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**First record of *Drosophila parthenogenetica* and *D. neomorpha, cardini* group, Heed, 1962 (*Drosophila*, *Drosophilidae*), in Brazil.**

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The *cardini* group of the Quinaria section of the genus *Drosophila* is composed of 16 Neotropical species. The two focal species of this report, *Drosophila parthenogenetica* and *D. neomorpha*, are included in this group, and are placed in the *cardini* subgroup with seven other species: *D. polymorpha*, *D. cardinoides*, *D. cardini*, *D. neocardini*, *D. acutilabella*, *D. bedicheki* and *D. procardinoides* (Heed and Krishnamurthy, 1959; Heed, 1962; Heed and Russell, 1971; Wilder *et al.*, 2003). The group is characterized by medium-sized flies with a shiny thorax (Vilela *et al.*, 2002). Different species of the group display different abdominal pigmentation patterns (Hollocher *et al.*, 2000a, b), different degrees of divergence and population structure (Wilder *et al.*, 2003; Wilder *et al.*, 2004) as well as different abilities to colonize multiple niches (Rhode and Valente, 1986; Machado *et al.*, 2001). We have been investigating the southern Brazilian Drosophilid communities since the 1990's as a system for understanding the impact of territorial range expansions. In this article, we report the movement of two species of the *cardini* group, *D. parthenogenetica* and *D. neomorpha*, into Brazil and discuss the potential impacts of these range expansions may have on the existing Drosophilid communities.

Drosophilid samples were obtained from collections of adults over fruit and /or vegetable baits in traps in both Atlantic and Amazonian forests in Brazil. The Atlantic sites were within forest in a secondary stage of regeneration at Caldas da Imperatriz (27° 44.480'S; 48° 48.440'W), Joinville (26° 17.150S; 49° 01.00'W), and Campeche Island (27° 41.310' S; 48° 28.880'W), all in the Brazilian State of Santa Catarina. This region is within the transition between subtropical and temperate climatic zones (Moreno, 1961), and, thus, has well-defined seasons with temperatures near 0°C commonly measured during the winter months (June, July, and August) and temperatures reaching 40°C during the summer (December, January and February). The Amazonian sites were at Caxiuana station (S 01° 44.258'; W 51° 27.352') within the northern Brazilian State of Pará, Urucu in the state of Amazonas (S 08° 17.150'; W 35° 0.200') and Alta Floresta in the state of Mato Grosso (S 09° 53.020'; W 56° 14.380'). These central and northern Brazilian regions exist within a tropical climate such that there are not well-defined seasons, with temperatures varying between 25°C and 40°C all year.

At the Atlantic forest site from Caldas da Imperatriz and Campeche Island, we caught three and ten individuals of *D. parthenogenetica*, respectively. This collection brought together *D.*

*willistoni*, *D. polymorpha*, *D. sturtevantii*, *D. sellata*, *D. malerkotliana*, *D. simulans*, *Zaprionus indianus*, *D. griseolineata*, *D. atrata*, species from *repleta* and *tripunctata* groups, as well as other less frequent species of Drosophilidae. At the Amazonian forest sites we found two, five and four individuals of *D. parthenogenetica* at the sites in Para, Mato Grosso, and Amazonas States, respectively. In Figures 1 and 2 we show, respectively, the external terminalia, and the *aedeagus* (male internal terminalia) of *D. parthenogenetica* (compared with Heed and Russell, 1971).

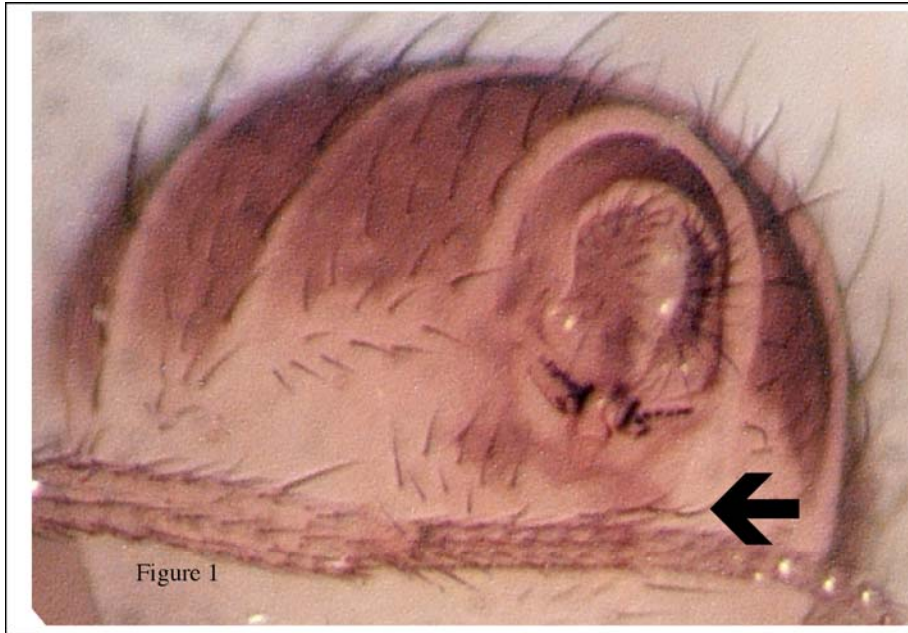


Figure 1. Frontal view of the external terminalia of *D. parthenogenetica*. Notice that the *surstyli* (arrow) are not in a goggle-shape as in *D. polymorpha* (Vilela *et al.*, 2002), but in a hook-shape.



Figure 2. Frontal view of the external terminalia of *D. neomorpha*. Notice the shape of the *surstyli* (arrow). They are composed of two separated rows of teeth, the anterior being the shorter of the two.

Twenty individuals of *D. neomorpha* were detected in the Amazonian rainy season samples (April, 2002) at Para State and five at Mato Grosso. They were caught together with *D. willistoni*, *D.*

*paulistorum*, *D. tropicalis*, *D. equinoxialis*, *D. sturtevanti*, *D. prosaltans*, *D. sellata*, *D. malerkotliana*, *D. simulans*, *Zaprionus indianus*, as well as *D. parthenogenetica* and other less frequent species of Drosophilidae. *D. neomorpha* was also collected at Santa Catarina State in a Joinville Atlantic Forest sample (May, 2003) together with *D. willistoni* subgroup, *D. simulans*, *D. guarani* subgroup, *D. saltans* subgroup, *D. polymorpha*, *D. neocardini*, as well as other less abundant species of Drosophilidae. In Figures 3 and 4 we show, respectively, the external terminalia and the *aedeagus* of *D. neomorpha* (compared with Heed and Russell, 1971; Vilela *et al.*, 2002).

Figure 3. a: Lateral view of the *aedeagus* of *D. parthenogenetica*, where it is possible to visualize the wide membranous sheath (arrow) in both ventral and dorsal sides; 3b: Lateral view of the *aedeagus* and *aedeagal apodema* of *D. parthenogenetica*; bar = 0.1mm.

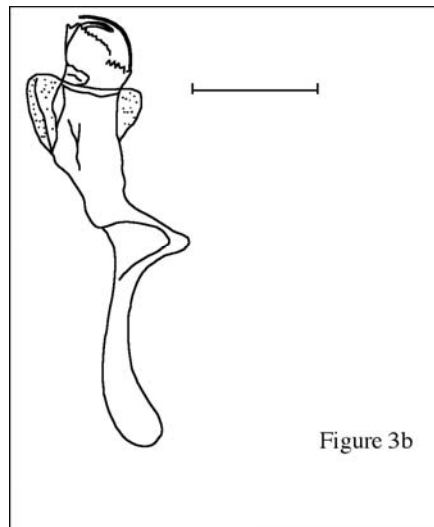


Figure 3b

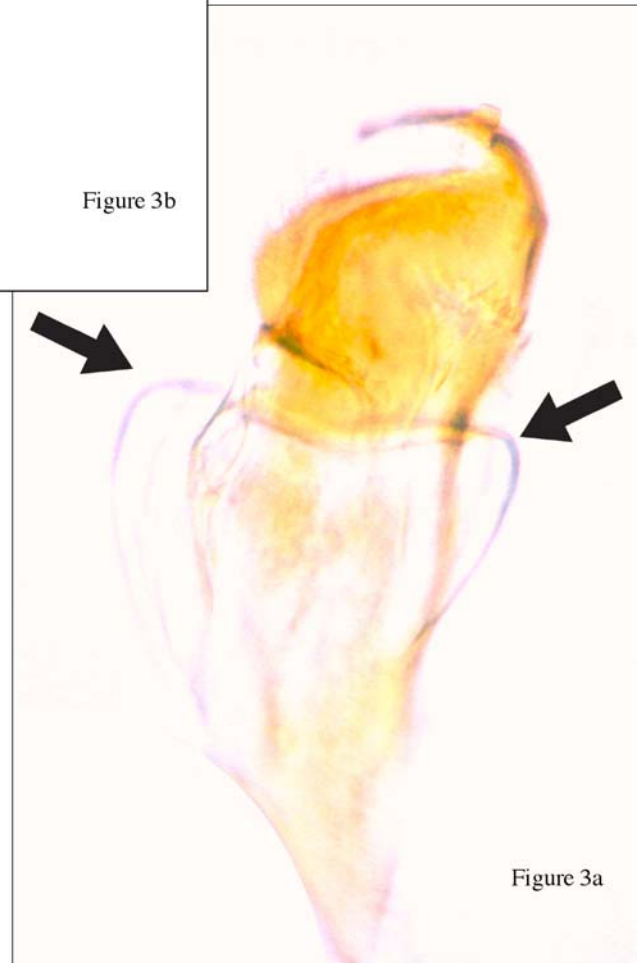


Figure 3a

Previous reports indicated that both of these species were distributed from central Mexico to northern South America (Heed, 1963). Therefore, our collections of *D. parthenogenetica* and *D. neomorpha* from the Amazonian and especially the Atlantic forest sites represent significant range expansions for both species. Our initial detection of these two species was made via sequencing of the mitochondrial *cytochrome B* and adjacent DNA regions (Brisson *et al.*, unpublished data) as well as mitochondrial

*16S rRNA* and the nuclear gene *yellow* (Penton, *et al.*, unpublished data). In addition, we confirmed their species identifications by male terminalia analysis.

