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SIMILARITIES AND DIFFERENCES IN ALTITUDINAL VERSUS LATITUDINAL VARIATION FOR MORPHOLOGICAL TRAITS IN *DROSOPHILA MELANOGASTER*

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Understanding how natural environments shape phenotypic variation is a major aim in evolutionary biology. Here, we have examined clinal, likely genetically based variation in morphology among 19 populations of the fruit fly (*Drosophila melanogaster*) from Africa and Europe, spanning a range from sea level to 3000 m altitude and including locations approximating the southern and northern range limit. We were interested in testing whether latitude and altitude have similar phenotypic effects, as has often been postulated. Both latitude and altitude were positively correlated with wing area, ovariole number, and cell number. In contrast, latitude and altitude had opposite effects on the ratio between ovariole number and body size, which was negatively correlated with egg production rate per ovariole. We also used transgenic manipulation to examine how increased cell number affects morphology and found that larger transgenic flies, due to a higher number of cells, had more ovarioles, larger wings, and, unlike flies from natural populations, increased wing loading. Clinal patterns in morphology are thus not a simple function of changes in body size; instead, each trait might be subject to different selection pressures. Together, our results provide compelling evidence for profound similarities as well as differences between phenotypic effects of latitude and altitude.

KEY WORDS: **Altitude, body size, cell number, clines, latitude, ovariole number, wing loading.**

Organisms often have to cope with substantial environmental change across space and time. Local adaptation along environmental gradients, for example, often results in the formation of clines, that is, genetic and phenotypic gradients across geography (Huxley 1938). Clines across latitude (presumably mainly driven by temperature), for instance, have been well documented for numerous traits in a wide range of ectotherms, including numerous species of the genus *Drosophila*. Traits that vary with latitude in *Drosophila melanogaster* include, for example, developmental rate (James and Partridge 1995; van't Land et al. 1999); larval growth efficiency (Robinson and Partridge 2001); body size (David and Bocquet 1975; Coyne and Beecham 1987; Capy et al. 1993; Imasheva et al. 1994; James et al. 1995; van't Land et al. 1999); ovariole number (David and Bocquet 1975; Capy et al. 1993); egg size (Azevedo et al. 1996); starvation, cold, and heat resistance (DaLage et al. 1990; Karan et al. 1998; Hoffmann et al. 2002; Frydenberg et al. 2003); ethanol tolerance (Cohan and Graf 1985); metabolic rate (Berrigan and Partridge 1997); and diapause incidence (Schmidt et al. 2005). Latitudinal clines in *Drosophila* have also been documented at the genetic level, for example, at the level of allozyme, DNA, and inversion polymorphisms (Mettler et al. 1977; Knibb 1982; Oakeshott et al. 1982; Gockel et al. 2001; Sezgin et al. 2004; Anderson et al. 2005; Hoffmann and Weeks 2007; Turner et al. 2008; Fabian et al. 2012; Kapun et al. 2013). The pervasive similarities in clinal variation across continents and species suggest that latitudinal clines are, at least partly, the result of spatially varying selection (Endler 1977; Fabian et al. 2012), with temperature being considered the major selective agent (Stalker and Carson 1947; David et al. 1977; Partridge et al. 1994).

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Unlike latitudinal clines, clines across altitude have received considerably less attention, especially in *Drosophila* (reviewed in Hodkinson 2005; Pitchers et al. 2013). Because mean temperature decreases as a function of both increasing latitude and altitude, altitudinal clines have often been postulated to mirror latitudinal clines, at least qualitatively (Hopkins 1938; Stevens 1992; Lencioni 2004; Pitchers et al. 2013). Indeed, similar to the effects of latitude on body size, positive altitudinal clines for body size have been identified in *D. buzzatii* (Dahlgaard et al. 2001; Sambucetti et al. 2006), *D. robusta* (Stalker and Carson 1948), and *D. takahashii* (Parkash et al. 2005), and for wing size (a proxy of body size) also in *D. melanogaster* (Pitchers et al. 2013). The prediction that altitudinal clines mirror latitudinal clines has, however, not always been borne out. While some studies have found that flies are larger at higher elevations (see above), others have not (cf. Mani 1968; Hodkinson 2005; Dillon et al. 2006; Pitchers et al. 2013). Studies of wild-caught flies, for example, have typically failed to detect differences in body size across altitudinal gradients (reviewed in Dillon et al. 2006). Yet, because body size is a highly plastic trait that can be influenced by several factors (e.g., developmental temperature, nutrient uptake), it is difficult to draw firm conclusions based on field observations alone. Thus, whether altitudinal clines for body size represent a general pattern that applies to most ectotherms or whether such clines may be taxon specific, for instance reflecting specific ecological demands and physiological constraints of particular species, is largely unclear. More generally, our understanding of altitudinal variation in morphological and life-history traits in *Drosophila* remains limited.

Apart from the altitudinal decrease in temperature, whose rate is approximately 6–7°C per km (Finlayson-Pitts and Pitts 1999), there also exist many other biologically important abiotic factors that change with altitude: for example, a decrease in atmospheric pressure and partial pressure of atmospheric gases; and an increase in (both incoming and outgoing) radiation and a higher fraction of ultraviolet-B radiation (Körner 2007). Decreased gas pressure, especially of oxygen, might affect metabolic rate, growth rate, and survival of insects (Dillon et al. 2006). Moreover, reduced air density at high elevation can strongly compromise flight performance (Dillon and Frazier 2006). Increased radiation at high elevation produces large differences between atmospheric and surface temperatures, allowing for the existence of microhabitats with relatively higher temperatures as compared to microhabitats in high-latitude environments (Mani 1968). Furthermore, unlike high-latitude temperate biota, high-altitude tropical biota do not face any reduction in season length (Körner 2000). In addition to these factors, another major characteristic of altitudinal gradients is that they entail considerable environmental change across very short horizontal distances (Hodkinson 2005). This in turn can lead to high levels of gene flow among adjacent areas, rendering major altitudinal genetic differentiation only possible under conditions of rather strong selection (Blanckenhorn 1997). Together, these diverse factors might all contribute to differences in adaptation to altitudinal versus latitudinal gradients but the extent of such differences is not yet well understood.

Here, our main aim was to systematically examine similarities and differences in latitudinal and altitudinal variation for morphological traits among natural populations of *D. melanogaster* of diverse geographic and climatic origin. We measured a suite of morphological traits (thorax length, wing area, wing loading, ovariole number, and ovariole index [the ratio of ovariole number to body size]) in 19 populations of *D. melanogaster* originating from markedly different altitudes and latitudes across Africa and Europe. Since previous studies have found that thermal evolution in the laboratory causes differences in body size due to variation in cell size (Cavicchi et al. 1985; Partridge et al. 1994), we also analyzed the relative contribution of cell number versus cell size to body size (using wing area as a proxy; Partridge et al. 1994). By examining both ancestral African and derived European populations, our study extends previous work investigating the cellular basis of variation in body size along the Australian and South American clines (James et al. 1995; Zwaan et al. 2000). Moreover, to the best of our knowledge, no intraspecific studies of the cellular basis of altitudinal variation in body size have been performed to date; thus, potential differences in cell number versus cell size with respect to altitude remain unknown.

