same direction as allometric variation, and the moderately correlated direction of adaptation to altitude may be mediated by this effect, but is less consistent. By comparison the case for temperature as a mediator of the latitudinal cline appears stronger. One explanation for this pattern would be if the direction of allometry was consistent among populations, but altitudinal adaptation had proceeded in different directions.

Contingency of Altitudinal Variation?

In order to ascertain the consistency of altitudinal wing shape variation between populations, we reanalysed the main dataset in 2 ways: grouping populations by latitude into a more westerly group and a more easterly group (Figure 1), and also grouping populations by country of origin for Cameroon & Ethiopia (see Methods). Altitude-related shape change is detectable in both the western and eastern groups (Figure 5(a)), but is greater in magnitude in the easterly group of populations (regression slope of 0.003) than in the westerly group (regression slope of <0.001). While some of the lines generated from the western and eastern populations were collected at different times (and thus vary with respect to lab age of the lineages), there was little evidence for a substantial age effect in the model (see methods). One of the advantages of studying variation in a multivariate fashion is that we can also address alignment; effects of similar magnitudes may be present in different phenotypic directions. In this case the vector correlation between the modelled altitude effects for easterly and westerly groups analysed separately was small at 0.14 (CI 0.00 – 0.58), indicating that the directions of these vectors are poorly aligned (Figure 5(c)). By contrast, the other shape effects were consistent between easterly and westerly population groups: sexual shape dimorphism effects were strongly correlated at 0.92 (CI 0.82 – 0.94), and the directions of allometry were even more strongly correlated at 0.95 (CI 0.90 – 0.97).

We subset the data in a further way; using the data from the two countries (Ethiopia in the east, Cameroon in the west – Figure 1(a)) that contributed more than one population to the dataset. Altitudinal shape clines still appeared divergent when we modelled them within country-of-origin (Figure 5(b)), with the slopes of their altitudinal clines being an order of magnitude apart (0.01 for Ethiopia, 0.001 for Cameroon). Moreover, the directions of these two country-specific altitudinal shape effects were almost as divergent as those of the overall easterly and westerly population groups, with a vector correlation of 0.21 (CI 0.00 – 0.60, Figure (c)). As above, the effects of sexual shape dimorphism (0.86 CI 0.70 – 0.91) and of allometry (0.94 CI 0.87 – 0.96) were very closely aligned between these populations. Thus, the magnitude and direction altitudinal shape clines seem to be somewhat region-

specific, whereas the magnitude and direction of sexual shape dimorphism and allometry appear to be consistent across the continent.

Discussion

How do we go from an observed association between an environmental gradient and a phenotype to putative agents and targets of selection? Clinal phenotypic variation is often correlated with latitudinal environmental variation; however it is difficult to both determine the direct targets of selection (such as body size, locomotory performance or thermal tolerance) for the organism and to identify the agents of selection (temperature, humidity). One approach is to find other macro-environmental gradients (such as altitude) with some overlap of putative agents of selection (such as temperature) and look for correspondence in the phenotypic traits. Such data provides important evidence linking putative agents and targets of selection. However, this leads to the additional question of addressing when the effects across gradients (altitude and latitude) are in fact similar, which can be particularly problematic for univariate traits. In this study we investigated the influence of altitudinal and latitudinal clines for multivariate wing morphology in populations of *D. melanogaster* from Sub-Saharan Africa. Wing size is associated with latitude, altitude and temperature, but with a univariate metric it is challenging to establish whether these effects are actually equivalent. Using a fifty-eight dimensional representation of wing shape, we demonstrate that while shape co-varies with both altitude and latitude, the explicit effects on shape are only weakly related. If wing shape and size are direct targets of selection, this suggests that different sets of selective agents are acting on them across each gradient with only a subset (including factors such as temperature) being common across them. The multivariate approach of comparing the direction of the vectors of effects (the vector correlations) is a sensitive measure that allows for far more compelling inferences about the degree of similarity of the association between the putative environmental variable and the phenotype than simple univariate measures.

While both altitudinal and latitudinal predictors appear to influence (or at least co-vary with) shape and size, the magnitude of the influence and the extent of variance accounted for is surprisingly small (Tables 2 – 5). Indeed, the influence of genetic variance (as estimated by line effects) and sexual dimorphism are the most strongly predictive factors in the model. The sexual dimorphism is not surprising since sexual dimorphism is well-reported across the whole *melanogaster* subgroup (Gidaszewski et al. 2009). The genetic (line) effects may be an over-estimate, as the isofemale lines used in this study have likely undergone various degrees of inbreeding, thus uncovering the phenotypic effects of many recessive alleles (unlikely to be phenotypically expressed in large natural populations). When we manipulated rearing temperature, this factor was also an important predictor – particularly for wing size (Table 4). This also fits well with the widespread finding of thermal plasticity for *Drosophila* morphology, and in particular for size related traits (e.g James et al. 1997; Bitner-Mathe & Klaczko 1999; Dahlgaard et al. 2001).