Since *D. parthenogenetica* was initially described as native to Central America and northern South America (Heed, 1963) and now has been found in the state of Santa Catarina in Brazil, we hypothesize that it has expanded its range via northern South America to northern and southern Brazil. We have been making extensive detailed collections in the Atlantic forests of Santa Catarina for the last five years and analyzing the species collected using both internal male terminalia

morphology and chromosomal polymorphism (De Toni *et al.*, 2001b, c). We have not found *D. parthenogenetica* in these collections previously. Therefore, it is likely that this species has arrived in southern Brazil in the last five years or was at a density that was below detection previously. We project that the species will continue southward into the most southern Brazilian state (Rio Grande do

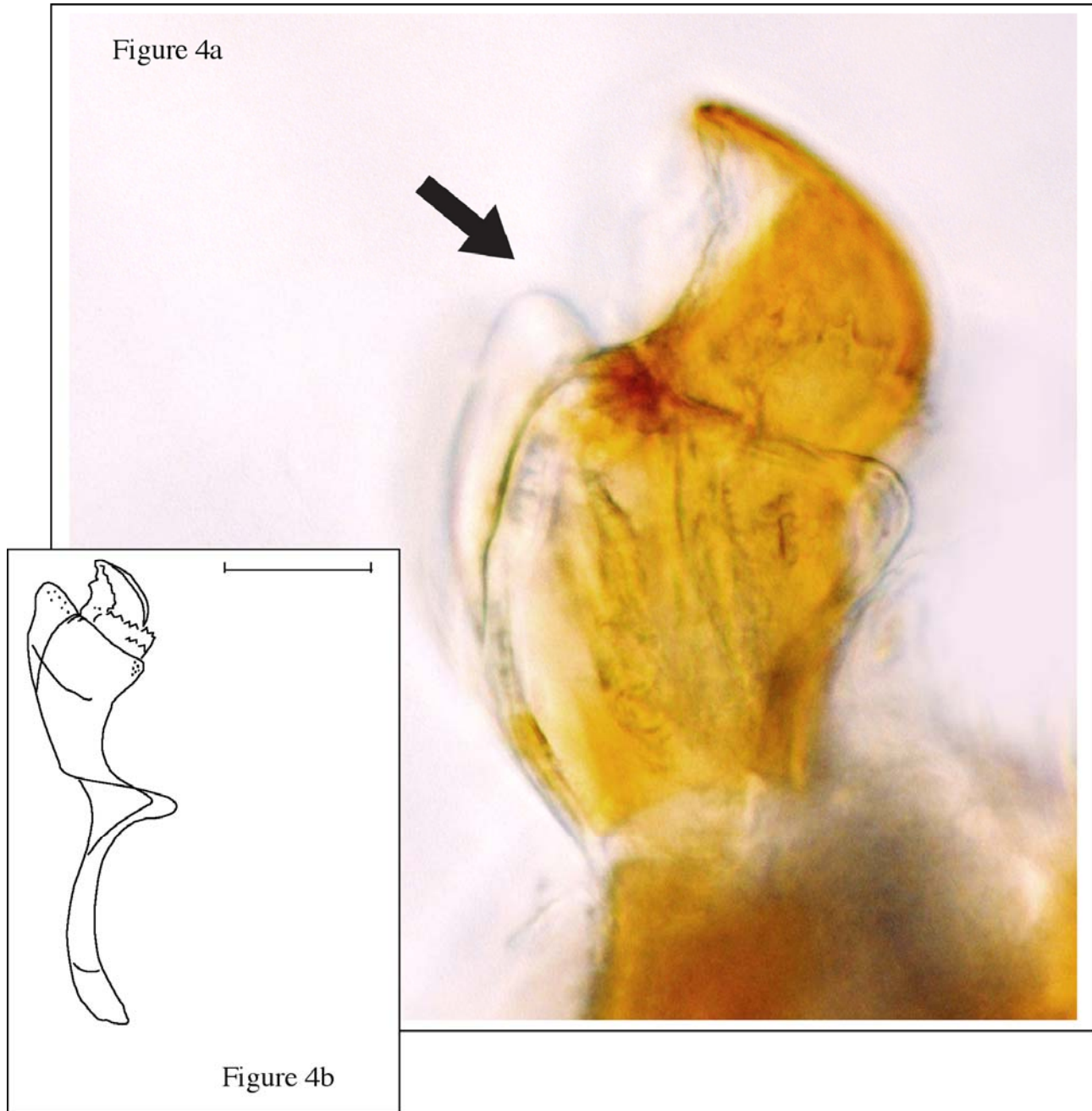


Figure 4. a: Lateral view of the *aedeagus* of *D. neomorpha*. The arrow points to the angle formed between the ventral membranous sheath and the sharp end at the top of the “head” of the *aedeagus*. This angle is smaller than the one observed in *D. polymorpha* (Heed and Russell, 1971) which is almost 180°; 4b: Lateral view of the *aedeagus* and *aedeagal apodema* of *D. neomorpha*; bar = 0.1mm.

Sul), because of the lack of barriers and the continuity of the habitat between these states. Other species of Drosophilidae, such as *D. malerkotliana* (Sene *et al.*, 1980; Martins, 2001) and *Zaprionus indiannus* (De Toni *et al.*, 2001a; Castro and Valente, 2002), have been described first in the Santa Catarina State and after some months were then detected in Rio Grande do Sul.

*D. neomorpha* is also not reportedly a species native to Brazil (Heed, 1963). We hypothesize that it arrived in the northern Brazilian states recently, and we expect that it already occupies a large portion of the Amazon forest, sharing an ecological niche with *D. polymorpha*, its closest relative. Vilela *et al.* (2002) described the geographical distribution of *Drosophila* of the *cardini* group in morphoclimatic domains of Brazil with very detailed drawings of male terminalia. We believe that the *D. polymorpha aedeagus* of a holotype collected in Bertioga, Sao Paulo State (Southeast region), described in Vilela *et al.* (2002) is actually a *D. neomorpha aedeagus* according to our comparisons made with Heed's drawings (1963). However, interestingly, the *epandrium* and the surstyli are very similar to those of *D. polymorpha*. This combination of morphological characters could be evidence of introgression or it could reflect a wide range of morphological variability in *D. neomorpha* previously undescribed. We favor the latter hypothesis, because multiple molecular markers have consistently placed these morphologically unique individuals into the *D. neomorpha* species categories (Penton, *et al.*, unpublished data). Given this result, it is important that the specimens of *D. polymorpha* collected earlier in various regions of Brazil should be re-examined to determine if any are in fact *D. neomorpha*.

Throughout the years, populations of the four species of the *cardini* group (*D. polymorpha*, *D. cardinoides*, *D. cardini* and *D. neocardini*) that are commonly distributed throughout Brazil have been studied to elucidate the characteristics that allow them to exploit various habitats, such as chromosomal inversions (De Toni *et al.*, 2001b, c) and abdominal pigmentation (Da Cunha, 1949; Heed, 1963; Rhode and Valente, 1986; Valente and Araújo, 1991; Machado *et al.*, 2001; Brisson *et al.*, 2004). It appears that *D. parthenogenetica* and *D. neomorpha* are now sympatric with these four species that have traditionally been found throughout Brazil. *D. parthenogenetica* and *D. neomorpha* occupy very similar, if not identical, ecological niches that overlap with *D. polymorpha* and *D. neocardini*, and both exhibit similar abdominal pigmentation adaptations to temperature and humidity (Penton *et al.*, unpublished data). This raises questions regarding how and when this territory expansion occurred and what route it took. Of special concern is whether their presence represents significant new competitive interactions with their previously resident close relatives.

Interestingly, the recent expansion of *D. parthenogenetica* and *D. neomorpha* into Brazil is part of a greater influx of new arrivals into the south Brazilian Atlantic forest *Drosophila* community over the last few decades. This trend includes the invasion and expansion of *D. malerkotliana* with the subsequent displacement of the *D. willistoni* group species in certain parts of their ranges (Sene and Val, 1977; Martins, 2001) and the first register of six new species from the *Sophophora* subgenus (Doge *et al.*, 2004). It is evident that the equilibrium of these *Drosophila* guilds has been recently disrupted, yet the long-term impact this will have is yet to be revealed. Further studies of the dynamics of these Drosophilid communities will be important to understand what new interactions may be occurring between the different species and the evolutionary and ecological factors that may be critically important in shaping diversity in these Amazonian and Atlantic forests.

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### **Mortality patterns in *Drosophila buzzatii* lines selected for wing length and developmental time.**

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## **Introduction**

Intrinsic mortality patterns become evident when the external mortality factors are removed as in laboratory rearing conditions. Mortality can be affected directly or indirectly by selection regimes (e.g., Foley and Luckinbill, 2001). Besides, genetic correlations among traits are a central feature in quantitative genetics and also provide a parameter to measure indirect responses to selection in other traits, sharing genes with the true target of selection (Roff, 1996). This approach is fruitful in experiments aimed to address indirect effects of artificial selection.

In the present study, we investigated the intrinsic mortality patterns in *Drosophila buzzatii* lines selected for wing length (WL) and developmental time (DT) (Cortese *et al.*, 2002). These lines were previously selected for body size and developmental time, two traits involved in a trade off. Three sets of lines were selected for reduced developmental time, the first was also selected for shortened wing length (S lines), *i.e.*, in the same direction of the trade off, the second was selected for increased wing length, *i.e.*, in the opposite direction of the trade off (L lines), and finally F lines were not selected for wing length. In response to selection, the lines have dramatically diverged for both traits (Cortese *et al.*, 2002), with S lines showing the strongest response. However, no response was observed for developmental time in L lines, whereas wing length in F lines, did not diverge from the control. These results are consistent with the hypothesis that both traits are related in a trade-off (Cortese *et al.*, 2002) and now we evaluate the indirect effect of artificial selection on mortality. All selection lines were compared for age-specific mortality rate at 25°C in order to test whether or not they responded to selection on either body size or developmental time.

## Materials and Methods

### *Selection lines and wild stocks*

Selection lines used in this study were described in detail in Cortese *et al.* (2002). Directional selection was applied on wing length and/or developmental time for 68 generations in 4 replicated lines according to the following regimes: (i) fast development and large wing length (L lines), (ii) fast development and small wing length (S lines), (iii) fast development (F lines), and (iv) overall control lines with no selection (C lines). In all cases 10 males and 10 females were used as parents of the next generation (see Cortese *et al.*, 2002 for details on the selection protocol).

We analyzed the original population by collecting a new sample of adult flies in June 2002 in the same locality from which selection lines were derived (Buenos Aires Province, Argentina). Two replicate lines were established from the base stock. The G3 outbred laboratory generation of these newly collected flies (W lines) was used in comparisons with G70 laboratory generation of selection lines and their among-replicates-within-lines hybrid stocks as a general control for inbreeding and artificial selection.

### *Longevity assessment*

Ten vials, each containing ten 1-day-old individuals (100 total), were set up for each replicate of all treatments at 25°C and in a 12 h light 12 h dark cycle. Flies were transferred to fresh vials every 48 hours, and old vials were examined for deaths. The number of vials was gradually reduced as deaths occurred, maintaining adult density as close as possible to 10 individuals per vial, with the prevailing sex ratio.

