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DIFFERENT CELL SIZE AND CELL NUMBER CONTRIBUTION IN TWO NEWLY ESTABLISHED AND ONE ANCIENT BODY SIZE CLINE OF *DROSOPHILA SUBOBSCURA*

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Abstract.—Latitudinal genetic clines in body size occur in many ectotherms including *Drosophila* species. In the wing of *D. melanogaster*, these clines are generally based on latitudinal variation in cell number. In contrast, differences in wing area that evolve by thermal selection in the laboratory are in general based on cell size. To investigate possible reasons for the different cellular bases of these two types of evolutionary response, we compared the newly established North and South American wing size clines of *Drosophila subobscura*. The new clines are based on latitudinal variation in cell area in North America and cell number in South America. The ancestral European cline is also based on latitudinal variation in cell number. The difference in the cellular basis of wing size variation in the American clines, which are roughly the same age, together with the similar cellular basis of the new South American cline and the ancient European one, suggest that the antiquity of a cline does not explain its cellular basis. Furthermore, the results indicate that wing size as a whole, rather than its cellular basis, is under selection. The different cellular bases of different size clines are most likely explained either entirely by chance or by different patterns of genetic variance—or its expression—in founding populations.

Key words.—Body size cline, cell number, cell size, colonization, *Drosophila subobscura*.

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Body size clines, with size positively correlated with latitude, are often found in ectotherms (Partridge and French 1996). Some cosmopolitan species belonging to the genus *Drosophila* have been found to produce parallel wing area clines on different continents (e.g., Gilchrist and Partridge 1999; Huey et al. 2000; Zwaan et al. 2000). Common garden-rearing experiments with *Drosophila melanogaster* have revealed a genetic basis for the size differences found along the clines (e.g., Coyne and Beecham 1987; Imasheva et al. 1994; James et al. 1995, 1997; Van't Land et al. 1999).

Temperature is the most probable selective factor causing these latitudinal clines in size. Latitude is consistently correlated with average, minimum, and maximum temperature but not with other factors that could influence size, such as humidity or rainfall (Zwaan et al. 2000). Furthermore, caged laboratory populations of *Drosophila melanogaster* kept at different temperatures evolve genetically different sizes, with larger flies in the ''cold'' selection lines (Anderson 1973; Cavicchi et al. 1985; Partridge et al. 1994).

In *D. melanogaster*, wing area can change through changes in cell size, cell number, or both. Several studies (e.g., Robertson 1959; Cavicchi et al. 1985; Partridge et al. 1994) have found that laboratory thermal selection lines differ in wing area entirely as a consequence of a difference in cell size. Latitudinal clines, on the other hand, show variation in wing area based mainly on cell number, with cell size contributing at most only a small amount (James et al. 1995, 1997; Pezzoli et al. 1997; Zwaan et al. 2000).

The difference in the cellular bases of wing area differences in latitudinal clines and laboratory thermal selection lines requires explanation, especially if both are due to thermal selection. Is the cell size difference that we have seen in thermal selection lines an early stage in the evolution of body size that eventually will evolve into a cell number difference (see also Partridge and French 1996)? If this is the explanation, then we would expect to see clines based on cell size in nature when a latitudinal wing size cline is established for the first time.

To test this idea, we examined the cellular basis of three wing size clines in *D. subobscura*: Europe, North America, and South America. *Drosophila subobscura* is endemic to Europe, where latitudinal clines in several traits, including body size, are observed (Misra and Reeve 1964). This species has recently colonized South America, with a first report in 1978 (Brncic et al. 1981), and North America, with a first report in 1982 (Beckenbach and Prevosti 1986). After colonization, the North and South American populations underwent significant genetic differentiation from the original European colonizers in a number of different traits: allozyme polymorphism (Prevosti et al. 1983; Balanya and Serra 1994), lethal allelism (Sole et al. 2000), chromosomal polymorphism (Prevosti et al. 1985, 1988; Ayala et al. 1989; Mestres et al. 1994), DNA polymorphism (Latorre et al. 1986; Rozas et al. 1990; Rozas and Aguade 1991) and quantitative traits (Budnik et al. 1991). Nonetheless, the first survey, conducted using flies collected in 1986 and 1988, failed to show any latitudinal size cline on either continent (Pegueroles et al. 1995). A second survey conducted by Huey and Gilchrist (Huey et al. 2000; Gilchrist et al. 2001) in North America, with flies collected in 1999, did find a wing length cline, with genetically larger flies at higher latitudes.