Our second aim was to examine how morphological traits change in relation to variation in body size. Changes in body size might be achieved by altering either cell number or cell size, or by a combination of the two (cf. Arendt 2007). Because latitudinal differences in body size in *Drosophila* seem to be predominantly driven by variation in cell number (James et al. 1995; Pezzoli et al. 1997), we used two methods to investigate the effects of manipulating cell number on covariation among morphological traits. First, we examined individuals that had developed at different larval densities. Larval crowding affects nutrient uptake, which in turn causes changes in body size via changes in cell number (Robertson 1959). Second, we used genetic manipulation of the insulin signaling pathway in the larval ring gland (the site of production of the steroid hormone ecdysone), an intervention that affects body size via changes in cell number due to an antagonism between ecdysone and insulin signaling (Colombani et al. 2005; Mirth et al. 2005); this allowed us to test directly how changes in cell number affect morphological traits and their interrelationships.

Finally, we asked whether and how variation in ovariole index (a trait which we find to be clinal) affects early fecundity, a major fitness component.

Table 1. Populations used in this study. See Materials and Methods for further details[1].

Population	Locality	Latitude	Longitude	Altitude	Collector	Date	Number of isofemale lines
South Africa	Paarl	−33.72	18.96	94	H. van Schalkwyk	March 2012	30
South Africa	Phalaborwa	−23.93	31.12	420	R. Corbett-Detig	July 2010	10
Madagascar	Antananarivo	−18.93	47.52	1276	J. David	March 2008	8
Zimbabwe	Harare	−17.86	31.03	1490	C. F. Aquadro and C. I. Wu	1990	10
Zambia	Siavonga	−16.53	28.72	578	R. Corbett-Detig	July 2010	30
Tanzania	Uyole	−8.92	33.44	1800	L. Nsemwa	December 2009	12
Rwanda	Gikongoro	−2.49	28.92	1927	J. Pool	January 2009	16
Gabon	Franceville	−1.63	13.58	350	B. Ballard and S. Charlat	March 2002	10
Kenya	Thika	−1.05	37.08	1631	J. Pool	January 2009	10
Cameroon	Oku	6.25	10.43	2169	J. Pool	April 2004	10
Ethiopia	Dodola	6.98	39.18	2492	J. Pool	December 2008	5
Ethiopia	Gambella	8.25	34.58	525	J. Pool	December 2011	14
Ethiopia	Fiche	9.8	38.73	3050	J. Pool	December 2011	14
Egypt	New Cairo	30.03	31.47	300	E. Nasser	September 2006	10
Portugal	Évora	38.57	−7.91	300	C. Soussa	September 2004	14
Switzerland	Zürich	47.36	8.55	408	L. Wilfert	August 2007	14
Austria	Vienna	48.21	16.37	484	P. Klepsatel	October 2010	30
England	Royal Tunbridge Wells	51.13	0.27	116	D. Obbard	August 2007	18
Sweden	Uppsala	59.85	17.63	7	L. Wilfert	August 2007	20

[1]We thank the different original collectors mentioned above, and C. Schlötterer and J. Pool for making these strains available to us.

Our results suggest that there exist both profound similarities as well as important differences in altitudinal versus latitudinal variation for morphological traits in *D. melanogaster*. Notably, we find strong evidence that clinal patterns in morphology are not simply due to changes in body size.

Materials and Methods

FLY POPULATIONS

We used 19 populations of *D. melanogaster* from different latitudes (range: −33.72°–59.85°) and altitudes (range: 7–3050 above mean sea level [AMSL]) from both Africa and Europe; notably, six populations were from high-altitude (all >1500 m AMSL; Table 1). All populations were kept in the laboratory as isofemale lines for different periods of time (see Table 1). To avoid potential effects of inbreeding (Tantawy 1957), we crossed isofemale lines from each population in a combinatorial fashion, resulting in at least 10 different among-line crosses per population. From these crosses, we obtained F1 individuals for phenotypic measurements. Flies were raised at 25°C (12:12 L:D), at a medium egg density (50 eggs per vial; eggs were collected by allowing flies to lay eggs directly into vials during —one to two hours; any excess eggs were removed) on standard cornmeal agar yeast (2% yeast) medium with 20 mg of active dry yeast sprinkled on top. In addition, we also used Austrian and Zambian flies that were kept as mass-bred populations at a population size of approximately 1500–2000 adults; flies were maintained on standard cornmeal agar yeast medium in bottles (60 mm diameter/130 mm height) with overlapping generations (generation time two to three weeks) at room temperature (∼25°C). The Austrian outbred population was established with approximately 200 freshly collected females and males; the Zambian population was initiated with 600 flies, that is, 10 females and 10 males from each of 30 isofemale lines. Both populations were kept in the laboratory for 18 months prior to our experiments. For further details, see Klepsatel et al. (2013b).

MEASUREMENT OF MORPHOLOGICAL TRAITS

To measure morphological traits, we used nine- to 10-day-old adult flies (at least 23 females and 23 males per population; randomly chosen from a pool created by using the same number of individuals from each cross in each population) from each of the 19 populations. Whenever possible, all traits were measured on the same individuals. Measurements of thorax length (mm) and wing area (mm^2) were performed with a stereo dissecting microscope (Leica M205FA, Leica Microsystems GmbH, Wetzlar, Germany), with a digital camera (DFC 300 FX) attached to it and using the Leica Application Software (LAS). Thorax length was measured from the base of the most anterior humeral bristle to the posterior tip of scutellum on the left side of the fly (French et al. 1998). For wing area measurements, the left wing was removed and mounted between two microscope slides. The wing contour

was traced and the area measured using LAS (also see Klepsatel et al. 2013b). Wing loading was calculated as (thorax length)^3/(wing area) (Starmer and Wolf 1989). To estimate cell number, we used a Leica DM5500 microscope and the same camera and LAS as mentioned above. Cell size was estimated by calculating the number of trichomes in a 0.01 mm^2 sampling square area on the dorsal wing surface, chosen by eye, equidistant from the fourth longitudinal vein, the posterior cross vein, and the fifth longitudinal vein (McCabe and Partridge 1997; Zwaan et al. 2000). Because each epidermal cell secretes one bristle, the number of bristles corresponds to the number of cells (cf. Dobzhansky 1929). The total number of cells was estimated by dividing the wing area by the estimated cell size. Ovaries were dissected in water, and the number of ovarioles was counted using a stereo dissecting microscope; ovariole number was calculated as the sum of the number of ovarioles from both ovaries. To describe the relation between ovariole number and body size, we also estimated an "ovariole index," which is defined as the ratio of ovariole number to (thorax length)^3 (cf. Klepsatel et al. 2013b).