We adopted analytical demographic techniques in order to investigate mortality differences among treatments based on maximum likelihood techniques (*e.g.*, Carey, 2001). There are several advantages in the use of maximum likelihood estimates for hypothesis testing and model fitting of experimental mortality data (Pletcher, 1999; Driver, 2001). Mortality curves often bring more information than standard analysis aimed to compare means and maximum lifespan, as they allow the discrimination of two populations that, in spite of sharing a mean lifespan, may differ in their initial mortality rate and/or their increment of mortality with age. Using a maximum likelihood approach we estimated the parameters of the function of deaths accumulation for each selection treatment assuming the following the model:

$$\mu_x = e^{bo+cx} / (1 + e^{bo+cx})$$

where  $\mu_x$  is the proportion of dead individuals in the cohort at age  $x$ ,  $x$  is the age in days,  $e$  is Euler's constant,  $bo$  is the first constant shaping the curve and  $c$  is the second parameter of the estimation, the rate of increasing with age. With this approach it is possible to estimate instantaneous mortality rates without making any assumption regarding the underlying mortality model. The function is sufficiently general as to behave as a logistic or exponential curve depending on the values of two parameters:  $bo$  primarily associated with intrinsic mortality factors affecting the initial stages of adult life, and  $c$  mainly related to the temporal increment of the function. However, it should be noted that both parameters contribute to the general shape of the function. The first derivative of the estimated death accumulation function would correspond mathematically to the mortality rate function.

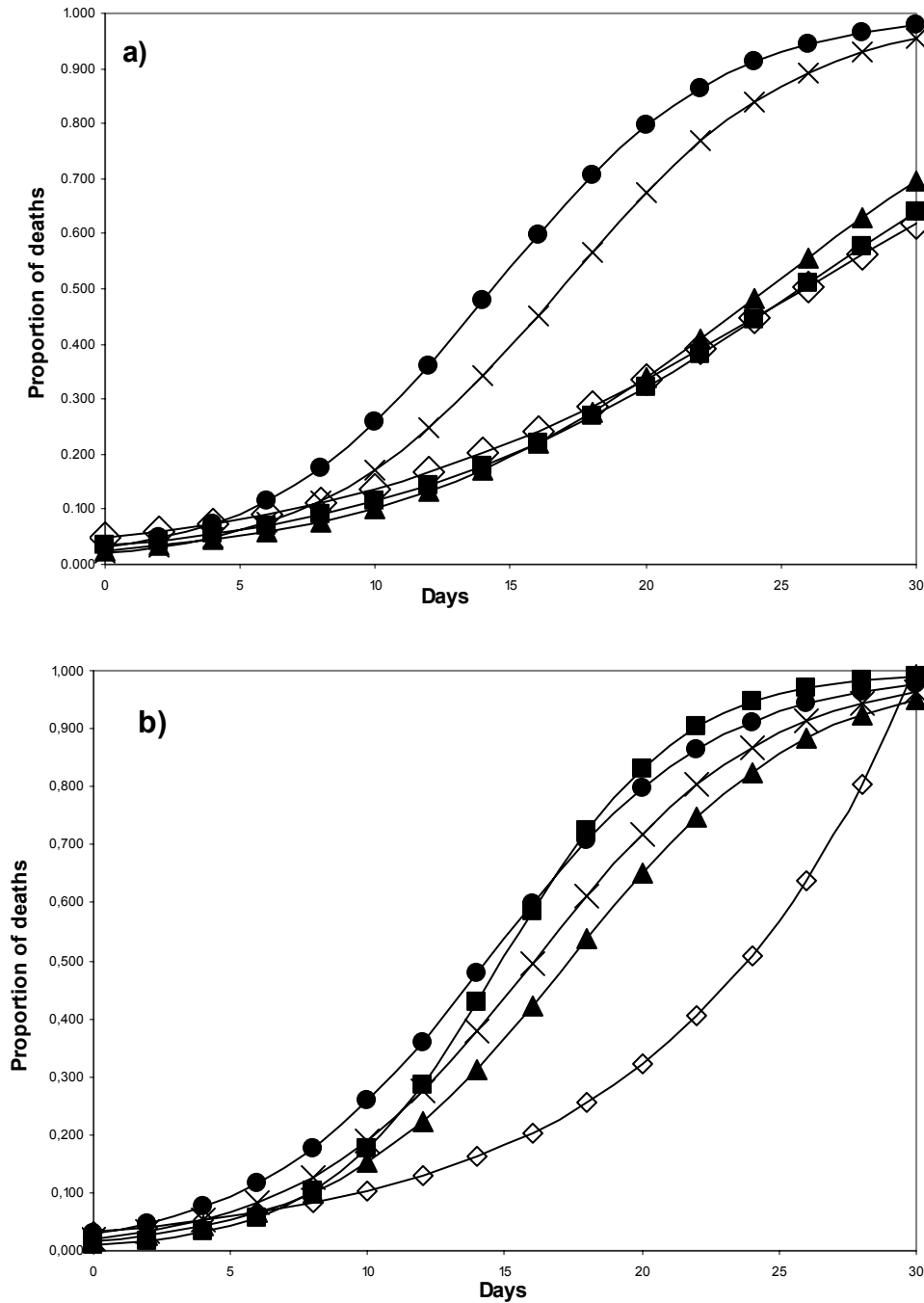


Figure 1. Death accumulation functions for (a) males and (b) females of wild flies (W -◇-). Control lines (C -●-), selected for large wings and fast development (L -▲-); for small wings and fast development (S -×-) and for fast development (F -■-) (see text for details).

The maximization procedure was performed using Statistica (Statsoft, 1995). Individual death events in a time interval were denoted as “1” and individuals surviving the interval as 0’s.

In this analysis, replicates within treatments were pooled since the statistical power is proportional to the sample size and is low for cohorts with less than 50 individuals (Pletcher, 1999). The age of each individual fly of each replicate was corrected by adding or subtracting the difference between the median of the corresponding replicate and the general median of the corresponding

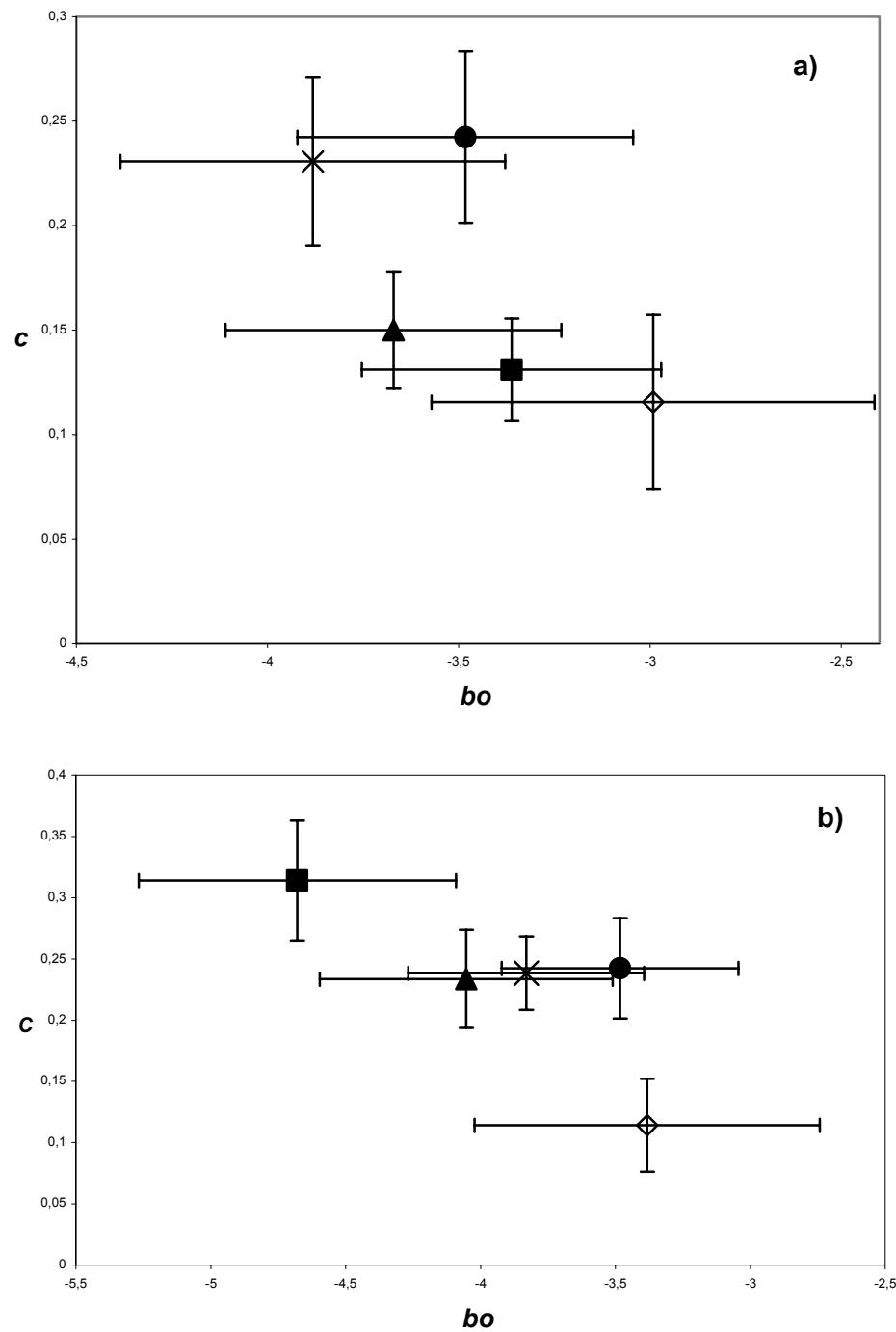


Figure 2. Estimated parameters of mortality for (a) males and (b) females of wild flies (W -◇-). Control lines (C -●-), selected for large wings and fast development (L -▲-); for small wings and fast development (S -×-) and for fast development (F -■-) (see text for details).

treatment. The intention of this pooling procedure was to emphasize common features shared among replicates within treatments that differentiate treatments.

95% confidence intervals of the parameters of the functions were also obtained by means of the optimization procedure; therefore, hypothesis testing is direct.

## Results

In Figure 1 we plotted the death accumulation function for each treatment. In males, differences among treatments for the *bo* parameter were not significant, but the functions estimated for each treatment can be discriminated in two distinctive groups according to the values of parameter *c* (Figure 2). On one hand, C and S lines displayed curves with a faster increase in the proportion of deaths (implying higher mortality rates), which is in total agreement with the fact that these lines S have a shorter lifespan as compared to L and F lines. C and S also presented a deceleration of the death accumulation rate at higher ages, a feature observed neither in L and F lines nor in wild flies (Figure 1). In females, the 95 % confidence intervals of *bo* estimated for C and F lines did not overlap (Figure 2). Regarding parameter *c*, F lines exhibited a rather extreme value suggesting that this treatment had an increased death accumulation rate with age when compared to other treatments. Finally, wild females presented a significantly lower *c* value (Figure 2).