In both experiments there was a significant trend for wings to be larger in samples originating from higher altitudes (Figures 2(b) & 3(b), Tables 2 & 4), though this trend was more pronounced in those flies reared for the temperature manipulation experiment. The modelled wing size difference between lowest and highest altitude populations – an altitude difference of 2.2km – was 0.15mm^2 (credible interval $0.11 - 0.20\text{mm}^2$) from the primary experiment (with much larger numbers of lines sampled), as compared to 0.40mm^2 (CI $0.20 - 0.59\text{mm}^2$), from the temperature manipulation experiment (this is equivalent to ~4% or ~9% size increase respectively). This stronger trend in the temperature manipulation experiment may be due to better control of larval density: Imasheva & (Bubliy 2003) showed that stress due to larval crowding in *D. melanogaster* can lead to an increase in

phenotypic variance, particularly of size-related traits, and they point out that this finding is in agreement with variance effects of temperature stress (Bubliy & Loeschcke 2002; Bubliy & Loeschcke 2000). Our results for temperature qualitatively fit this pattern, with a slight decrease in variance (from 0.16mm^4 to 0.13mm^4) associated with the comparison between the more stressful 18°C treatment and the less stressful 24°C group. There was no evidence of a reduction in variance overall between the two experiments however, as wing size in the main dataset had a comparable variance at 0.14mm^4 . Altitude accounted for $\sim 1\%$ of the variation in wing size in the main dataset (Table 2), but for slightly more ($\sim 7\%$) in the experiment where rearing temperature, where density was also somewhat more controlled.

Analyses of both datasets revealed significant associations between wing shape and altitude of origin, in addition to the influence of sexual dimorphism, wing size (allometry) and in the main dataset; significant variation associated with latitude and longitude (see Tables 3 & 5). The temperature manipulation dataset also showed an effect of rearing temperature treatment, both as a main effect and in interactions with sex and altitude. The fraction of the total shape variance accounted for by altitude was not large (between $\sim 1\%$ and 7%) in both cases. This is in line with the level of morphological variation accounted for by altitude in *D. mediopunctata* (Bitner-Mathe & Klaczko 1999). The variation associated with temperature in the temperature-controlled dataset was larger (between 15% and 31%) than that accounted for by altitude. The vectors of shape change associated with altitude are extremely similar between the temperature treatments, with a vector correlation of 0.98 (CI $0.92 - 0.99$; Figure 3(c)). That said, there is evidence that the effect of temperature on wing shape may be partially mediating the altitudinal effect, since the two directions of shape variance are somewhat correlated (vector correlation 0.20 credible interval were $0.01-0.49$).

By contrast, there were some strong correlations among some effect vectors (Figure 4), particularly between latitude and rearing temperature, as well as between size and rearing temperature. The only vector correlations that we encountered that were stronger than these were between the effects of a single factor in different groups (see results), and in a 'phenotype space' that has variance in 58 dimensions, close alignment of vectors by chance is unlikely. The latitude-temperature vector correlation was estimated at 0.66 (CI $0.19 - 0.80$). While this is far from conclusive, it provides evidence supporting the suggestion that temperature is the strongest candidate for the selective agent responsible for latitudinal size clines in *Drosophila* (Bubliy & Loeschcke 2005). The vector correlation of size and temperature effects was 0.75 (CI $0.68 - 0.80$), suggesting that the larger wings associated with lower temperature (Figure 3(b)) differ in shape predominantly along the allometry vector; i.e. wing shapes differ because they differ in size. Also notable is the very close alignment of the allometry vectors for shape when males and females were modelled separately – their vector correlation was 0.95 (CI $0.91 - 0.97$). That is not to say that wing shape is not dimorphic – shape dimorphism accounts for between 67% and 71% of the variance in the main dataset and between 22% and 39% in the temperature manipulation dataset – rather that changes in wing size seem to affect male and female wing shapes in a similar fashion. The shape vectors associated with altitude, size and sex show a moderate level of correlation in both experiments, though the size-sex effect correlation was less consistently estimated than that for sex-altitude (Figure 4). Considered across both experiments, the clinal shape variation associated with altitude shows some similarity to the variation associated with allometry, sexual dimorphism, temperature and the latitudinal cline, but none of these relationships is strikingly strong. This pattern could indicate that there are some characteristics of altitudinal shape variation that do not overlap with the other effects we were able to quantify here, and the possibility that we are actually measuring an amalgam of a number of independent clines may exacerbate this by contributing to the large credible interval on our estimates of correlations relating to altitude.