RELATIONSHIPS AMONG MORPHOLOGICAL TRAITS AND EFFECTS OF CELL NUMBER

To examine how morphological traits are interrelated, and to investigate how cell number affects covariation among traits, we used two approaches. First, we examined individuals raised at different larval densities throughout development, resulting in adults with substantial variation in body size. To make sure that our results are general and robust, and to account for potential confounding effects of inbreeding versus outbreeding, we picked two markedly distinct groups of flies for measurements: an outbred population from Austria and an inbred isofemale line from Ethiopia (Dodola). To produce substantial variation in adult size, flies from both groups were reared at either medium (50 eggs per vial) or high larval density (Austria: 150–200 eggs per vial; Ethiopia: 150 eggs per vial) at 25°C and 12:12 L:D on a cornmeal agar yeast (2%) diet with active yeast. To collect eggs from the Austrian population, we placed approximately 500 adults into a population cage (390 × 280 × 280 mm) at room temperature (~25°C) and allowed females to oviposit for one hour on dishes containing standard cornmeal agar yeast medium (with active yeast sprinkled on top). Eggs were allocated to vials by excision and transfer of pieces of egg-laying medium (either containing 50 eggs or ≥ 150 ≤ 200 eggs). For the low egg density treatment, we adjusted the number of eggs to 50 per vial by carefully removing any excess eggs from small pieces of medium. For the high egg density treatment, we used larger pieces of medium containing more eggs; because at this higher density, eggs were sometimes damaged during the excision and transfer process, we transferred more eggs (approximately 200) than we aimed for (150) to compensate for any loss due to damaged eggs. Ethiopian flies, on the other hand, laid eggs directly into vials, and eggs were typically quite evenly distributed on the egg-laying substrate, which made removal of excess eggs and adjustment of egg density easier. After 10 days, we measured 30 females from the Austrian population and 20 females from the Ethiopian line for all traits, as described above. Note that, to obtain a broad range of size classes, females were specifically selected from a larger group of flies (Austria: $N = 60$; Ethiopia: $N = 82$) whose thorax length we had measured previously.

Second, to test the effect of increased cell number on morphological traits, we used the binary GAL4>UAS transgenic system to drive expression of UAS-*PTEN*, an inhibitor of insulin signaling, in the ring gland using a ring gland-specific GAL4 driver, *P0206*-GAL4 (experimental transgenic genotype, T: *y w*; *P0206*-GAL4>UAS-*PTEN* flies). This manipulation suppresses growth of the prothoracic gland (the part of the ring gland that produces ecdysone) and the corpus allatum (another part of the ring gland) during the larval period, which in turn causes reduced ecdysone levels, delayed onset of metamorphosis and increased adult size (Mirth et al. 2005). Normally, ecdysone signaling acts to inhibit peripheral insulin signaling, leading to reduced body size, but in *P0206*-GAL4>UAS-*PTEN* flies, this downregulation of insulin signaling by ecdysone is relaxed, thus causing size to increase (Colombani et al. 2005). As controls, we used females from a cross between *y w*; *P0206*-GAL4 females and *y w* males (control 1 = C_1) and a cross between UAS-*PTEN* females and *y w* males (control 2 = C_2). All genotypes were reared at constant 23°C because higher temperatures might increase the proportion of precocious L2 puparia (Mirth et al. 2005). Traits were measured on these flies (20–25 females per genotype) as described above; for estimating cell size and cell number, we measured 10 females per genotype.

RELATIONSHIP BETWEEN OVARIOLE INDEX AND FECUNDITY

To determine the effect of variation in ovariole index on fecundity, we used flies from an outbred temperate (Austrian) and an outbred tropical (Zambian) population and manipulated their size and ovariole number by exposing them to different growth temperatures. Because fecundity is highly sensitive to inbreeding, we deliberately chose to compare two outbred populations from two geographically and climatically distinct regions. To initiate experimental populations, we collected eggs as described above. Eggs were allocated to vials at medium density (50 eggs per vial), and vials were randomly allocated to either of two temperature treatments (five to 10 vials per population and treatment): 18°C or 25°C (both with 12:12 L:D). Upon emergence, we individually placed single adult females from these treatment groups together with two males from the same group into vials ($N = 60$–70 females at both 18°C and 25°C); vials contained standard

cornmeal agar yeast (2%) medium with approximately 10 mg of active yeast sprinkled on top and were kept at 25°C on a 12:12 L:D cycle. Adults were transferred daily to fresh vials, dead or escaped males were replaced, and eggs were counted using a stereo dissecting microscope. For each female, we expressed fecundity as the cumulative number of eggs laid during first 10 days of adulthood. Flies that died during the experiment were excluded from analysis. After 10 days, we measured female thorax length and determined the number of ovarioles as described above.

CLIMATE DATA

To relate variation in morphological traits to climatic conditions experienced by populations in their natural environments, we obtained the following climate data: mean annual temperature (mean of average monthly temperatures), average seasonal temperature, mean temperature of the hottest month, mean temperature of the coldest month, temperature difference between the hottest and coldest month of the year, temperature difference between the hottest and coldest month of the season, mean monthly precipitation, mean monthly precipitation during the season, and season length (in months) for all locations of origin (±20 km) from the World Meteorological Organization (worldweather.wmo.int) and from www.climatedata.eu (Table S1). We defined "season" as the period during which the mean monthly temperature did not fall below 12°C; we used this thermal limit because development of *D. melanogaster* is not possible below 12°C (David and Clavel 1966; Cohet et al. 1980).

STATISTICAL ANALYSES

We first analyzed the effects of latitude and altitude on variation in morphological traits (population means) using multiple linear regressions with one categorical factor (sex), two continuous variables (latitude, altitude), and two interactions (sex × latitude; sex × altitude). Due to collinearity between altitude and latitude in our data (variance inflation factor, VIF > 6.0), the effect of altitude was analyzed only for populations from localities with latitudes ranging between 30°N and 30°S; note that this latitudinal range included all high-altitude populations. Likewise, to analyze the effect of latitude, we excluded all populations from localities with elevations >1500 m AMSL. This procedure decreased VIF below 2.0. Since we did not detect an effect of hemisphere on the relation between latitude and any of the traits (data not shown), we expressed latitude as absolute latitude. We excluded effects of longitude from all analyses (except for factor analysis, see below) because longitude did not have a significant effect in any analysis (not shown).

Second, to further analyze potential interrelationships, and to discriminate between direct and indirect effects of altitude and latitude on thorax length, wing area, and ovariole number, we used path analysis (Wright 1934; Mitchell 1993) implemented in the R package plspm (Sanchez 2013; R version 2.12.2). Path analysis is a statistical method for describing dependencies among two or more variables, based on a linear system of equations (Olobatuyi 2006). Confidence intervals for path coefficients were estimated by bootstrapping ($N = 1000$). Path coefficients were compared between females and males using bootstrap *t*-tests implemented in plspm (see Sanchez 2013).

Third, to reduce the number of climatic and geographic variables, and to explain variation in morphological traits in relation to them, we performed factor analysis with principal component (PC) factoring and varimax rotation (Johnson and Wichern 2007; Abdi and Williams 2010). Note that we included latitude, longitude, and altitude in this analysis because these geographic variables not only describe climatic effects but also other potentially important effects such as day length, resource abundance, and air density (for which we had no independent estimates). We extracted the first three factors with eigenvalues >1.0 (explaining 89.5% of the total variance); eigenvalues >1 indicate that the linear combinations of variables (PCs) account for more variance than the original variables and are thus used as a criterion for deciding which PCs should be retained for analysis. We subsequently used these three factors in multiple regression analyses of morphological traits with one categorical (sex), three continuous variables (factors 1, 2, and 3) and three interactions (sex × factor 1, sex × factor 2, and sex × factor 3). The benefit of PC factoring is twofold: first, it reduces the number of different (and potentially correlated) predictors to a much smaller number of explanatory variables whose combination explains most of the variance in the data; second, and more importantly, it might uncover effects of biologically relevant predictors that are normally "hidden" within composite variables (e.g., "altitude" is a composite variable consisting of several factors that covary with elevation, such as temperature, air pressure, etc.).