Among selection lines, S presented the lowest mean longevity and higher mortality rates in late ages in both sexes. The selection protocol applied to S lines differs from L only in the direction of selection for wing length and from F lines which were only selected for reduced developmental time. Thus, selection for developmental time or wing length can not account *per se* for mortality differences. Selection for shortened developmental time did not produce the same effect in the mortality pattern in males of F lines as observed in S lines, though the high value of the *c* parameter in F females is highly suggestive of a sex-dependent response.

Thus, it seems that mortality differences among selection lines may be the outcome of the simultaneous selective pressure on wing length and developmental time.

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### Use of elliptic Fourier descriptors for quantification of male genitalia morphology of cactophilic *Drosophila*.

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## Introduction

The aedeagus is the intromittent organ of male *Drosophila*. Aedeagus morphology is considered a main diagnostic character to species recognition in Diptera, and it is especially relevant in the *repleta* group (Vilela, 1983). Due to the morphological complexity that it presents, its study as a quantitative character presents a methodological challenge.

*Drosophila buzzatii* and *D. koepferae* are two cactophilic sibling species belonging to the *buzzatii* cluster of the *repleta* group. Otherwise sinomorphic, they are distinguishable by the genitalia

of their males. Conspicuous shape differences between *D. buzzatii* and *D. koepferae* male genitalia preclude the possibility of capturing the organ geometry by means of an adequate number of reliable homologous landmarks. However, outline methods can still be performed as long as the edge or contour of the organ can be considered homologous across individuals (Adams *et al.*, 2004).

I present an evaluation of the application of a geometric morphometrics methodology to assess the morphological variations that explain the interspecific differences in shape of the aedeagus in two sibling *Drosophila* species.

## Materials and Methods

15 isofemale lines of each species were analyzed. They represent the fourth generation in laboratory conditions of flies originally collected in sympatric populations in Suyuque region, western Argentina.

Flies were reared in “semi-natural media” of one of their natural cactus host, *Opuntia sulphurea*. For the preparation of the “semi-natural” media, pieces of cactus were mixed in a blender and 6 grams poured into glass vials. Vials were then autoclaved and after cooling each vial was inoculated with 0.1 ml of the corresponding fermenting juice obtained from naturally occurring rots.

Four replicate vials were set for each isofemale line and first instar larvae were seeded at a constant density of 30 larvae per vial. All cultures were maintained at  $25 \pm 1^\circ\text{C}$  with a 12:12 light:/dark photoperiod until the emergence of adults. Adult flies were simultaneously collected from all lines. Aedeagi were dissected with entomological needles from 2 to 5 males of each replicate and boiled in a solution of 10 % KOH for 15 minutes to remove fatty tissues. Aedeagi were mounted on a slide in Hoyer’s medium and incubated overnight at  $50^\circ\text{C}$ . Slides were photographed with a digital camera mounted on a microscope at  $100\times$  magnification. A total of 647 aedeagus were dissected, 302 for *D. buzzatii* and 345 for *D. koepferae*.

Table 1. Eigenvalues and proportion of variance explained by the effective Principal Components (Principal Components whose proportion is larger than  $1 / \text{Number of analyzed components}$ ).

	<i>Eigenvalue</i>	<i>Proportion of explained variance (%)</i>	<i>Cumulative explained variance (%)</i>
<i>PC1</i>	0.03202	52.4	52.4
<i>PC2</i>	0.01126	18.4	70.8
<i>PC3</i>	0.00413	6.8	77.5
<i>PC4</i>	0.00265	4.3	81.9
<i>PC5</i>	0.00165	2.7	84.6
<i>PC6</i>	0.00133	2.2	86.7
<i>PC7</i>	0.00102	1.7	88.4
<i>PC8</i>	0.00092	1.5	89.9
<i>PC9</i>	0.00072	1.2	91.1
<i>PC10</i>	0.00065	1.1	92.2
<i>PC11</i>	0.00054	0.9	93.0
<i>PC12</i>	0.00047	0.8	93.8

As a flat chitinous organ, the aedeagus can be effectively described in shape and size in two dimensions when flattened with a cover slip. Thus, I employed a description of the organ outline by means of elliptic Fourier descriptors (EFDs) (Kuhl and Giardina, 1982) as a proper resolution to the shape quantification problem (Liu *et al.*, 1996). This is a type of analysis in which differences in  $x$  and  $y$  coordinates of an outline are fit separately as functions of arc length by Fourier analysis, so the outline is decomposed

into a weighted sum of sine and cosine functions (harmonics). This procedure provides a descriptive function that can reproduce the original outline of the organ at any desired degree of precision, depending on the number of harmonics used. I considered 30 harmonics for outline description. The

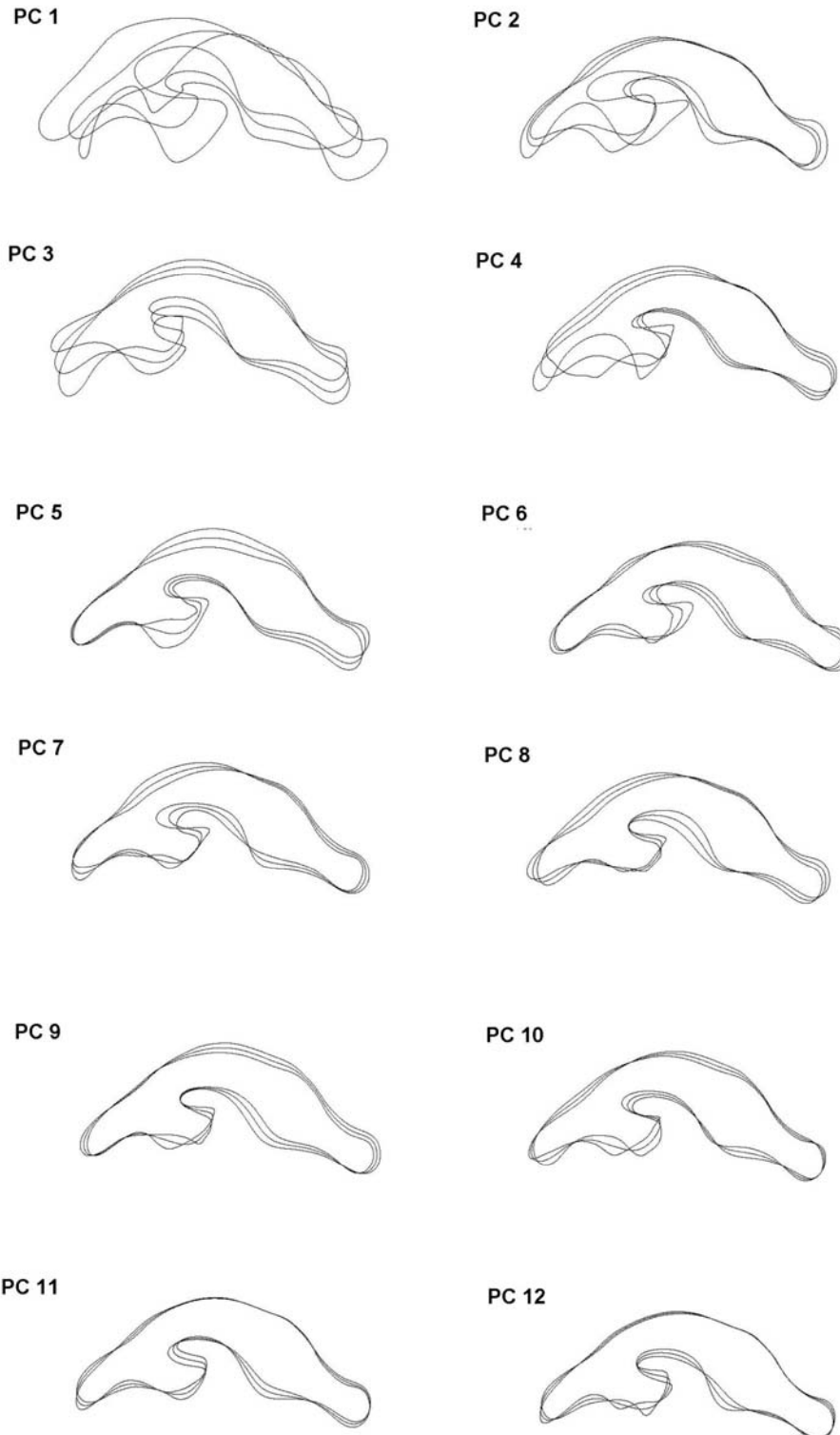


Figure 1. Aedeagus morphology accounted for each Principal Component. Mean outline morphology and  $\pm$  two standard deviations were superimposed for visualization of the portions of the organ varying in each axis.

four coefficients for each harmonic were estimated. These coefficients were used in a Principal Components Analysis in order to assess morphological variation reducing the number of variables considered. Shape (Principal Components) scores and log values of size were used as multivariate morphological variables in posterior analyses. Organ outline descriptions were obtained using SHAPE package (Iwata and Ukai, 2002), a public domain group of programs available at <http://cse.naro.affrc.go.jp/iwatah/shape>. These programs also provide information of the organ size. All outlines were rescaled to have a unit area to ensure size independence. The starting point for tracing the contour is also standardized with respect to the major axis of the shape.

To calculate the repeatability of the process, 30 aedeagi of each species were photographed and their outlines were digitalized 3 times. Repeatability was measured as the intraclass correlation coefficient (Lessels and Boag, 1987). I used one-way ANOVA with measurements nested within individuals as replicates to test for differences in size and shape calculation between digitalization events.

A discriminant-function analysis was performed to determine the degree to which the samples can be maximally distinguished. "Species" was the grouping variable with 2 levels (*D. buzzatii* and *D. koepferae*), and the PC scores were the independent variables. Genitalic size was included in a second examination. *A priori* classification probabilities were set equal for all groups regardless of their sample sizes.

## Results and Discussion

Principal Components Analysis established 12 independent Principal Components (Table 1) whose proportion of explained morphological variability is larger than 1/ (Number of analyzed components). The remaining components account for less than 7% of the total variation and, consequently, are less likely to be biologically meaningful. I present a visualization of the morphological variation considered by each principal component in Figure 1. It is worth mentioning that in spite of being rearranged independent morphological variables, the PC scores should be analyzed simultaneously with multivariate analyses for a proper testing of morphological differences.