Huey and Gilchrist (Huey et al. 2000; Gilchrist et al. 2001) found that the increase in wing length with latitude in the European cline was associated with a relative lengthening of

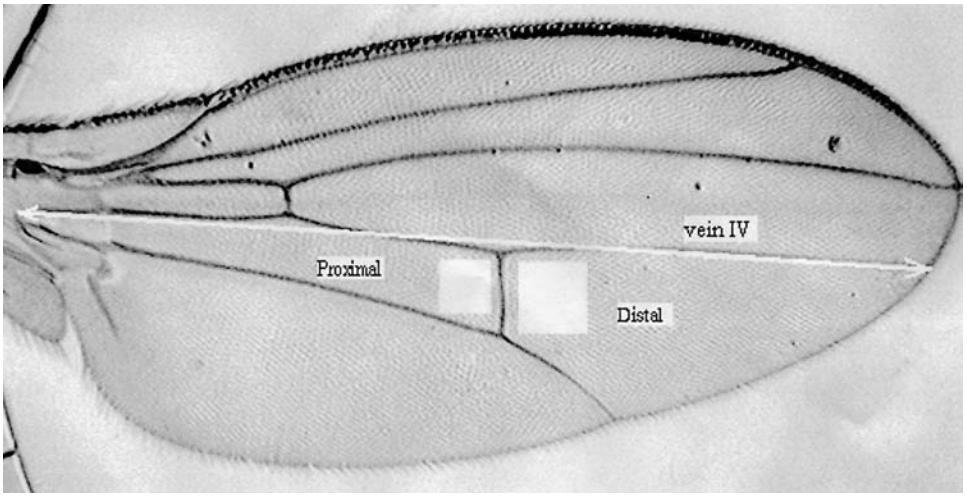

FIG. 1. Wing. The two white squares, left and right of the posterior crossvein, represent the area where cell area measurements were taken. The white line is the length of vein IV. In Huey et al. (2000), vein IV was measured from the base of the vein to the crossvein and from the crossvein to the wing border.

the basal portion of vein IV, whereas the increase in North America was associated with an increase in the distal portion of the same vein (see Fig. 1). Preliminary results (G. W. Gilchrist, unpubl. obs.) indicate that in South America both segments of vein IV increase in length with latitude. These findings suggest that total wing size or one of its cellular components, rather than the size of a particular wing region, may be the target of selection. Assessing the cellular basis of the latitudinal variation could provide evidence on whether wing size itself or its cellular components are a target of selection. At present the adaptive significance of evolutionary size increase in the high latitude populations is not understood.

MATERIALS AND METHODS

The wings used for this study were described in Huey et al. (2000) and Gilchrist et al. (2001). In brief, North American flies collected in 1997 (April and May) from 11 localities, European flies collected in 1998 (May) from 10 localities, and South American flies collected in 1999 (November) from 10 Chilean localities were raised in population cages (10 flies per sex from each of 15 to 25 isofemale lines) for five or six generations in common laboratory conditions at 20°C, then one generation was reared under controlled density of 50 flies per vial. The eclosing flies were collected and the wings mounted on tape on slides (Table 1).