Fourth, we analyzed relationships between morphological traits by using simple linear regressions. Note that, to estimate the relative contribution of cell number and cell size to variation in wing area, trait values were log transformed (Robertson 1959; Stevenson et al. 1995; Zwaan et al. 2000); the value of the slope of a simple regression between log-transformed data for wing area versus cell number or cell size determines the proportion of the variance in wing area that is due to covariation with cell number or cell size, respectively (see Zwaan et al. 2000).

Fifth, we calculated pairwise correlations between morphological traits and fecundity using Spearman rank correlations, followed by Bonferroni–Holm correction for multiple testing.

Finally, we examined among-population (group) variation for ovariole index and egg production rate per ovariole using two-way analysis of variance (ANOVA), including "population" and "temperature" as fixed factors and the "population" by "temperature" interaction, followed by Tukey's HSD (honestly significant

difference) post-hoc tests. Unless stated otherwise, all analyses were performed with JMP version 10.0.0 (SAS, Raleigh, NC).

Results

ALTITUDINAL AND LATITUDINAL VARIATION IN MORPHOLOGY

Altitude had a significant effect on all morphological traits: elevation was positively correlated with thorax length, wing size, ovariole number, cell number, and cell size but negatively correlated with wing loading (multiple regressions; Table S2). The correlation between altitude and ovariole index was negative and became significant after the nonsignificant effect of latitude was removed ($F_{1, 10} = 5.74$; $P = 0.036$). Despite a positive relationship between altitude and cell size, altitudinal variation in wing area was mainly driven by variation in cell number in both sexes (females: slope $s = 0.90 \pm 0.06$, $P < 0.0001$; males: $s = 0.91 \pm 0.05$, $P < 0.0001$; corrected for latitude) but not significantly so by cell size (females: $s = 0.09 \pm 0.06$, $P = 0.2$; males: $s = 0.09 \pm 0.05$, $P = 0.11$; corrected for latitude).

Latitude was positively correlated with wing size, ovariole number, ovariole index, and cell number (multiple regressions; Table S3), suggesting that altitude and latitude have similar positive effects on wing size, ovariole number, and cell number, but an opposite effect on ovariole index (latitude: positive, altitude: negative) (Figs. 1 and 2). Similar to the effects of altitude, latitudinal variation in wing area was predominantly affected by variation in cell number in both sexes (females: $s = 0.94 \pm 0.11$, $P < 0.0001$; males: $s = 0.96 \pm 0.15$, $P < 0.0001$; corrected for altitude).

To discriminate between direct and indirect effects of altitude and latitude on thorax length, wing area, and ovariole number, we performed path analysis (Fig. 3; Table S4). A comparison of path coefficients for thorax length and wing area did not reveal any significant differences between the sexes (Table S5). Both altitude and latitude had positive effects on thorax length in females, which in turn affected wing area. Altitude had an additional positive effect on wing area, suggesting that high-altitude flies have relatively larger wings as compared to flies from high latitudes. Moreover, latitude had a stronger effect on ovariole number than altitude.

EFFECTS OF CLIMATIC AND GEOGRAPHIC VARIABLES ON MORPHOLOGY

Next, we analyzed the effects of climatic and geographic variables by factor analysis, using the first three factors with eigenvalues >1.0 (together explaining 89.5% of the total variance). The first factor explained 34.0% of the total variance and was positively related to season length and negatively related to temperature differences; note that this factor was also positively correlated with altitude but negatively correlated with latitude (Table S6). This factor might thus be interpreted as a measure of "environmental stability," both in terms of annual temperature fluctuations and season length. The second factor, which explained 30.2% of the total variance, might be thought of as describing the effects of temperature, that is, mean annual and mean seasonal temperatures (Table S6). Finally, the third factor explained 25.3% of the total variance and was related to the amount of rainfall (Table S6). Multiple regression analyses with morphological traits revealed that the first factor (environmental stability) was positively correlated with wing area but negatively correlated with wing loading and ovariole index (Table S7; the negative relation with ovariole number was marginally nonsignificant, $P = 0.056$). The second factor (temperature) scaled positively with wing loading but negatively with wing area, cell number, ovariole number (the negative relation with thorax length was marginally nonsignificant, $P = 0.064$). Finally, the third factor (rainfall) was positively correlated with cell size but negatively correlated with wing loading. Together, these analyses suggest that, in addition to temperature, there are other important environmental variables that affect morphological variation across latitude and altitude.

RELATIONSHIPS AMONG MORPHOLOGICAL TRAITS AND EFFECTS OF CELL NUMBER

To analyze how morphological traits change in response to variation in body size, we used two experimental approaches aimed at maximizing variation in adult body size.

First, we studied covariation among morphological traits in flies from an outbred Austrian population and from a single isofemale line derived from an Ethiopian high-altitude population which both had been raised at different larval densities (see Materials and Methods). For both populations, we found positive linear relationships between thorax length and wing area, wing loading and ovariole number (Table S8). The increase in wing area was mainly due to an increase in cell number (Table S8). The slopes of the regression between log(wing area) and log(cell number) (Fig. S1) and log(cell size) (Fig. S2) did not differ among populations (F-test for parallelism: log(wing area) vs. log(cell number): $s = 0.67 \pm 0.002$, $F_{1, 46} = 0.38$, $P = 0.54$; log(wing area) vs. log(cell size): $s = 0.34 \pm 0.002$, $F_{1, 46} = 0.45$, $P = 0.5$). Similarly, the slope of the regression between thorax length and ovariole number did not differ among populations (F-test for parallelism: $s = 60.04 \pm 42.31$, $F_{1, 45} = 0.02$, $P = 0.88$) (Fig. S3). In contrast, populations differed for the slopes between thorax length and wing area (Austria: $s = 2.09 \pm 0.25$; Ethiopia: s = 3.46 ± 0.21; F-test for parallelism: $F_{1, 46} = 14.06$, $P = 0.0005$) (Fig. S4) and between thorax length and wing loading (Austria: $s = 1.04 \pm 0.1$; Ethiopia: $s = 0.62 \pm 0.06$; F-test for parallelism: $F_{1, 46} = 9.86$, $P = 0.003$) (Fig. S5).