Coefficients of repeatability were high and significant. Regarding organ size, *Drosophila buzzatii* presented repeatability values of 0.87 and *D. koepferae* of 0.92 ( $P < 0.0001$  in both cases). Repeatability coefficients for shape were calculated for each Principal Component. They ranged from 0.79 to 0.95 for *D. buzzatii* and from 0.83 to 0.96 for *D. koepferae* ( $P < 0.005$  in all cases).

Discriminant factors separated both species consistently. Reclassification of individuals allowed a 100% accuracy of correct attributions regardless of the exclusion or inclusion of organ size as an additional variable. Consequently, it can be stated that the EFDs contain enough information of the original shape as to ensure total discrimination of the species. Large proportions of the general variation of aedeagi morphology was assessed and quantified without measuring distances or establishing landmarks and, what is more important, without assuming homology for particular portions of the organ studied. The derived Principal Components Scores can be further used as multivariate morphological variables to test shape differences among samples.

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**Differential body expression of isoenzymatic loci in adults of the cactophilic species *Drosophila antonietae* (Diptera: Drosophilidae).**

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### **Abstract**

Isoenzyme electrophoresis has been used in populational and evolutionary research by the fact that it provides a direct way to evaluate the genetic variability of populations through the direct product of the gene. The aim of this work was to provide a tool related to the isoenzymatic polymorphism, making easier the data analysis in *Drosophila* natural population genetic approaches. In general, all isoenzymatic *loci* showed differences in the expression over the body. Therefore, in cases of isoenzyme/DNA combined analyses it is suggested DNA extraction of the head, and using the remaining thorax and abdomen for the isoenzymatic assays. Our results also suggest that not only evolutionary processes but also the enzyme physiological function should be included in the discussion of allelic frequency alterations considering these *loci* in natural populations. Key-words: Isoenzymes, Differential Expression, *Drosophila*, natural populations.

### **Introduction**

The electrophoresis technique has been used by evolutionary and population geneticists since 1966 because it offers a way to evaluate the population genetic variation through the direct product of gene expression. Using specific substrates, this technique allows the researcher to detect some molecules with similar catalytic activity, *i.e.*, isoenzymes, from an impure sample containing thousands of different proteins.

Markert and Möller (1959) defined isoenzymes (or isozymes) for the first time, and later the Biochemical Nomenclature Commission of the International Union of Pure and Applied Chemistry and International Union of Biochemistry (1977) proposed that isozyme should be restricted to the multiple molecular forms of an enzyme that have similar catalytic activities, occur in a species, and are the result of a structural gene expression.

These genes coding proteins have two important properties for geneticists: 1) some are polymorphic (Ford, 1940, *apud* Ford, 1953), that is, two or more discontinuous types occur simultaneously in such proportions that the rarest can not be maintained merely by recurrent mutation; 2) the alleles are generally codominants, which allow us to relate the observed phenotypes with the genotypes (Pasteur *et al.*, 1988).

Several works have been done on protein and isoenzyme polymorphisms using different organisms, including *Drosophila*. Since they were detected, a certain amount of effort was dispended to relate the variation found in natural populations with several environmental aspects and also with enzymes structure and functions. The isoenzymatic approach is very important for population genetic

variability studies. However, the recent advances in the molecular genetic field showed that the amount of variation in DNA sequences is higher than that found with isoenzyme and, therefore, could be more informative. More recently, it has been proposed the combined data analysis of isoenzyme loci, DNA sequencing and morphology produce better results about the history and genetic structure of populations (Estoup *et al.*, 1998; Dufresne *et al.*, 2002).

Thus, the aim of this work is to detect isoenzymatic differential tissue expression in *Drosophila antonietae* in order to: 1) support this species natural population data analysis using isoenzymatic genetic marker; and 2) provide the pattern of isoenzymatic tissue expression as a tool for future works with combined isoenzyme/DNA/morphology data. *Drosophila antonietae* is a South America endemic cactophilic species that belongs to the *D. buzzatii* cluster of the *D. repleta* group. It is found mostly associated with the *Cereus hildmaniannus* cactus, using its decaying cladodes as egg laying sites. Several aspects of *D. antonietae* biology still need to be elucidated, including some involving isoenzymes and their application in population genetics (Manfrim and Sene, 2005).

Table 1. Qualitative analysis of isoenzymatic expression pattern in the body of individuals from *Drosophila antonietae* natural populations. – expression not detected; + = very low expression; ++ = low expression; +++ = medium expression; ++++ = high expression; +++++ = very high expression.

	head	thorax	abdomen
Adh-1	+	++++	+++++
Adh-2	+	++++	+++++
Est-1	+	++	+++++
Est-2	+++	+++++	++++
Gpdh	–	+++++	+
Hk-1	–	–	+++++
Hk-2	+	+++	+++++
Hk-3	–	+++++	–
Idh	+	+++	+++++
Lap	+++	+++++	+
Mdh	–	+++++	+++++
Me	+	++++	+++++
Pep	–	+++	+++++
Pgm	–	+++++	+++++

## Material and Methods

*Drosophila antonietae* adults were collected in four localities where *Cereus hildmaniannus* cactus occurs: Serrana (21° 15' S, 47° 34' W); Sertãozinho (21° 10' S, 48° 05' W); Santa Maria da Serra (22° 34' S, 48° 12' W); and Itirapina (22° 16' S, 47° 48' W).

Enzymatic expression pattern was researched qualitatively in three different adult body regions: head, thorax and abdomen. Individual (1 head, 1 thorax and 1 abdomen) and three specimens (3 heads, 3 thoraxes and 3 abdomens) samples were submitted to gel electrophoresis and isoenzymatic loci analysis according to Mateus and Sene (2003).

## Results and Discussion

Table 1 summarizes the results of isoenzymatic body expression patterns based on the individual and three specimens body part analysis. Thorax and abdomen showed expression of 13 out of 14 loci studied as no *Hk-1* activity in the thorax and *Hk-3* activity in the abdomen were detected. Some enzymes presented specific expression pattern detected by activity in only one body part, as *Gpdh* and *Hk-3* in the thorax and *Hk-1* in the abdomen (Figure 1). In general, differences in the pattern of expression all over the body were detected for all loci.

These findings demonstrate that the isoenzymatic expression pattern observed has a direct relation with their function. *Hk-3* and *Gpdh*, for example, are related with the fly in insects (Castanheira and Contel, 1995), therefore it was expected to detect higher activity of these enzymes in the thorax. Thus, it became clearer that populational variability analyses of these loci require almost the whole individual. In this way, we suggest to proceed a DNA extraction from the head, leaving the remaining parts for isoenzymatic approaches in cases of combined analysis of this marker with DNA markers. These results also suggest that the enzyme physiologic function, as well as evolutionary factors, should be part of the discussion in the allelic frequency modifications in natural populations.

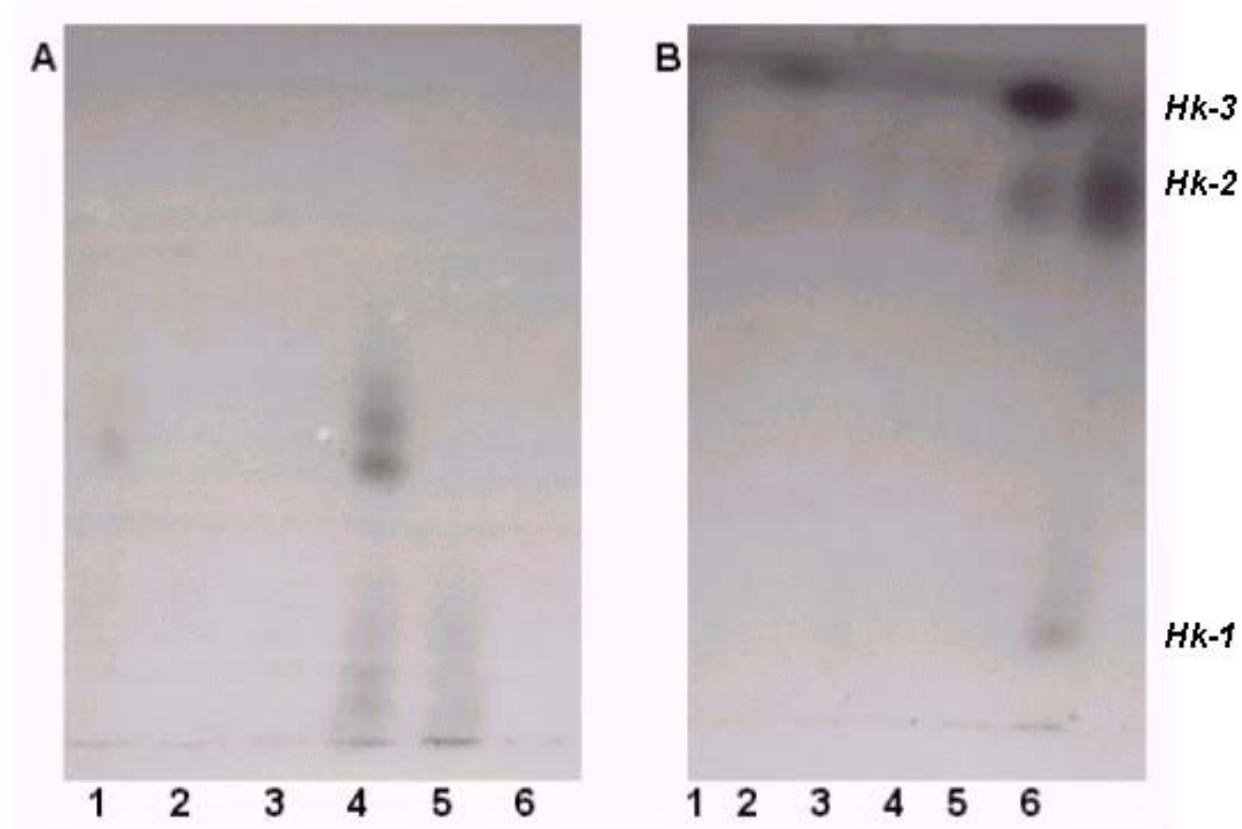


Figure 1. Starch gel (14%) electrophoretic pattern of *Drosophila antonietae* adults from natural populations. A, 1-GPDH; B, HK. 1 = one head; 2 = one thorax; 3 = one abdomen; 4 = three heads; 5 = three thorax; 6 = three abdomens.

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### **Lack of correlation between reproductive diapause and life span in *Drosophila melanogaster*.**

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Many insects and other invertebrate species enter reproductive diapause, a hormonally mediated arrest of reproductive development, to improve survival during unfavorable environmental conditions (Tauber *et al.*, 1986). In *Drosophila melanogaster*, diapausing females are characterized by immature ovaries and reduced yolk in their oocytes (King *et al.*, 1956). Ovarian diapause is induced by low temperature and short day length, and is proximally controlled by reduced synthesis of juvenile hormone in the *corpus allatum* (Saunders *et al.*, 1990).