In the present study, the left or right wings of 20 females and 20 males were measured for each population. Occasionally fewer flies per population were available for measurement, but always at least fourteen flies per sex were scored. The wings were measured using a microscope with camera lucida attachment and graphic table at 10×40 magnification. Cell density varies across the surface of the *Drosophila* wing. However, concordant differences in the cell area between different parts of the wing blade are found for differences between both individuals and populations (see Delcour and Lints 1966; Partridge et al. 1994; Pezzoli et al. 1997). The proximal and distal part of vein IV showed a different lengthening pattern with latitude in the European and North American clines (Huey et al. 2000; Gilchrist et al. 2001). For this reason two different sampling areas in the region between veins IV and V were examined. These areas have been pre-

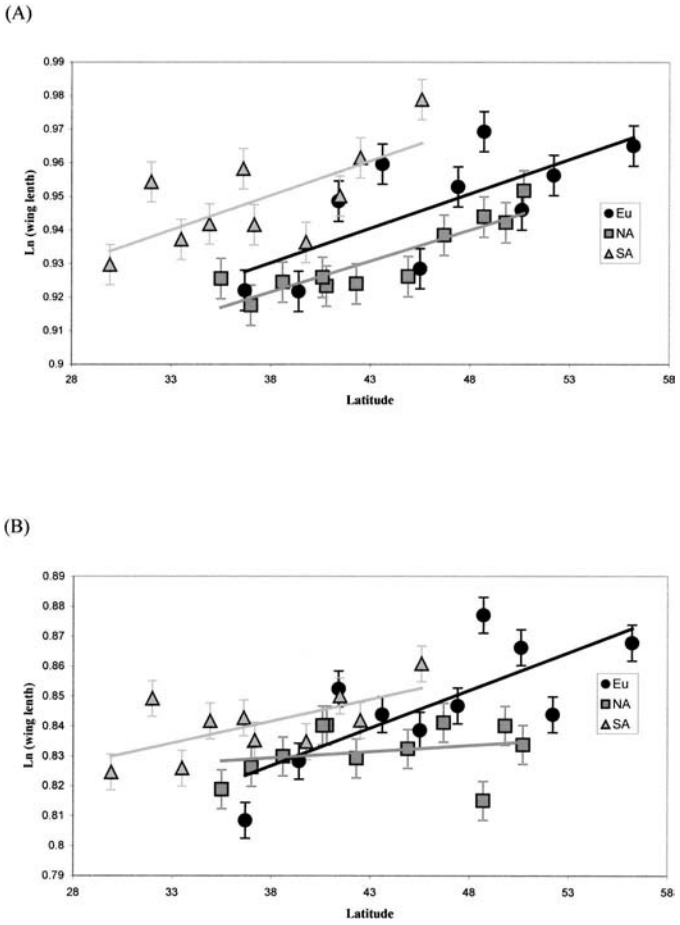

FIG. 2. Regression of ln (wing length) on latitude. (A) females; (B) males.

TABLE 1. Localities of collection. Name and latitude (decimal degrees) of each population.

Europe 1998	Latitude N
Arhus, Denmark	56.2
Leiden, The Netherlands	52.2
Lille, France	50.6
Gif-sur-Yvette, France	48.7
Dijon, France	47.4
Lyon, France	45.5
Montpellier, France	43.6
Barcelona, Spain	41.4
Valencia, Spain	39.4
Malaga, Spain	36.7
North America 1997	Latitude N
Port Hardy, BC, Canada	50.7
Peachland, BC, Canada	49.8
Bellingham, WA	48.7
Centralia, WA	46.7
Salem, OR	44.9
Medford, OR	42.3
Eureka, CA	40.8
Redding, CA	40.6
Davis, CA	38.6
Gilroy, CA	37.0
Atascadero, CA	35.5
South America (Chile) 1997	Latitude S
Coyhaique	45.58
Castro	42.50
Porto Montt	41.47
Valdivia	39.77
Laja	37.17
Chillan	36.62
Curico	34.92
Santiago	33.50
Illapel	32.00
LaSerena	29.92