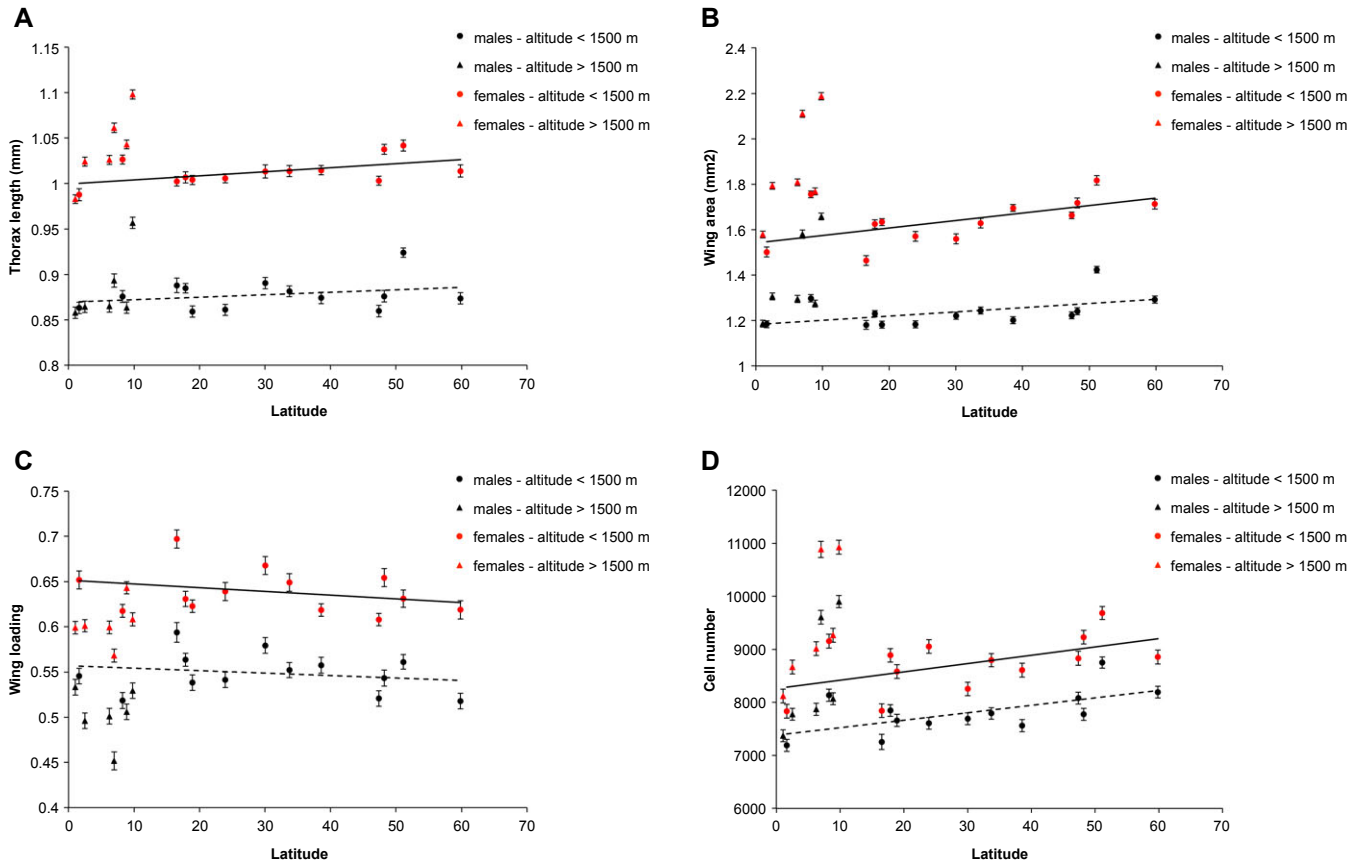

Figure 1. **Relationship between latitude and (A) thorax length, (B) wing area, (C) wing loading, (D) cell number. Black symbols: males, red symbols: females. Lines (solid line: females; dashed line: males) represent simple linear regression lines for the effect of latitude for populations with elevations below 1500 m AMSL (solid line: females; dashed line: males; thorax length: R^2 (males) = 0.08, R^2 (females) = 0.29; wing area: R^2 (males) = 0.24, R^2 (females) = 0.34; wing loading: R^2 (males) = 0.05, R^2 (females) = 0.09; cell number: R^2 (males) = 0.37, R^2 (females) = 0.28). The partitioning of the data into low versus high altitude is shown for the purpose of illustration only; for details of the multiple regression analyses using both latitude and altitude as predictor variables, see main text and Supporting Information File.**

Second, we examined the effects of increased body size via increased cell number on morphological traits by genetically manipulating insulin signaling in the ring gland during larval development (T: thorax length = 1.110 ± 0.007 mm, C_1: 1.040 ± 0.008, C_2: 1.051 ± 0.008; ANOVA: $F_{2,75} = 25.11$, $P < 0.0001$; Tukey's HSD: $P < 0.05$; $T > C_1 = C_2$) (Fig. S6A). Our results are consistent with the notion that flies with decreased insulin signaling in the ring gland are larger because they have more cells (cf. Colombani et al. 2005): as compared to the controls, experimental flies had a larger wing area (T: 2.02 ± 0.023 mm^2, C_1: 1.799 ± 0.023, C_2: 1.884 ± 0.021; ANOVA: $F_{2,60} = 23.95$, $P < 0.0001$; Tukey's HSD: $P < 0.05$; $T > C_2 > C_1$) (Fig. S6B). This effect was due to a larger number of cells (cell number: T: 9820.9 ± 149.2, C_1: 9053.1 ± 149.2, C_2: 9266.8 ± 149.2; $F_{2,27} = 7.06$, $P = 0.003$; Tukey's HSD: $P < 0.05$; $T > C_2 = C_1$), without any changes in cell size (cell size: T: 196.4 ± 3.2 μm^2, C_1: 190.6 ± 3.2, C_2: 200.5 ± 3.2; ANOVA: $F_{2,27} = 2.47$, $P = 0.1$; Tukey's HSD: $P < 0.05$; $C_2 = T = C_1$) (Fig. S6C) (also cf. Colombani et al. 2005). Despite having larger wings, these flies also exhibited increased wing loading (T: 0.683 ± 0.011, C_1: 0.620 ± 0.011, C_2: 0.621 ± 0.010; $F_{2,60} = 11.55$, $P < 0.0001$; Tukey's HSD: $P < 0.05$; $T > C_2 = C_1$). Moreover, experimental transgenic flies also had more ovarioles than controls (T: 48.6 ± 0.8, C_1: 37.9 ± 0.8, C_2: 43.3 ± 0.8; $F_{2,72} = 41.52$, $P < 0.0001$; Tukey's HSD: $P < 0.05$; $T > C_2 > C_1$) (Fig. S6D), but not when relative differences in body size were taken into account (ovariole index: T: 35.4 ± 0.7, C_1: 33.8 ± 0.7, C_2: 37.3 ± 0.7; ANOVA: $F_{2,72} = 7.07$, $P = 0.0016$; Tukey's HSD: $P < 0.05$; $C_2 = T = C_1$; $C_2 > C_1$). These results suggest that higher ovariole number, larger wing area and higher wing loading are consequences of increased body size via an overall increase of cell number.

OVARIOLE INDEX

One of the major differences between the effects of altitude and latitude was that flies from high-altitude populations had a relatively fewer ovarioles per body size (i.e., lower ovariole index).