Several observations suggest that there might be a causal connection between life span extension and the tendency to enter reproductive diapause. In the nematode *Caenorhabditis elegans*, many single-gene mutations that extend life span function in the dauer pathway, which regulates entry into a semi-dormant and non-reproductive state (Guarente and Kenyon, 2000). Diapausing *D. melanogaster* exhibit negligible actuarial senescence (Tatar and Yin, 2001), perhaps because of elevated stress resistance and reallocation of resources to somatic maintenance (Tanaka, 1997). Populations of *D. melanogaster* that have been artificially selected for long life exhibit delayed oocyte maturation compared to un-selected controls (Carlson and Harshman, 1999), a condition that bears qualitative similarities to full reproductive diapause.

We tested the hypothesis that reproductive diapause is related to variation in adult longevity in *Drosophila melanogaster*. Experimental material consisted of 47 inbred and recombinant inbred lines described by Curtsinger and Khazaeli (2002), which are known to exhibit a wide range of mean adult life spans. Flies were maintained on standard agar-yeast-molasses-cornmeal medium on a two-week generation schedule and kept at 24°C, with constant illumination and 65-70% relative humidity in a walk-in-incubator. Survival was studied in mixed-sex population cages under non-diapausing conditions. Flies for survival experiments were reared in half-pint bottles under controlled larval density using the method of Fukui *et al.* (1993). Approximately 250 males and 250 females from a cohort of newly emerged flies collected over a 24-hour period were transferred into a 3.8 liter population cage specially designed for longevity measurements (Fukui and Kirscher, 1993; Promislow *et al.*, 1996). The mouth of each cage was covered with a fine mosquito netting and inverted over an 11cm. diameter disk of cooked medium. Forty-seven population cages were established, one for each RI line and each parental inbred line. Flies were transferred without anesthesia to clean cages every ten days, and medium was replaced every other day. Dead flies were removed by suction, and numbers were recorded daily until the last death. Population cages were assigned to random locations in a walk-in-incubator with constant illumination and 65-70% relative humidity at 24°C. Longevity assays of all lines were performed simultaneously to minimize environmental variations.

For the diapause assay, 50-60 females emerging within a three-hour period were collected under light CO<sub>2</sub> anesthesia at the same time that flies were collected for survival tests. Female flies were transferred into 8-dram glass vials with standard cornmeal food, in groups of five. Vials were immediately placed in an incubator at 11°C and 10h : 14h light : dark regime for 16 days. Flies were

transferred into clean vials with fresh food chilled to 11°C every six days. At 16 days post-eclosion, flies were anesthetized with CO<sub>2</sub> and dissected under a light microscope on a drop of Drosophila Ringer solution (140 mM NaCl, 5 mM KCl, and 1.4 mM CaCl<sub>2</sub>) using a fine stainless steel needle. Diapause was scored as an "all or none" character in individual flies by examining the vitellogenic and previtellogenic oocytes using the criteria of King *et al.* (1956).

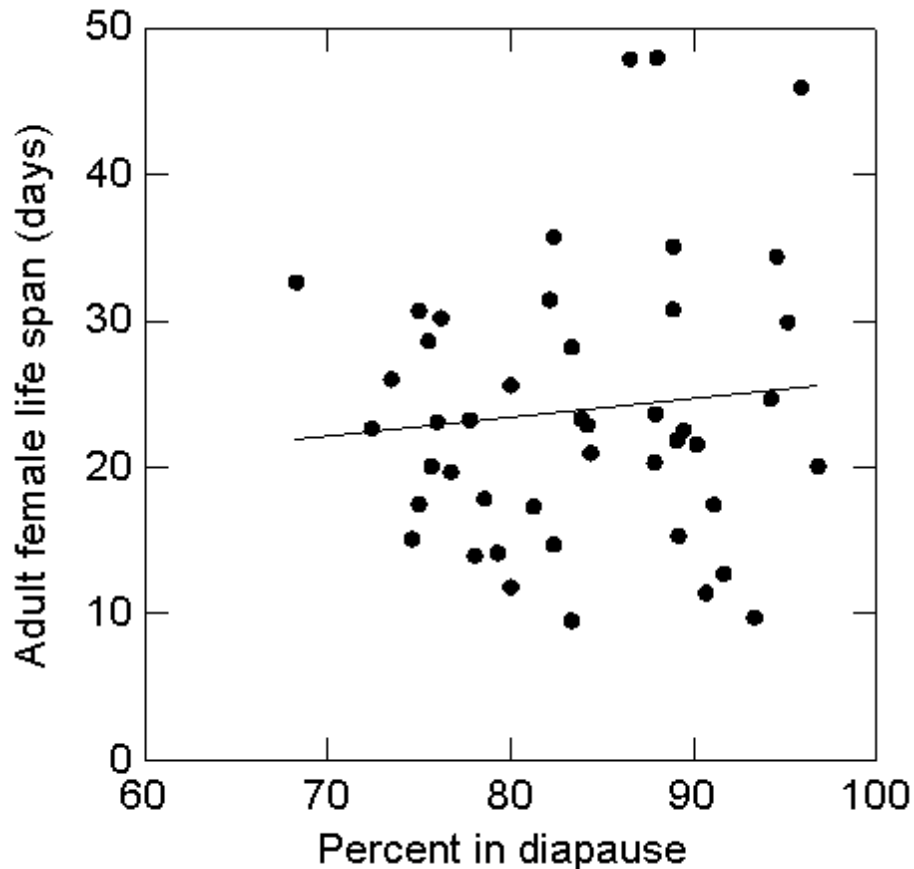


Figure 1. Correlation between line means for female adult life span, measured in days, and percentage of females from each line that entered reproductive diapause in response to low temperature and short daylight. The correlation is positive, but not statistically significant ( $r = 0.11$ ,  $P = 0.48$ ). One outlier genotype (44% diapause and an average life span of 24 days) is not shown, and does not alter the correlation.

The pooled mean adult life span for non-diapausing females was 23.5 days (SD = 9.1 days); for males the pooled mean was 29.4 days (SD = 11.1 days). Line means for male and female life spans are highly correlated ( $r = 0.82$ ,  $P < 0.001$ ). Overall, 83% of females entered diapause under experimental conditions. The percentages of diapausing females observed in each line varied from 44% to 98%. The lowest line is an outlier. The correlation between life span and tendency to diapause, shown in Figure 1, is positive but not statistically significant ( $r = 0.11$ ,  $P = 0.48$ ). The correlation remains non-significant when we remove one clear outlier with 44% diapause ( $r = 0.10$ ,  $P = 0.51$ ). For a more qualitative analysis, we stratified line means according to female life spans and asked whether quartiles differ in percentage of females in diapause. Observed percentages are 84.0,

80.9, 82.1, and 84.4 for shortest to longest life span quartiles respectively, and are not significantly different (Student's  $t < 1.0$ ,  $P > 0.2$  in all cases).

We conclude that while inbred and recombinant inbred lines of *D. melanogaster* exhibit substantial variation in average adult life spans, and in the tendency to enter reproductive diapause in response to environmental cues, the two metrics are not significantly correlated. This suggests that life span extension is achieved in these lines by mechanisms that are unrelated to reproductive diapause.

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### The *cadmus*<sup>BG02608</sup> mutation interferes with embryonic muscle morphology.

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Specific muscle fibers are distinguished by size, shape, innervation and attachment to the epidermis of each muscle, all properties with clear counterparts in vertebrate development. Diversity in muscle identity begins in *Drosophila* with the specification of two types of myoblasts: founder cells (FCs), which are Ras signaling dependent and contain information required for morphogenesis of a given muscle, and fusion-competent myoblasts (FCMs), which are Notch signaling dependent and fuse to a FC to become entrained to a particular muscle program (reviewed in Chen and Olson, 2004). To better understand muscle diversity and morphogenesis, a large-scale gene expression analysis identified 83 genes differentially expressed in FCs and FCMs (Artero *et al.*, 2003). In this study, the *Drosophila* gene *cadmus* (*cdm*) was found upregulated in FCMs and, hence, predicted as a putative Notch signaling target. The *cadmus* locus is localized in 90F5 and only one transcript has been described for the gene, which encodes a 971 amino acid protein. The protein structure reveals importin-beta and armadillo-like domains (Flybase; Drysdale *et al.*, 2005). The presence of these domains, and the characterization of its human ortholog, the Importin 13 protein (Mingot *et al.*, 2001), shows that *cadmus* may be a protein transporter implicated in cytoplasm-nucleus import. Members of the importin-beta/karyopherin-beta family act as carriers for many nuclear trafficking processes. Importin-beta binds cargo in the cytoplasm, the complex moves through the pore and cargo is released in the nucleus on binding of Ran-GTP to importin-beta.

Based upon its predicted function as a nuclear import factor, one would expect a general cellular requirement for *cadmus* in all embryonic tissues. However, attempts to detect expression of *cadmus* by *in situ* hybridization and by crossing the gene trap insertion BG02608 (see below) to a UAS-lacZ reporter failed. Nevertheless, a developmental gene expression time course study

(Arbeitman *et al.*, 2002) reported higher than average expression of this gene from 0 to 10 h of embryonic development, thus confirming that *cadmus* is indeed expressed in the embryo.

Upregulation of *cadmus* in FCMs implicates the gene in muscle development, most likely during muscle morphogenesis. Previous mutational analysis on *cadmus* revealed that initial specification of the muscles appeared normal by FC marker analysis, but specific morphogenetic defects existed. Their description, however, was partial and restricted to the *cadmus*<sup>BG02608</sup> allele (Artero *et al.*, 2003). Here, we further describe the embryonic phenotype of the *cdm*<sup>BG02608</sup> mutation in homozygous mutant embryos and in trans to a deficiency that removes the gene.

Table 1. Phenotype of muscle LT3 in homozygous mutant *cdm*<sup>BG02608</sup> embryos.

Genotype	LT3 muscle <sup>a</sup>			
	Hemisegments <sup>b</sup>	Absent	Hemisegments <sup>b</sup>	LT3 < LT2
Wild type	111 (16)	1 (0.9%)	110 (16)	11 (10%)
<i>cdm</i> <sup>BG02608</sup>	105 (8)	2 (1.9%)	103 (8)	29 (28%)

<sup>a</sup>LT3 < LT2 means that both attachment sites of LT3 are closer together than LT2.

<sup>b</sup>Number of hemisegments and, in parentheses, of embryos examined. Abdominal hemisegments A1-A7 were scored.