viously used in the analysis of cell size/cell number variation. They can be located independent of wing allometry and wing area changes and are regions of relatively low variation in cell density. The two sampling areas were considered proximal and distal, referring to the crossvein and the landmarks used by Huey and Gilchrist (Huey et al. 2000; see Fig. 1). The number of trichomes in two 500- μm^2 sampling squares within each sampling area was counted and cell area was calculated as (500/no. trichomes). Two measurements were taken for each sampling area and the average was used for statistical analysis. Because cell area is variable across the wing blade, it was not possible to infer total cell number in the wing, and a total cell number index was used. The length of vein IV was used to represent wing length; the index was calculated as wing length^2/cell area; again two indices were calculated, one using the distal cell area and one using the proximal cell area, because of the different behavior of the two segments of vein IV in Europe and North America. Vein IV itself was measured using an ocular micrometer on a 10x eyepiece on a dissection microscope, at 4x magnification.

The five characters measured (wing length, distal and proximal cell size, distal and proximal cell number index) were analyzed separately. For each trait, we used a standard linear model to estimate the regression coefficients simultaneously by nesting latitude inside sex and continent. This yields an estimate of the slope for each continent-by-sex subset of the data. We tested for parallel regression slopes using a standard ANOVA comparison of slopes test. Type-III sums of squares were used for all ANOVAs to compensate for the unequal sample sizes. An ANCOVA was used to test for sex effects. Data were normally distributed in all cases (Shapiro-Wilks *W*-test). In addition, plots of residuals versus latitude revealed homoscedasticity and therefore no transformation was deemed necessary.

RESULTS

Wing Length

Ln(wing length) was regressed on latitude, nested within sex and continent, to produce individual estimates of the slopes for females and males in North America, South America, and Europe. All slopes but one, North American males, were significant and positive (Fig. 2 and Tables 2, 3). A comparison of slope tests revealed that all the slopes in the three continents were homogeneous; the main effects on size were due to sex and latitude. Our data show that, by 1999, a latitudinal wing length cline had evolved in South America.

Cell Size

The regression of proximal cell size and distal cell size was analyzed using the same nested design as for ln(wing length). For proximal cell size, with the exception of North American males, no significant regression with latitude was found; the trend for both sexes in Europe and South America is negative, whereas it is positive in North America, and a linear model for comparison of slopes revealed significant difference between continents, detected by significant interaction between continent and latitude (Fig. 3A, B and Tables 2, 3).

For distal cell size, the regression analysis on latitude (Fig. 3C, D and Tables 2, 3) was negative but not significant for European flies. North American females exhibited positive and significant cline, whereas in males the trend is positive but not significant. The South American flies yielded a similar pattern to that in Europe with a negative but not significant trend in distal cell size for both sexes. A comparison-of-slopes test detected significant difference between continents, revealed by significant interaction between continent and latitude. No main effect was applicable.

For both proximal and distal cell size the deletion of the interaction terms involving sex in Table 3 resulted in the main effects of sex becoming highly significant in all cases, with females having bigger cells on all continent and across all latitudes (results not shown).

Cell Number Index

The regression slopes for proximal and distal cell number index were estimated using a nested model similar to that for cell area and ln(wing length). For the proximal index (Fig. 4A, B and Tables 2 and 3) both sexes in Europe and females in South America showed a significant positive regression coefficient. North American flies did not show a significant regression in either sex. A comparison-of-slopes test showed significant differences between continents revealed by a sig-

TABLE 2. Linear model estimates.