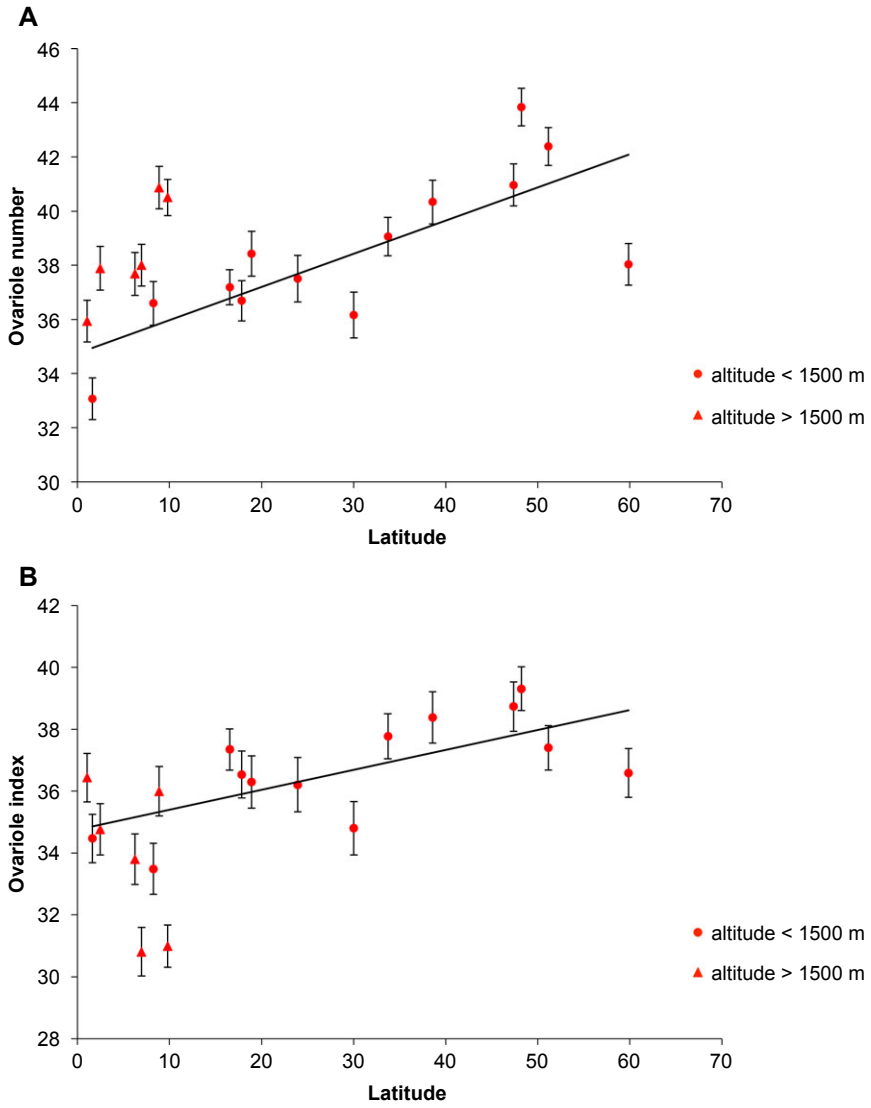

Figure 2. Relationship between (A) latitude and ovariole number and (B) latitude and ovariole index. Lines represent simple linear regression lines for the effect of latitude for populations with elevations below 1500 m AMSL (ovariole number: $R^2 = 0.58$; ovariole index: $R^2 = 0.45$). The partitioning of the data into low versus high altitude is shown for the purpose of illustration only; for details of the multiple regression analyses using both latitude and altitude as predictor variables, see main text and Supporting Information File.

To examine how variation in ovariole index might affect fitness, we analyzed its effects on early fecundity. Because thorax length, ovariole number and fecundity are known to be positively associated with each other (also see Tables S8 and S9), we hypothesized that ovariole index might influence the rate of egg production per ovariole. In support of this prediction, ovariole index was negatively correlated with the rate of egg production per ovariole in all four experimental groups (Fig. 4; Table S9). In contrast, there was no significant relationship between ovariole index and fecundity (Table S9). This suggests that flies with relatively few ovarioles per unit body size have an increased rate of egg production per ovariole. However, when we compared groups that had developed at different temperatures (18°C vs. 25°C), and which thus differed in ovariole index (Austria 18°C = 34.8 ± 0.5; Austria 25°C = 40.8 ± 0.5; Zambia 18°C = 31.8 ± 0.6; Zambia 25°C = 37.6 ± 0.5; ANOVA: $F_{3, 277} = 52.4$, $P < 0.0001$; Tukey's HSD: $P < 0.05$; Austria 25°C > Zambia 25°C > Austria 18°C > Zambia 18°C), we found that the rate of egg production per ovariole was significantly different (i.e., higher) only for Austrian flies reared at 18°C (Austria 18°C = 20.2 ± 0.3 eggs per ovariole; Austria 25°C = 18.2 ± 0.3; Zambia 18°C = 18.9 ± 0.3; Zambia 25°C = 18.5 ± 0.3; ANOVA: $F_{3, 277} = 8.82$, $P < 0.0001$; Tukey's HSD: $P < 0.05$; Austria 18°C > Zambia 18°C = Zambia 25°C = Austria 25°C) (Fig. S7; Table S10). Ovariole index is therefore a good predictor of the rate of egg production per ovariole, but only for individuals that have developed under the same thermal

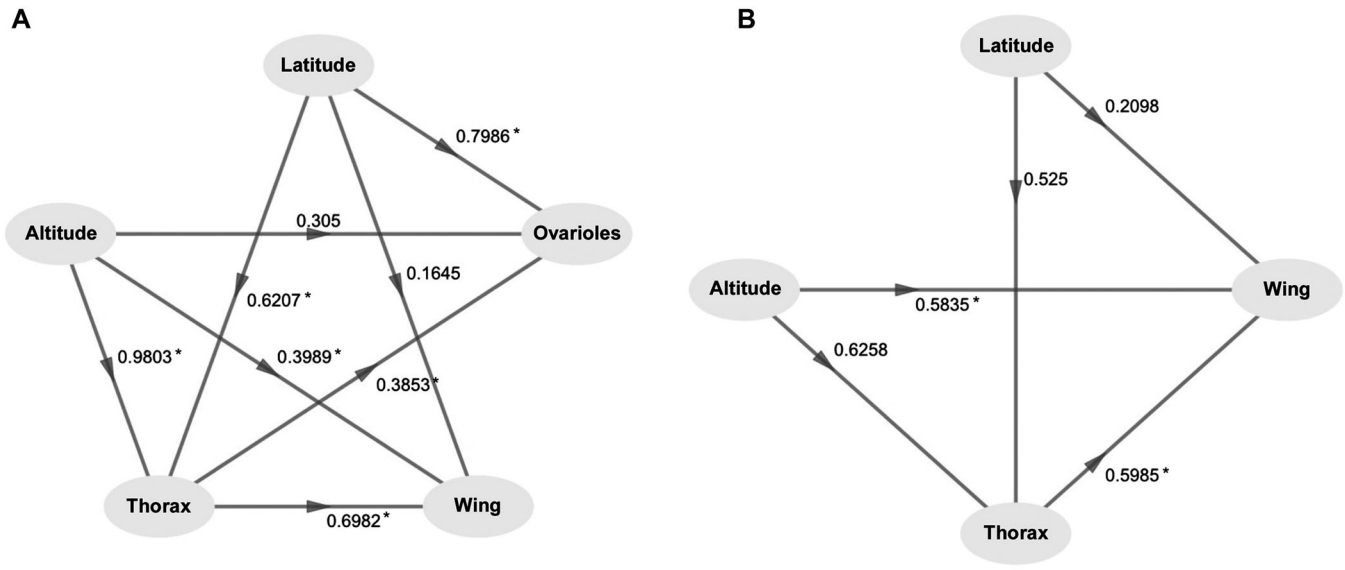

Figure 3. Path model of the effects of altitude and latitude on thorax length, wing area, and ovariole number. (A) Females, (B) males. Values with asterisks are significantly different from zero (see Table S4). See text for further details.