*Drosophila genetics*: OrR embryos were used to detect the wild-type muscle pattern. A gene trap insertion 27 nucleotides downstream of the transcription start site of *cadmus* (*cdm*<sup>BG02608</sup>) was used throughout this work. The insertion is predicted to cause a null mutation (Lukacsovich *et al.*, 2001). However, a second site mutation on this chromosome might also exist. To rule out this possibility, we collected embryos that are trans-heterozygous for *cdm*<sup>BG02608</sup> and Df(3R)DG4, which is a deficiency that deletes the locus, with relevant stocks balanced over *TM3 Sb Ubx-lacZ*.

*Histology techniques*: Immunocytochemistry in embryos was performed as described (Rushton *et al.*, 1995), with antibodies pre-absorbed against fixed wild-type embryos. Antibody dilutions were 1:1000 for anti-myosin heavy chain (MHC; Kiehart and Feghali, 1986) and 1:2000 for anti- $\beta$ -Galactosidase (Promega) antibodies. Biotinylated secondary antibodies were used in combination with Vector Elite ABC kit (Vector Laboratories, CA) at 1:200 dilution. Specimens were embedded in Araldite and images captured using an Axiocam camera (Zeiss) using Adobe Photoshop software.

## Results and Discussion

The embryonic phenotype of *cadmus* mutant embryos was studied by immunocytochemistry with anti-MHC antibodies. In these experiments, we found that in *cdm*<sup>BG02608</sup> homozygous mutant embryos lateral transverse 1 to 4 (LT1-4; nomenclature according to Bate, 1993), and dorsal transverse 1 (DT1) muscles were frequently shorter than wild type (Figure 1A,B). Moreover, muscles in *cdm*<sup>BG02608</sup> mutant embryos often looked truncated as if had failed to grow towards their normal epidermal attachment sites (Figure 1B, asterisks), and displayed abnormal shapes. LT4, for example, which normally shows a kink, often had a straight morphology in *cadmus* mutant embryos (Figure 1B, black arrowheads). Because the *cadmus* phenotype had variable expressivity, we scored one aspect of the phenotype: LT3 muscles significantly shorter than LT2. The analysis of 103 hemisegments from *cadmus* mutant embryos revealed 29 examples (28%) of said phenotype. In comparison, 110 hemisegments from wild-type embryos showed only 11 examples (10%) of the phenotype. Worth noting is that even though LT3 muscles were occasionally shorter than LT2 in wild-type embryos, they rarely looked truncated as in *cadmus* mutants thus making our estimate of

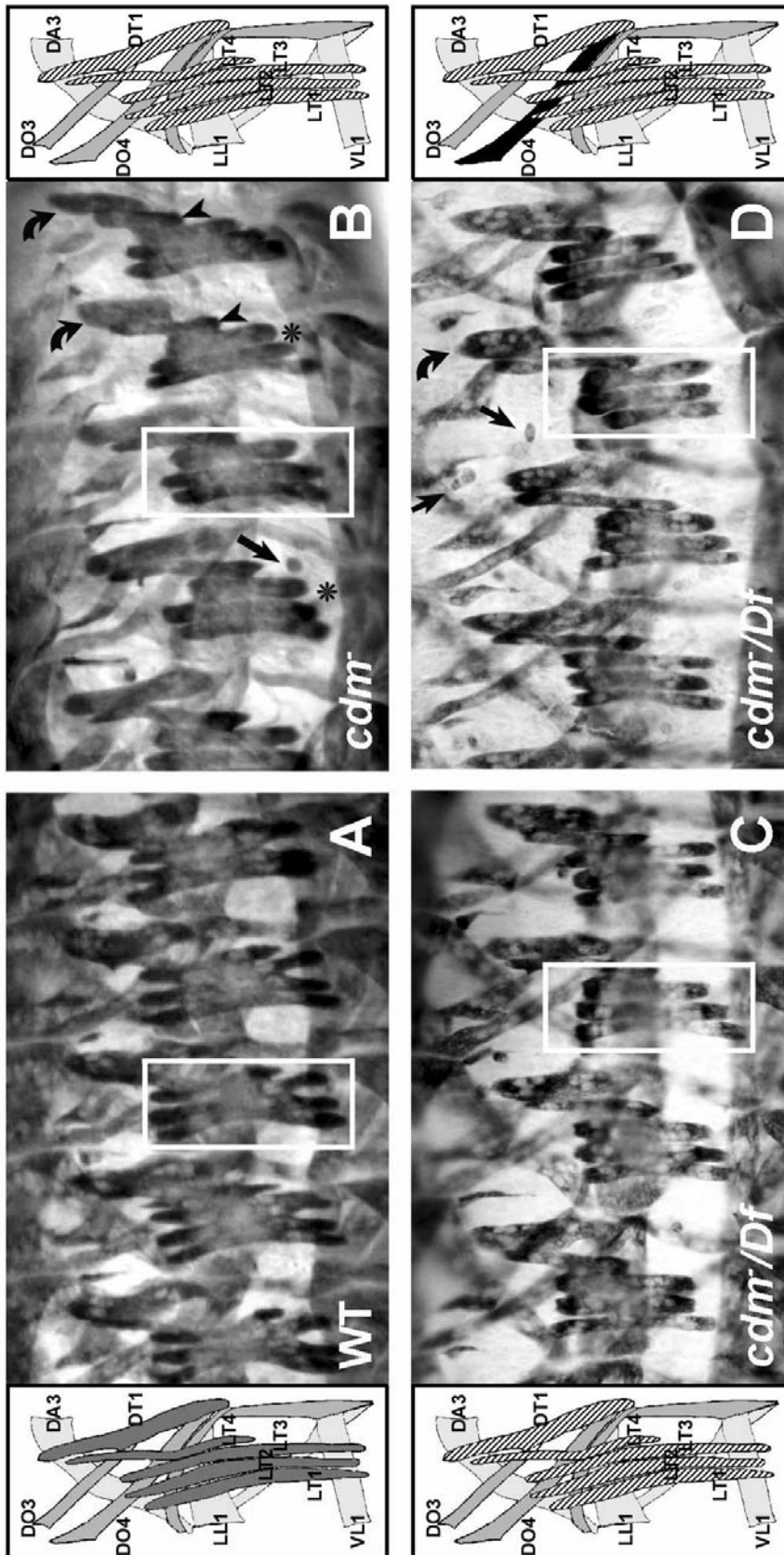


Figure 1. Muscle phenotype of *cadmus*<sup>BG02608</sup> mutation reveals morphology defects. Lateral views of late stage 16 embryos stained with anti-MHC antibody. (A) Wild-type embryos showing normal musculature. (B) *cdm*<sup>BG02608</sup> homozygous mutant embryos. (C, D) Embryos with the genotype *cdm*<sup>BG02608</sup>/*Df(3R)DG4*. A diagrammatic representation of visible muscles is included within each panel with muscles showing morphological abnormalities striped and muscles missing in black. Note that, when compared to the superimposed box, LT1-3 muscles are smaller in *cadmus* mutant embryos. Asterisks denote muscles that apparently failed to reach both their epidermal attachment sites. Bent arrows (DT1) and black arrowheads (LT4) indicate examples of aberrant morphologies whereas arrows mark examples of unfused myoblasts.

10% of LT3 muscles shorter than LT2 a very conservative estimate. The percentage of LT3 muscles showing the phenotype in *cadmus* mutant embryos is highly significant in a Fisher exact test ( $P = 0.001$ ). Although we have not explored the ultimate reason for the smaller size of *cadmus* mutant muscles, we propose that a partial myoblast fusion block may contribute since *cadmus* was upregulated in FCMs, which normally contribute mass to the muscle, and we detected unfused myoblasts in *cadmus* mutant embryos. In addition, *cadmus* mutant muscles seem to fail to grow towards their normal attachment sites further contributing to their reduced size.

To discard the possibility that we were detecting the phenotypic effect of a second site mutation in the BG02608 gene trap chromosome, we stained embryos of the genotype *cdm<sup>BG02608</sup>/Df(3R)DG4* with anti-MHC antibody to reveal their muscle pattern. In these embryos we detected a slightly stronger phenotype with even shorter muscles (compare boxes in Figure 1A-D), unfused myoblasts and occasional lack of muscles such as dorsal oblique 4 (DO4; Figure 1D). These results strengthen the conclusion that the observed phenotype is due to disruption of *cadmus* and not to a second site mutation, and establish *cadmus* as a gene relevant to muscle morphogenesis during *Drosophila* muscle development. It remains to be investigated if the human *cadmus* ortholog Importin 13 plays a similar role in vertebrate myogenesis.

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### Reproductive isolation in the *Drosophila longicornis* species complex.

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The *Drosophila repleta* group currently includes ca 100 species divided into the *fasciola*, *repleta*, *hydei*, *mulleri*, and *mercatorum* subgroups (Vilela, 1983; Wasserman, 1992). Within the large *mulleri* subgroup, the *longicornis* species cluster was established based largely on chromosomal similarities, as well as external and male genital morphology, and included *D. longicornis*, *D. pachuca*, *D. propachuca*, *D. mainlandi*, and the undescribed species “from Sonora” (Wasserman and Koepfer, 1977; Wasserman, 1982). More recently, several other species have been suggested to be close relatives of this group, including *D. hexastigma*, *D. spenceri*, *D. huckinsi*, and *D. huichole*, based on morphology, allozyme, chromosome, or DNA sequence data (Richardson *et al.*, 1977; Wasserman, 1992; Durando *et al.*, 2000; Etges *et al.*, 2001). One widespread species, *D. hamatofila*,

has been tenuously aligned with the *longicornis* complex based on nuclear and mtDNA sequence variation (Durando *et al.*, 2000; Oliveira *et al.*, 2005), but its affiliation with other *mulleri* subgroup species based on chromosomal gene arrangements is unclear (Wasserman, 1992). A recent review of the systematic relationships of these species based on four mtDNA regions, a description of the *longicornis* species complex, and a compilation of the geographical distribution of these species based on collecting records from the last 60 years, suggest that these species may form a monophyletic group, but phylogenetic resolution remains incomplete (Oliveira *et al.*, 2005). Further, all of these species, except *D. hexastigma* and *D. spenceri*, share the ancestral *Opuntia* cactus breeding habit, and there is a report of *D. hamatofila* using barrel cactus in central Arizona (Wasserman, 1992).

Wasserman and Koepfer (1977) demonstrated that crosses among *D. longicornis*, *D. pachuca*, and *D. propachuca* produced low frequencies of fertile females and sterile males in the laboratory, but no hybrids have been observed in nature. These species were not resolvable at the molecular level (Oliveira *et al.*, 2005), and cannot be differentiated on the basis of male genital morphology (Vilela, 1983) suggesting that reproductive isolation has evolved rapidly without mtDNA or genital differentiation. Since these species are sympatric, Wasserman and Koepfer (1977) concluded that gene flow among them was still possible in nature, but reproductive isolation would also be expected in this case, *i.e.*, if they are becoming differentiated. In this report, we extend these interspecific mating tests to include nine species in the *longicornis* complex in order to understand more about the relationships between the “biological” and “phylogenetic” nature of these species.