Continent	Sex	Intercept ± SE	Slope ± SE	*t*-value (slope)
Wing length				
Europe	F	0.85 ± 0.027	0.0020 ± 0.00060	3.52***
North America		0.85 ± 0.029	0.0019 ± 0.00070	2.82**
South America		0.87 ± 0.026	0.0021 ± 0.00070	2.96**
Europe	M	0.73 ± 0.027	0.0025 ± 0.00060	4.22***
North America		0.81 ± 0.028	0.0004 ± 0.00060	0.65
South America		0.79 ± 0.026	0.0015 ± 0.00070	2.10*
			R^2: 0.9686	
Proximal size				
Europe	F	268.87 ± 26.204	−0.90 ± 0.564	−1.60
North America		167.95 ± 28.146	1.02 ± 0.651	1.56
South America		241.82 ± 25.481	−0.62 ± 0.677	−0.91
Europe	M	225.42 ± 26.651	−0.41 ± 0.573	−0.71
North America		141.46 ± 27.451	1.26 ± 0.632	2.00*
South America		204.27 ± 25.481	−0.27 ± 0.677	−0.39
			R^2: 06448	
Proximal index				
Europe	F	19526.89 ± 3717.254	219.71 ± 79.988	2.75**
North America		32395.61 ± 3992.795	−39.56 ± 92.318	−0.43
South America		22713.71 ± 3614.786	215.92 ± 96.032	2.25*
Europe	M	18019.56 ± 3780.702	190.24 ± 81.341	2.34*
North America		33287.16 ± 3894.167	−141.65 ± 89.703	−1.58
South America		23591.82 ± 3614.786	116.86 ± 96.032	1.22
			R^2: 0.6559	
Distal size				
Europe	F	272.71 ± 23.935	−0.67 ± 0.515	−1.29
North America		164.02 ± 25.709	1.30 ± 0.594	2.18*
South America		253.31 ± 23.275	−0.74 ± 0.618	−1.19
Europe	M	242.48 ± 24.344	−0.60 ± 0.524	−1.15
North America		170.48 ± 25.074	0.80 ± 0.578	1.39
South America		219.63 ± 23.275	−0.46 ± 0.618	−0.74
			r^2: 0.7290	
Distal index				
Europe	F	19450.69 ± 3175.131	181.44 ± 68.323	2.66*
North America		32159.92 ± 3410.487	−61.28 ± 78.854	−0.78
South America		21194.66 ± 3087.607	231.43 ± 82.027	2.82**
Europe	M	16328.97 ± 3229.326	202.19 ± 69.478	2.91**
North America		29611.56 ± 3326.243	−83.83 ± 76.621	−1.09
South America		21362.75 ± 3087.607	145.37 ± 82.027	1.77*
			R^2: 0.7256	

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

nificant interaction between continent and latitude. No main effect was applicable.

For the distal index we found similar results (Fig. 4C, D and Tables 2 and 3), with both sexes in Europe and South America giving a positive regression with latitude, and North American flies not showing a significant regression with latitude. The same results were found for the comparison-of-slopes test, with significant differences between continents due to a significant interaction between continent and latitude. Again no main effect was applicable.

For both proximal and distal cell number index the deletion of the interaction terms involving sex in Table 3 resulted in the main effects of sex becoming highly significant in all cases, with females having more cells than males on all continents and across all latitudes (results not shown).

DISCUSSION

The most important result of this work is the finding that the two newly established North American and South American wing area clines in *Drosophila subobscura* differed in the cellular basis of the latitudinal variation. The North American cline was based on cell size whereas the South American cline was based on cell number. The ancestral European cline was also based on cell number. Cell size showed a positive regression with latitude in North American female flies, whereas South American and European flies showed a positive cline with latitude in both cell number indexes. The slopes for ln(wing length), reflecting overall size, were positive and significant, with the exception of North American males. Thus, parallel wing size clines are present on all three continents. Our data show that latitudinal size clines in nature can differ in their cellular basis, as previously observed (Zwaan et al. 2000).

It is interesting to note that in all continents, independent of the cellular mechanism determining the cline, for all latitudes, an increase in both cell size and cell number explains the bigger wing size of females.