Where wing size has been found to show (latitudinal) clinal variation previously (e.g. Capy et al. 1993; Imasheva et al. 1994; James & Partridge 1998; Huey et al. 2000; Hallas et al. 2002), evidence has suggested temperature as the selective agent responsible (Reeve et al. 2000; Norry et al. 2001). Whereas temperature-related size effects could also be involved with altitudinal variation, there are other potential selective agents at work – in particular reduced air density and partial pressure of oxygen – and in any case the lack of an overall pattern relating body size to latitude in *Drosophila* (reviewed by Dillon et al. 2006) suggests that other factors are likely to be important also. Notably, Dillon & Frazier (2006) found that low temperature and low air pressure interacted to reduce the locomotor performance of *D. melanogaster* – especially flight – more than would be predicted by the effect of either factor measured in isolation. Our finding that shape displays altitudinal clinal variation after controlling for size and allometry would fit with a hypothesis of adaptation to maintain wing function (either in flight and/or sexual signalling) under the altered atmospheric conditions at altitude. However, more direct tests would be needed to be confident that this is the case. It is clear that we still do not know the agents of selection, and indeed the direct targets (i.e. the vectors of shape change for the wings that are directly under selection) that generate the pattern of clinal variation.

The populations from which our lines are derived are quite widespread geographically, spanning much of the latitude between the tropics and most of the longitude of equatorial Africa. Given this spread it is perhaps surprising that the variation jointly accounted for by latitude and longitude – i.e. among populations – is not larger, only 1 – 14% for size and 1 – 10% for shape (Tables 2 & 5). Though generally accounting for more variance than altitude, this inter-population variance is dwarfed by that accounted for by sexual dimorphism, genetic variation (line) and rearing temperature. This implies that – at least for wing morphology – there is a considerable amount of standing genetic variance and plasticity to be found within these populations. When we separated our main dataset into western and eastern groups (see Figure 1(a)) we found different magnitudes and directions of shape variance associated with altitude between the groups (Figure 5(a)). The correlation between the altitude effect in the two longitudinal groups was 0.14 (CI 0.00 – 0.58), indicating that these shape effects are quite different (Figure 5(c)), despite the similarity of sexual shape dimorphism and allometry across these two broad geographic groups. For those two countries (Cameroon in the west and Ethiopia in the East) where we had lines from more than one population, we were able to test for altitudinal effects within populations. This comparison revealed rather more variation associated with altitude in Ethiopia than in Cameroon (Figure 5(b)) and, the altitudinal shape effects estimated in these populations separately are also quite different (Figure 5(c)), with their vector correlation estimated at 0.21 (CI 0.00 – 0.60). The other shape effects modelled simultaneously with altitude; sexual dimorphism and allometry; were much more strongly correlated both between East and West groups and between Ethiopian and Cameroonian populations (>0.9 in both cases). Since being brought into the lab, the stocks may have evolved by lab-adaptation and/or drift. However since all stocks here have been maintained as isofemale lines, there is little genetic variance present within lines for selection or drift to act upon. Though divergent drift may account for some of the difference in our measured altitudinal effects, it seems unlikely given the pronounced similarity of the other shape effects measured. Indeed lab-adaptation has been shown to be quite repeatable in the lab (Santos et al. 2004; 2005; 2006), at least when temperature is the applied selective agent, so the divergence of the altitudinal effect vectors here may be another indication that there is more to altitudinal variation than the thermal cline alone. We can have less confidence, however, that the altitudinal shape clines we measured are adaptive. To confirm this we would need to recapitulate these patterns of variance by direct manipulation of putative agents of selection (e.g. Santos et al. 2004; 2006) or by making comparisons between clines that we can be confident are independent (e.g. on different continents: Huey et al. 2000; Zwaan et al. 2000; Paaby et al. 2010).

Here we found clinal variation in both size and shape associated with altitude, though this variation accounted for little of the total variance. The largest fraction of the variance we detected was within rather than between populations. The association of shape with altitude appears to be partly recapitulated by allometry and, to a lesser extent, by temperature. Interpretation of continent-wide patterns is complicated however, by the inconsistent directions of shape associated with altitude among populations – suggesting multiple clines with only limited convergence. Data from populations intermediate in location between some of those measured here, or from altitudinal clines on other continents, may clarify these findings in the future.