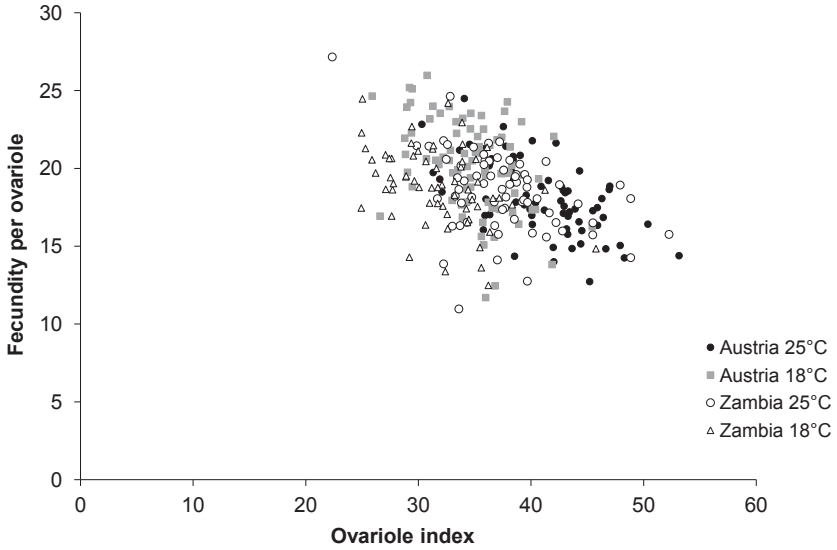

Figure 4. Relationship between ovariole index and fecundity for flies from Austria and Zambia, reared at 18°C and 25°C. See text for further details.

conditions; discrepancies across thermal conditions may be due to among-population differences in the effects of developmental temperature (significant interaction between "population" and "temperature" in Table S10).

Discussion

Here, we have examined altitudinal variation (from sea level to 3000 m altitude) and latitudinal variation (from approximately the southern to the northern range limit) for a suite of morphological traits by investigating 19 populations of *D. melanogaster* from Africa and Europe. Our principal findings are that (1) for most morphological traits the effects of altitude indeed mirror those of latitude (likely due to similar effects of temperature), as has been previously predicted, yet with some notable exceptions (see below); and that (2) clinal patterns of variation in morphology cannot be explained by clinal changes in body size alone.

ALTITUDINAL VARIATION IN MORPHOLOGY

Environmental factors that covary with altitude affected all morphological traits. Altitude was positively correlated with thorax length, wing area, ovariole number, cell number, and cell size

but negatively correlated with wing loading. Overall, altitude and latitude had similar effects on wing area, ovariole number and cell number, as has been previously postulated (Hopkins 1938; Stevens 1992; Lencioni 2004; Pitchers et al. 2013). Unlike the effects of latitude, however, high-altitude flies had relatively fewer ovarioles per unit body size (i.e., lower ovariole index), suggesting that, depending on the trait, altitude and latitude can have substantially different effects.

Traditionally, latitudinal clines for morphological traits, especially for body size, have been thought to be the result of thermal selection (Partridge and Coyne 1997). Similar clinal trends have been predicted and observed for the effects of altitude on body size, which qualitatively mirror those of latitude (Stalker and Carson 1948; Chown and Klok 2003). Our results confirm the existence of an altitudinal cline for body size in female and male *D. melanogaster* (both in terms of thorax length and wing area), and our analysis of climatic variables shows that the altitudinal increase in body size is correlated with an elevational decrease in temperature. Interestingly, high-altitude flies are phenotypically remarkably similar to flies reared at low temperatures: in both cases, flies are larger (Alpatov 1930; Imai 1934; David et al. 1994), exhibit lower wing loading (Starmer and Wolf 1989; David et al. 1994; Azevedo et al. 1998), have a lower ovariole index (Klepsatel et al. 2013b), and possess larger cells (Alpatov 1930; Robertson 1959; Azevedo et al. 2002). Similar phenotypic outcomes have also been found in laboratory natural selection experiments performed at low temperature (Anderson 1966, 1973; Cavicchi et al. 1985, 1989; Partridge et al. 1994). Together with our results, these data suggest that temperature is the major environmental factor responsible for clinal effects of altitude. Yet, these observations cannot rule out the existence of environmental factors other than temperature that might explain altitudinal patterns of phenotypic variation.

Indeed, several potentially important selective agents other than temperature are correlated with altitude and might thus underlie patterns of altitudinal variation. One such factor may be the partial pressure of atmospheric gases, which decreases with altitude. Oxygen pressure falls approximately linearly with altitude; at 5500 m, the level drops to about 50% of the value at sea level (Peacock 1998). Although the relative amount of oxygen does not change significantly with altitude, the lower atmospheric pressure at higher elevation causes oxygen to be less concentrated. Consequently, the amount of oxygen available to tissues is reduced, an effect known as high-altitude hypoxia (Frisancho 1975). Interestingly, hypoxia in *D. melanogaster* prolongs development and reduces body size (Frazier et al. 2001). This effect depends on temperature: flies reared under hypoxic conditions (10% oxygen) at 30°C exhibit a 30% reduction in thorax length as compared to flies under normoxic conditions (21% oxygen), whereas at 15°C they only show a 9% reduction in size (Frazier et al. 2001). In our study, Ethiopian high-altitude females (3000 m, oxygen availability is reduced by around 30% relative to sea level) had on average an approximately 7% larger thorax length than Ethiopian low-altitude flies (525 m). This suggests that altitudinal differences in body size might represent an example of countergradient variation, that is, patterns of geographic variation in which genetic differences among populations produce phenotypic effects that are opposite to those caused by environmental differences among populations (Conover and Schultz 1995).

High elevation also challenges flight performance, which is dramatically reduced by low air pressure (Dudley 2000; Dillon and Frazier 2006). This negative effect of low air density on performance is even more pronounced at lower temperatures (Dillon and Frazier 2006). Dudley (2000), for example, hypothesizes that high-altitude insects might exhibit lower wing loading to increase lift generated during flight. This idea is consistent with the observation that wing length increases relative to thorax length with increasing altitude in *D. robusta* (Stalker and Carson 1948). Similarly, we found a negative relationship between wing loading and altitude, suggesting that decreased wing loading might represent an adaptation to high-altitude environments.

Flies from higher elevations also had more ovarioles in our dataset. Several studies have documented a positive relation between ovariole number and early fecundity (David 1970; Klepsatel et al. 2013a). Moreover, in a previous study we found that Ethiopian high-altitude flies were larger, had more ovarioles, and were more fecund than Ethiopian low-altitude flies across a broad range of rearing temperatures in the laboratory (Klepsatel et al. 2013b). Because in their natural environment high-altitude flies might be relatively smaller (due to hypoxia) and thus have fewer ovarioles, their natural reproductive potential might actually be lower than (or similar to) that of low-altitude flies.