The situation presented by the North and South American

TABLE 3. Comparison of slopes test, type-III sums of squares. We cannot reject the null model (homogeneity of slopes) for wing length; however, all other traits show a significant interaction between continent and latitude. Sex, however, does not interact with either latitude or continent, suggesting the slopes of the sexes are parallel and can be analyzed via ANCOVA.

	df	Mean square	*F*-value	*P* (*F*)
Wing length				
Continent	2	0.0027	1.26	0.292
Sex	1	0.0284	13.27	0.001***
Latitude	1	0.0911	42.60	0.000***
Continent : Sex	2	0.0024	1.13	0.331
Continent : Latitude	2	0.0034	1.59	0.214
Sex : Latitude	1	0.0022	1.03	0.314
Continent : Sex : Latitude	2	0.0025	1.19	0.313
Residuals	50	0.0021		
Proximal Size				
Continent	2	12611.7	6.19	0.004 na
Sex	1	5547.1	2.72	0.105
Latitude	1	5.4	0.00	0.959
Continent : Sex	2	101.9	0.05	0.951
Continent : Latitude	2	9986.9	4.90	0.011*
Sex : Latitude	1	1021.8	0.50	0.482
Continent : Sex : Latitude	2	44.3	0.02	0.979
Residuals	50	2036.6		
Proximal index				
Continent	2	284537902.0	6.94	0.002 na
Sex	1	33044.0	0.00	0.977
Latitude	1	269060107.0	6.56	0.013 na
Continent : Sex	2	2770047.0	0.07	0.935
Continent : Latitude	2	268717224.0	6.56	0.003**
Sex : Latitude	1	45384977.0	1.11	0.298
Continent : Sex : Latitude	2	4780141.0	0.12	0.890
Residuals	50	40984634.0		
Distal size				
Continent	2	12246.5	7.21	0.002 na
Sex	1	1585.8	0.93	0.339
Latitude	1	113.0	0.07	0.798
Continent : Sex	2	683.9	0.40	0.671
Continent : Latitude	2	9454.0	5.56	0.007**
Sex : Latitude	1	20.6	0.01	0.913
Continent : Sex : Latitude	2	380.7	0.22	0.800
Residuals	50	1699.2		
Distal index				
Continent	2	249272726.0	8.34	0.001 na
Sex	1	14536132.0	0.49	0.489
Latitude	1	323093344.0	10.81	0.002 na
Continent : Sex	2	4676216.0	0.16	0.856
Continent : Latitude	2	234242077.0	7.83	0.001***
Sex : Latitude	1	6586313.0	0.22	0.641
Continent : Sex : Latitude	2	7432990.0	0.25	0.781
Residuals	50	29901981.0		

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; na, not applicable.

clines is unusual. The founding populations in the two continents are closely related genetically (Prevosti et al. 1983; Balanya and Serra 1994; Mestres et al. 1994; Mestres and Serra 1995), and the evolution of the clines has been monitored since colonization. Despite the fact that the establishment of body size clines in the Americas was expected (Pegueroles et al. 1995) and eventually found, the cellular mechanism underlying wing size differences is not the same in the two continents. The comparison between the Americas and the ancestral European population is also revealing. Comparison of the European and North American clines, with their different cellular bases, is consistent with the idea that the cellular basis of body size variation could change from cell size to cell number with time (Partridge and French 1996). However, the South American data are not consistent with this hypothesis. The newly established South American cline is based on cell number. Thus the hypothesis that the cellular basis of wing size difference evolves over time from cell size to cell number is not supported by our findings.

The relative lengths of the proximal and distal segments of vein IV differ in Europe and North America (as noted by Huey and Gilchrist in Huey et al. 2000; Gilchrist et al. 2001). Nonetheless, we found that cell size and cell number showed the same clinal pattern in both the proximal and distal segments of the wing. Our results hence suggest that thermal selection may target the whole wing rather than just one of its parts.

Wing area is positively correlated with body size as a whole (Reeve and Robertson 1952; Robertson 1959; Misra and Reeve 1964; Wilkinson et al. 1990). However, the cellular