Data and scripts

All raw data and R scripts to perform these analyses are available on Dryad (datadryad.org) via DOI:XXX.

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Figure 1.

(a) Map of approximate locations of the populations from which our lines are derived. (b) A representative wing image, with fitted B-splines. The colour-coded circles are the locations of the control points used to adjust the spline fit. (c) & (d) represent the spread of variation in our sample. (c) shows the grand mean shape, with circles indicating the positions of the landmarks and semi-landmarks extracted from the splines. The background blur is composed of the 112 within-line mean shapes from experiment 1. (d) shows the within-sex grand mean shapes, over a background blur composed of the 3008 individual wing shapes from experiment 1.

Figure 2.

The influence of altitude on wing morphology. (a) Wing shape, represented as model-adjusted shape score, plotted against altitude. Each point is the within-sex mean for a single line. The altitude-shape relationship is similar in males and females. The black line shows the altitude-shape relationship modelled with the sexes pooled, with the grey region indicating 95 random draws from the posterior distribution. (b) The relationship between altitude and wing size; points are within-sex line means, and the grey regions indicate 95 random draws from the posterior distribution. Altitude is associated with an increase in wing size in both sexes. (c) Visualisation of the variation represented by the model-adjusted shape score, scaled to 3x the difference between lowest and highest populations. Most of the changes seem to be localized to medial and proximal regions of the wings, including shifting of the crossveins and an expansion of the posterior lobe at high altitudes.

Figure 3.

The joint influence of thermal plasticity and altitude of origin on wing morphology (a) The altitude-shape relationship for both sexes and rearing temperatures. Points are within-sex line means. At 24°C the relationship is similar between males and females. This pattern changes at 18°C, with the altitude-shape relationship maintained in females but weakened in males. (b) The altitude-wing size relationship for both sexes at both rearing temperatures. Points are within-sex line means. Both sexes' wings are larger at 18°C, but whereas the slope of the altitude-size relationship is consistent between temperatures in males, females show a steeper relationship at 18°C than at 24°C. (c) Visualizations of the altitude-shape effect, calculated separately within each rearing temperature. Between them is their vector correlation (95% credible interval).

Figure 4.

Vector correlations between model-adjusted effects on shape. Points are vector correlations between posterior mean coefficient vectors for pairs of modelled effects, with lines representing 95% credible intervals on the vector correlations. Coefficients are calculated simultaneously from the full model for each experiment. While most vector correlations indicate weak to moderate relationships between these effects, notably rearing temperature and allometric scaling effects appear very similar, as well as rearing temperature and latitude of origin.

Figure 5.

Altitudinal co-variation with shape differs between populations from Eastern and Western Africa. As demonstrated with both the shape scores and vector correlations between altitudinal effects from Eastern and Western African populations, there appears to be largely distinct influences on shape. (a) Altitude and wing shape, represented as model-adjusted shape score calculated separately for western and eastern population groups (see Figure 1). Each point is the within-sex mean for a single line. (b) Altitude and wing shape, represented as model-adjusted shape score calculated separately for Cameroonian and Ethiopian populations. Each point is an individual. (c) Visualizations of the shape effects associated with altitude calculated separately as labelled. Between pairs of effects are their vector correlations (95% credible intervals)

Table 1

Summary of the abbreviations used for Populations, and the number of lines used in these experiments. See supplemental table for numbers of individuals measured in each case.

Pop. ID	Country of Origin	Collection Locality	Map Reference	Collection Date	Collector(s)	Altitude of Origin (m)	# Lines: Exp. 1	# Lines: Exp. 2
KO	Kenya	Molo	0.25S, 35.73E	Jan-09	J. Pool	2506	18	5
ED	Ethiopia	Dodola	6.98N, 39.18E	Dec-08	J. Pool	2492	7	-
CO	Cameroon	Oku	6.25N, 10.43E	Apr-04	J. Pool	2169	10	5
RG	Rwanda	Gikongoro	2.49S, 28.92E	Dec-08	J. Pool	1927	20	-
EZ	Ethiopia	Ziway	7.93N, 38.72E	Dec-08	J. Pool	1642	20	-
CN	Cameroon	Nkouondja	5.50N, 10.68E	Apr-04	J. Pool	1121	10	5
MW	Malawi	Mwanza	15.62S, 34.52E	Jul-01	B. Ballard	690	5	5
GA	Gabon	Franceville	1.65S, 13.60E	Mar-02	B. Ballard & S. Charlat	332	11	5
NG	Nigeria	Maiduguri	11.85N, 13.16E	Sep-04	D. Gwary & B. Sastawa	295	10	5
total # lines							111	30

Analysis of wing centroid size from the main dataset. The coefficients, credible intervals and pseudo-*p* values are from the generalized linear mixed model, and the *R*² statistics are from least-squares ANCOVA (see methods section). Both analyses used includes only additive effects, but the results are qualitatively unchanged if the analysis was run including interaction effects. The ‘focal *R*²’ values are for a model with the variable as the only predictor and the adjusted *R*² value is for the full model.