Importantly, we found a negative relation between altitude and ovariole index, indicating that high-altitude flies have fewer ovarioles per unit body size. This trend is opposite to the gradual increase in ovariole index we observed with increasing latitude. Our experimental test of the relationship between ovariole index and fecundity revealed that flies with a lower ovariole index have a higher rate of egg production per ovariole and might thus be able to partially compensate for the absolute decrease in ovariole number. Indeed, previous work has shown that when one of the paired ovaries in a female is surgically removed, the other ovary increases egg output by about 50% (Robertson 1957). Although the adaptive significance of a lower ovariole index remains unknown, having fewer ovarioles per unit body size might keep body weight relatively low and might thus in turn facilitate flight ability under conditions of low air density and low temperature. This is perhaps also supported by the fact that ovariole index and wing loading were positively correlated in our data (Pearson's correlation, $r = 0.46$, $P = 0.046$).

LATITUDINAL VARIATION IN MORPHOLOGY

Body size, a quantitative trait tightly connected to fitness, is expected to follow Bergmann's rule in multivoltine insects (reviewed in Blanckenhorn and Demont 2004). Although we failed to find a significant latitudinal cline for thorax length (a proxy of body size), numerous studies of *D. melanogaster* have documented the existence of a latitudinal cline for body size on several continents (David and Bocquet 1975; Coyne and Beecham 1987; Capy et al. 1993; Imasheva et al. 1994; James et al. 1995; van't Land et al. 1999). For wing area, another major proxy of body size, however, we did detect a significant increase with latitude. Unlike the effect of altitude, the latitudinal cline for wing area was not coupled to a decrease in wing loading. When we analyzed variation in wing loading as a function of different climatic and geographic variables, we found a positive relationship between wing loading and an explanatory factor related to mean annual and seasonal temperature (factor 2; "temperature"). This is in good agreement with the notion that lower wing loading may be an adaptation for flight capability in low-temperature environments (Dudley 2000; Frazier et al. 2008).

Consistent with previous studies (David and Bocquet 1975; Capy et al. 1993), we also observed a latitudinal cline in ovariole number, with high-latitude flies having more ovarioles than flies from lower latitudes. Increased ovariole number is typically associated with increased body size and higher fecundity in temperate populations of *D. melanogaster* (Bouletreau-Merle et al. 1982; Cooper et al. 2010; Klepsatel et al. 2013b), thus perhaps representing an adaptation to higher seasonal resource availability at high latitudes (Bouletreau-Merle et al. 1982; Huston and Wolverton 2009). Interestingly, the latitudinal increase in ovariole number was much steeper than the altitudinal cline for ovariole number, lending support to the notion that the evolution of higher ovariole number may be driven by resource abundance rather than by temperature (cf. Kambysellis and Heed 1971).

VARIATION IN CELL NUMBER AND CELL SIZE

Although cell size increased with altitude in our data, the altitudinal cline in wing area appeared to be mainly driven by an increase in cell number. Similarly, the latitudinal increase in wing area was predominantly caused by a higher number of cells, not changes in cell size. This is consistent with previous reports (Robertson 1959; De Moed et al. 1997), in particular with a study of latitudinal variation in wing area along the Australian cline (James et al. 1995). In contrast, Zwaan et al. (2000) found that the South American cline in wing area was mainly caused by variation in cell size; these authors speculate that wing size (rather than cell number or cell size) may be the direct target of selection. In *D. subobscura*, the European and South American clines in wing area are mostly explained by variation in cell number, whereas for the North American cline, the pattern seems to be mainly caused by variation in cell size (Calboli et al. 2003). Thus, clinal variation in wing area/size can be explained by variation in *either* cell number *or* cell size. Even though the reasons for these specific patterns (i.e., cell number or size) remain unclear, one possibility is that differences among continents in the relative cellular composition of the wing are due to founder effects (Zwaan et al. 2000; Calboli et al. 2003).

When we tested how varying cell number affects morphology (by manipulating insulin signaling), we found that an increase in cell number was positively associated with all morphological traits except for ovariole index and cell size. The observation that cell number affected ovariole number but not ovariole index is particularly noteworthy. During larval development, the number of ovarioles is thought to be determined by the number of terminal filaments (TFs), structures at the tip of the ovary, which consist of terminal filament cells (TFCs) (King et al. 1968). Studies of intra- and interspecific variation suggest that evolutionary changes in *Drosophila* ovariole number are caused by changes in the number of TFCs (Hodin and Riddiford 2000; Sarikaya et al. 2012). Similarly, nutrition-driven changes in ovariole number also seem to be determined by changes in TFC number (Sarikaya et al. 2012). Based on these observations, we hypothesized that larger flies with more cells might also have more ovarioles. Indeed, when we manipulated insulin signaling during larval development to produce larger flies with more cells, we found that these flies had a higher number of ovarioles but did not differ in ovariole index relative to controls. Similar to our manipulation of larval density, but unlike our measurements of natural populations, larger transgenic flies had larger wings and increased wing loading. This implies that the clinal patterns in morphology we have documented here are not just consequences of simple changes in body size via increased cell number. Instead, our findings indicate that different morphological traits might be driven by different selection pressures along geographical gradients. Moreover, even though our data support the idea that altitudinal clines often mirror latitudinal clines, our results suggest that this similarity is unlikely due to temperature alone and also that it does not hold for all traits. Clearly, there exist profound—but poorly understood—differences between the effects of altitudinal and latitudinal gradients on phenotypes that deserve future study.

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DATA ARCHIVING

The doi for our data is 10.5061/dryad.mf1qf.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Relationship between log(wing area) and log(cell number) in females from the Austrian outbred population and the Ethiopian isofemale line.
Figure S2. Relation between log(wing area) and log(cell size) in females from the Austrian outbred population and the Ethiopian isofemale line.
Figure S3. Relationship between thorax length and ovariole number in females from the Austrian outbred population and the Ethiopian isofemale line.
Figure S4. Relationship between thorax length and wing area in females from the Austrian outbred population and the Ethiopian isofemale line.
Figure S5. Relationship between thorax length and wing loading [(thorax length)^3/wing area] in females from the Austrian outbred population and the Ethiopian isofemale line.
Figure S6. (A) Measurements of morphological traits in *y w*; (B) *P0206*-GAL4UAS-*PTEN* females in comparison to controls: *y w*; (C) *P0206*-GAL4 and *y w*; (D) UAS-*PTEN* females.
Figure S7. Comparison of egg production rate per ovariole in Austrian and Zambian flies that developed at 18°C or 25°C.
Table S1. Climate data for different populations. See text for further details.
Table S2. Multiple regression analyses of the effects of altitude, latitude and sex on variation in morphological traits in populations from latitudes between 30°N and 30°S.
Table S3. Multiple regression analyses of the effects of altitude, latitude, and sex on variation in morphological traits in populations with elevations below 1500 m AMSL.
Table S4. Path coefficients and their 95% confidence limits from the path model of altitudinal and latitudinal effects on thorax length, wing area, and ovariole number.
Table S5. Comparison of path coefficients between sexes, based on bootstrap *t*-test. See text for further details.
Table S6. Factor loadings. See text for further details.
Table S7. Multiple regression analyses of the effects of the three factors with the largest eigenvalues (obtained from factor analysis) and sex on variation in morphological traits.
Table S8. Linear regressions between individual morphological traits.
Table S9. Spearman rank correlation coefficients (ρ) for pairwise correlations between morphological traits and fecundity, measured in females from the outbred Austrian and Zambian populations.
Table S10. Two-way analysis of variance (ANOVA) for ovariole index and egg production rate per ovariole.