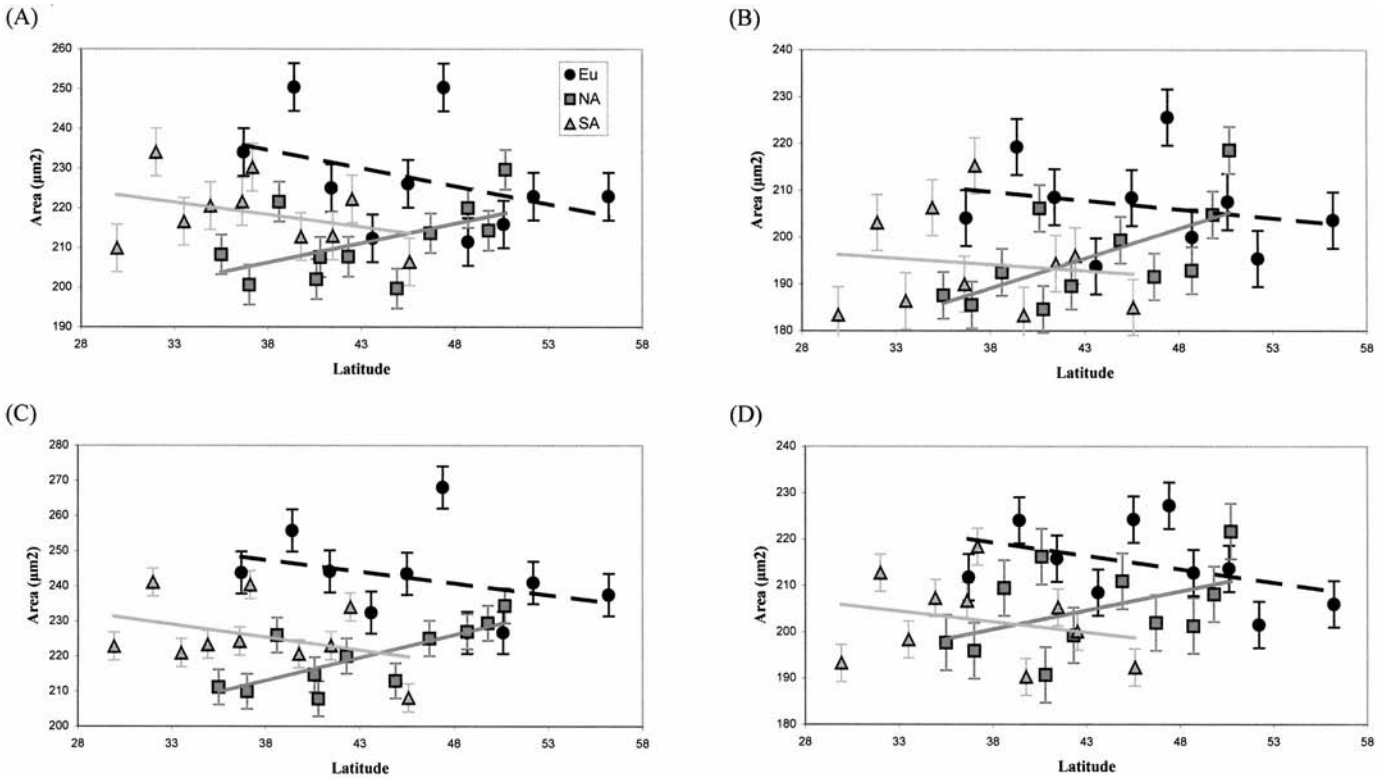

FIG. 3. Regression of cell size (± SE) on latitude. Area in square micrometers. Top panels, proximal cell size for (A) females and (B) males. Bottom panels, distal cell size for (C) females and (D) males.