Table 2

Effect	posterior mean	lower 95% cred. int.	upper 95% cred. int.	pseudo- <i>p</i> value	partial <i>R</i> ²	focal <i>R</i> ²	adjusted <i>R</i> ²
Altitude	7.11E-05	4.91E-05	9.46E-05	<0.001	<0.01	0.01	0.73
Sex	-5.64E-01	-5.78E-01	-5.49E-01	<0.001	0.71	0.67	
Latitude	1.41E-02	1.22E-02	1.59E-02	<0.001	0.05	0.01	
Longitude	-2.12E-03	-3.52E-03	-7.78E-04	<0.001	0.14	0.05	
Line	2.77E-02	1.60E-02	4.13E-02				
Residuals	3.88E-02	3.68E-02	4.07E-02				

Model summary table for least-squares MANCOVA on 58 principal components of shape from the main dataset, conducting using the line means. See results for description of the complete multivariate mixed model. The ‘focal R²’ values are for a model with the variable as the only predictor and the adjusted R² value is for the full model.

Table 3

Effect	df	residual df	Wilk's	F approx.	p value	partial R ²	focal R ²	model R ²
Centroid Size	58	155	0.13	17.99	<0.0001	0.02	0.15	0.26
Altitude	58	155	0.32	5.80	<0.0001	0.02	0.04	
Sex	58	155	0.19	11.28	<0.0001	0.06	0.18	
Latitude	58	155	0.47	2.96	<0.0001	0.01	0.03	
Longitude	58	155	0.37	4.47	<0.0001	0.02	0.03	
Residuals	212							

Analysis of wing centroid size measured from the temperature manipulation experiment. The statistics are from the generalized linear mixed model, with the exception of the R^2 measures; which are from least-squares ANCOVA (see methods section). The ‘focal R^2 ’ values are for a model with the variable as the only predictor and the adjusted R^2 value is for the full model.

Table 4

Effect	posterior mean	lower 95% cred. int.	upper 95% cred. int.	pseudo- <i>p</i> value	partial R^2	focal R^2	adjusted R^2
Altitude	1.81E-04	8.91E-05	2.72E-04	<0.001	<0.01	0.07	0.77
Temperature	-3.66E-01	-4.55E-01	-2.78E-01	<0.001	0.15	0.31	
Sex	-4.73E-01	-5.62E-01	-3.85E-01	<0.001	0.22	0.39	
Latitude	-3.54E-03	-1.66E-02	9.10E-03	0.58	0.01	0.03	
Longitude	-2.10E-04	-5.95E-03	5.57E-03	0.94	<0.01	<0.01	
Altitude x Temp.	-1.15E-04	-1.77E-04	-5.67E-05	<0.001	<0.01	0.31	
Sex x Altitude	-7.47E-05	-1.37E-04	-1.42E-05	0.02	<0.01	0.34	
Sex x Temperature	-9.23E-03	-1.41E-01	1.12E-01	0.89	0.00	0.71	
Sex x Temp. x Alt.	9.55E-05	1.08E-05	1.81E-04	0.03	0.02	0.59	
Line	2.32E-02	1.08E-02	3.84E-02				
Residuals	4.96E-02	4.40E-02	5.59E-02				

Model summary table for LS MANCOVA on 58 principal components of shape measured from the temperature manipulation experiment, conducted on line means. See results for description of the complete multivariate mixed model. The ‘focal R^2 ’ values are for a model with the variable as the only predictor and the adjusted R^2 value is for the full model.

Table 5

Effect	df	residual df	Wilk's	F approx.	p value	partial R^2	focal R^2	model R^2
Centroid Size	58	56.0	0.02	44.08	<0.0001	0.04	0.07	0.26
Altitude	58	56.0	0.17	4.84	<0.0001	0.02	0.04	
Temperature	58	56.0	0.04	21.16	<0.0001	0.03	0.04	
Sex	58	56.0	0.06	15.31	<0.0001	0.04	0.08	
Latitude	58	56.0	0.10	8.57	<0.0001	0.04	0.06	
Longitude	58	56.0	0.19	4.21	<0.0001	0.02	0.03	
Residuals	113							