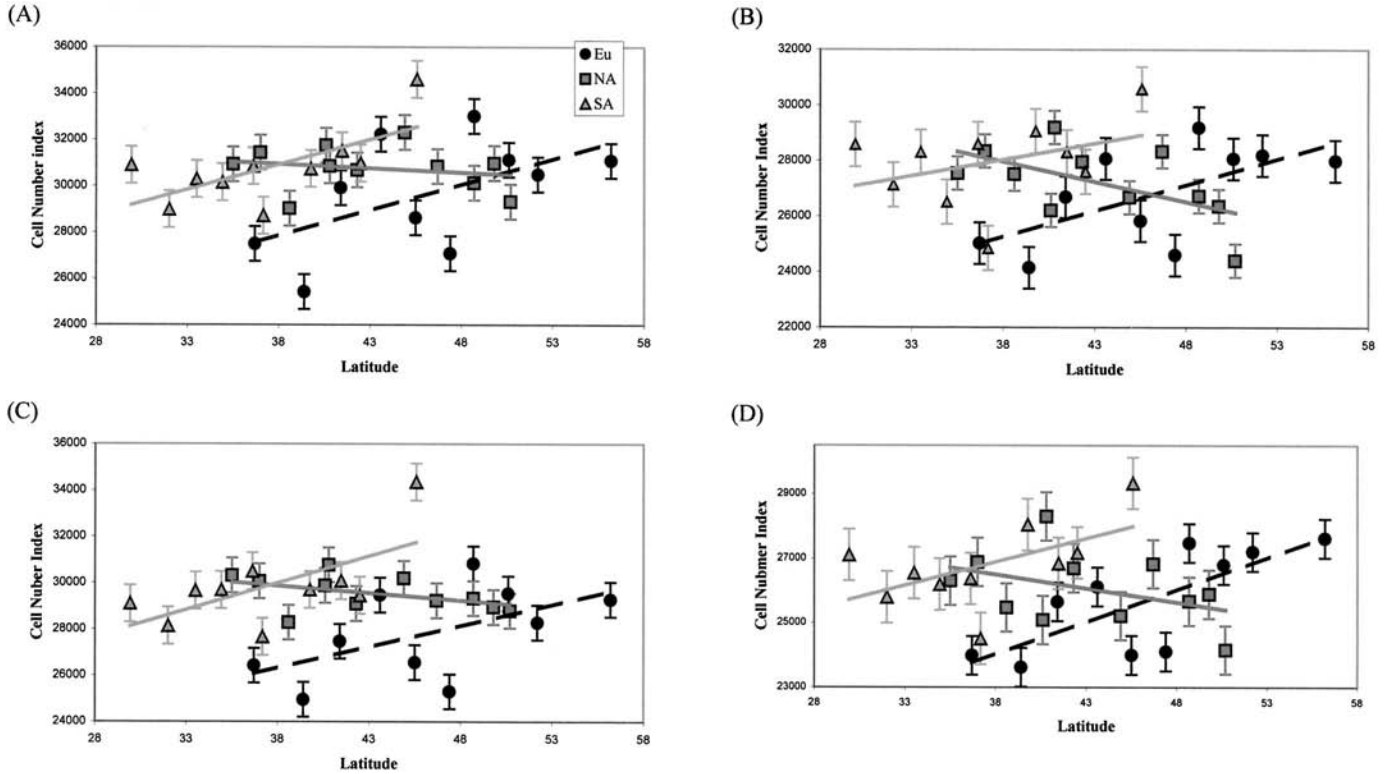

FIG. 4. Regression of cell number indexes (± SE) on latitude. Top panels, proximal cell number index for (A) females and (B) males. Bottom panels, distal cell number for (C) females and (D) males.

basis of variation in wing area is not always the same as that for other anatomical regions. Comparison of cline-end populations of a South American *D. melanogaster* size cline showed that the contribution of cell size differed in different organs (wing, eye, and proximal tarsal segments), with size variation between populations attributable to cell number for wing area and to cell size for eye and tarsal segments (Azevedo et al. 2002). The different cellular bases of the two newly established *D. subobscura* clines and the different cellular basis for clinal variation in the size of different body parts within a single cline all show that, rather than its cellular components, size per se or something genetically correlated with size is the target of selection.

The recent colonization of the Americas by *D. subobscura* is a singular chance to observe the evolution of metric traits in the field. It has allowed us to discount time since establishment as a likely cause of the different cellular bases of the response of body size to selection in different populations. Although we cannot completely rule out the hypothesis that the cellular bases of latitudinal size variation in the North and South American clines may not be caused by the same selective agents, a more parsimonious explanation is that pure chance or differences in the genetic composition of founding populations or the way that variation is expressed in different local environments must be responsible.

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