All interspecific crosses were performed with intraspecific control crosses simultaneously, and in most cases, reciprocal crosses were performed. All flies were cultured in 2 or 3 replicate vials on banana food (Brazner and Etges, 1993) supplemented with dried *Opuntia* powder. All adults used were virgins, aged for 10-12 days to insure sexual maturity. Crosses with groups of 10-20 adults of each species per vial were performed by WBH in 1993 and 2000, and those by WJE in 2001 (Table 1). Crosses involving *D. huckinsi* and *D. huichole* included fresh minced *Opuntia* tissue placed on the food surface, required for oviposition. All crosses were maintained for 2-3 weeks, observed every few days for evidence of offspring, and changed onto fresh media if any contamination was noted.

Crosses performed by each of the authors are indicated in Table 1 with *D. longicornis* complex species indicated by stock numbers and collection sites described in Oliveira *et al.*, (2005). All intraspecific crosses and within-species crosses involving different populations produced abundant offspring. Only *D. longicornis* × *D. mainlandi* crosses produced fertile F<sub>1</sub> females and sterile males (Table 1) consistent with *Drosophila mainlandi*'s status as a sister group to *D. longicornis*, *D. pachuca*, and *D. propachuca* (Durando *et al.*, 2000). A few other crosses produced some hybrid offspring (Table 1), but these were rare and usually found in single replicate vials. Even so, *D. mainlandi* was also involved in crosses with *D. pachuca* and *D. propachuca*. In all other crosses, no F<sub>1</sub> progeny were observed.

Thus, reproductive isolation in the *D. longicornis* complex is almost complete, except among *D. longicornis*, *D. pachuca*, and *D. propachuca* (Wasserman and Koepfer, 1977) and *D. mainlandi* (this report). *D. mainlandi* however is located in southern California and the entire Baja peninsula and so is allopatric to all species in the *longicornis* complex except it is overlapped by *D. hamatofila* in the northern part of its range and by *D. spenceri* in the cape region. Reasons for our inability to recover fertile F<sub>1</sub> female and sterile F<sub>1</sub> male hybrids between *D. longicornis*, *D. pachuca*, and *D. propachuca* in the laboratory are unknown, contrasted with Wasserman and Koepfer's (1977) few successful reciprocal crosses.

Vilela (1983, p. 68) commented “I was not able to find any remarkable differences among the male genitalia of the type specimens of *D. pachuca*, *D. propachuca*, and *D. longicornis*. Whether

Table 1. Crosses of *D. longicornis* complex species. Shaded cells indicate control intraspecific crosses that yielded abundant F<sub>1</sub> and F<sub>2</sub> generations. Dhuc = *D. huckinsi*, Dhui = *D. huichole*, Dpac = *D. pachuca*, Dpro = *D. propachuca*, Dspn = *D. spenceri*, Dson = *D. from Sonora*<sup>1</sup>, Dhex = *D. hexastigma*, Dion = *D. longicornis*, Dman = *D. mainlandi*

Locality	Species	Stock #	Dhuc	Dhui	Dpac	Dpro	Dson	Dspn	Dson	Dhex	Dion	Dion	Dion	Dion*	Dman	Dman
			A1040	A1040	E14.3	A961B	A877	A1010	A892	A1013	A1005	A1016	A1044	A956	A978	
San Francisco del Rincon, Guanajuato	Dhuc	A1040	WJE													
Ixtlan del Rio, Nayarit	Dhuc	A1042	WJE <sup>1</sup>	WJE												
San Francisco del Rincon, GTO	Dhui	A1040	WJE	WJE												
Chapingo, Mexico	Dpac	E14.3	WJE	WJE	WJE											
Chapingo, Mexico	Dpac	E14.10A			WJE											
Sta. Maria de Regla, Hidalgo	Dpro	A961B	WJE			WJE										
Meztitlan, Hidalgo	Dpro	A960			WJE	WJE										
Playa Azul, Michoacan	Dspn	A1010		WJE	WJE	WJE <sup>3</sup>	WJE	WJE								
El Fuerte, Sinaloa	Dspn	A1019			WJE	WJE		WJE								
Los Hornos, Sinaloa	Dson	A877				WBH	WBH	WJE								
Alamos, Sonora	Dson	A892	WJE	WJE		WBH	WBH	WBH	WJE							
Zapotitan de Salinas, Puebla	Dhex	A1013	WJE			WJE		WJE	WJE							
Navjoa, Sonora	Dion	A893					WBH	WBH			WBH					
Sta. Maria de Regla, Hidalgo	Dion	A961					WBH	WBH								
Tucson, AZ	Dion	A1005	WJE				WBH	WBH			WJE					
Las Vaquerias, Hidalgo	Dion	A1016	WJE	WJE			WBH	WBH			WJE	WJE				
Punta El Tecolote, San Luis Potosi	Dion*	A1044										WJE	WJE			
Santa Catalina Island, CA	Dman	A956	WJE	WJE <sup>5</sup>		WJE	WJE <sup>2</sup>	WBH	WBH	WBH	WBH			WJE		
Punta Agua Verde, BCS	Dman	A978	WJE		WJE <sup>1</sup>	WJE		WBH	WBH	WBH	WBH					WJE

<sup>1</sup> Determined to be *D. longicornis* from this cross; <sup>2</sup> Dpro F X Dman M F<sub>1</sub>, adults in one replicate; <sup>3</sup> Dpro F X Dspn M F<sub>1</sub>, pupae in one replicate; <sup>4</sup> Dman F X Dpac M F<sub>1</sub>, pupae only; <sup>5</sup> several dead larvae in both reciprocals; <sup>6</sup> F<sub>1</sub> fertile females, sterile males.

these forms have the same status remains an open question.” As these species were also not diagnosable based on mtDNA sequence variation (Oliveira *et al.*, 2005), they are likely very recent. Overall, these three closely related forms along with *D. mainlandi* are the only species in this group that still seem capable of hybridizing, albeit at low frequencies. Therefore, the biological and phylogenetic species concepts tend to be equally useful in understanding evolution and species divergence in the *D. longicornis* complex.

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### **Identification of genes regulating specification of adult muscle precursors in *Drosophila*.**

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### **Abstract**

Precursors of adult muscles in *Drosophila* (aP cells) display several properties (tightly attached to muscle fibres, quiescent, multipotent, able to self renewal, heterogeneous, essential in muscle regeneration) that make them similar to vertebrate satellite cells. Actually, little is known about the biology of satellite cells as their analysis is hampered by a limited number of specific molecular markers and by a lack of an experimental model. Thereby analysis of aP cells could contribute to understand the molecular mechanisms which regulate satellite cell number and renewal. All aP cells express myogenic bHLH transcription factor twist, and a subset of them co-expresses the homeobox gene *ladybird*. In order to isolate the evolutionarily conserved candidate genes that regulate the specification and proliferation of aP cells in *Drosophila*, we have performed a gain of function (GOF) screen. During this screen, candidate genes whose mesodermal overexpression leads to the modification of the number of aP cells were selected. Observed GOF phenotypes have led us to identification of an important number of candidate genes whose functions remain to be investigated. Amongst them, the EP insertions close to *tramtrack* (*ttk*) and *rhuboid* (*rho*) suggest a role for these genes in specification of aP cells.

## Introduction

### *Precursors of adult muscles in Drosophila*

At the end of *Drosophila* larval life (before metamorphosis), the majority of embryonic muscles are hystolized. However, a population of muscle stem cells, named adult muscle precursors (aP), persists that allows *de novo* formation of adult musculature. *Drosophila* aP cells have been identified as undifferentiated cells with persistent *twist* expression (Bate *et al.*, 1991). In the abdomen, there are 6 aP cells per embryonic abdominal segment, a single cell ventrally, pairs of cells laterally and three cells dorsally (Figure 1). During larval life, aP cells proliferate to form pools of myoblasts (ventral, lateral and dorsal clusters) from which specific abdominal adult muscles are formed. Several observations indicate that aP cells behave like vertebrate satellite cells and could therefore represent their invertebrate homologs. In fact, similar to the satellite cells, aP cells are quiescent during embryonic and larval life (Bate *et al.*, 1991) and become activated upon environmental activation. These cells are located close to embryonic muscles, which do not hystolize, and it is likely that muscle fibers that resist histolysis serve as template for adult muscle formation. Moreover aP cells are able to self renewal. As shown by laser ablation (Farrel and Keshishian, 1999), the loss of aP cells may be compensated by additional proliferation of aPs in neighboring segments and subsequent migration to the ablated region. Laser ablation (Farrell and Keshishian, 1999) and transplantation experiments (Roy and Vijay Raghavan, 1997) have also shown that aP cells are not committed to a particular muscle lineage, and the type of muscles they give rise to depends on environmental factors, such as growth factors and signals provided by surrounding tissue.

## Experimental Procedures

### *Gain of function screen*

To isolate novel genes implicated in aP cells specification, a gain of function screen has been performed (Bidet *et al.*, 2003) using the collection of the 2293 EP lines established by P. Rorth (Rorth, 1996). The EP-screening strategy takes advantage of the fact that P-elements insert preferentially into 5' regions of genes. Since the EP elements contain a series of UAS sequences, in the presence of a GAL4 driver, the genes located downstream to the insertion sites become inducible. This leads to the targeted, tissue-specific over expression. In our screen, we have chosen to cross the EP lines with the pan-mesodermal 24B-GAL4 driver (Brand and Perrimon, 1993) inducing candidate gene expression in the myogenic lineage, including the aP cells. In a wild type *Drosophila* embryo, there are 6 aP cells per abdominal hemisegment; thus, the over expression of a candidate gene, which induces a modification in this number, is easy to recognize.

### *Antibodies and immunostaining*

Embryos were collected from grape-juice agar plates, dechorionated, divitellinised and stained with the primary antibody: rabbit anti-Twi 1:1000 (provided by Siegfried Roth). Secondary antibodies diluted 1:300 are coupled with biotin and Streptavidin-DTAF diluted 1:300 was added. All the preparations were analysed using an Olympus Fluoview FV300 or an ZEISS LSM 510 Meta confocal microscope.

### *Drosophila strains*

The EP collection was supplied by the Szeged Drosophila Stock Centre. 24B-Gal4 effector line was amplified and virgin females were used to cross with males from individual EP lines. Ttk<sup>1</sup>,

UAS-DNDR and UAS-GFP.nls used in this study are referenced, respectively, as 4163, 5364 and 4775 in the Bloomington Stocks.

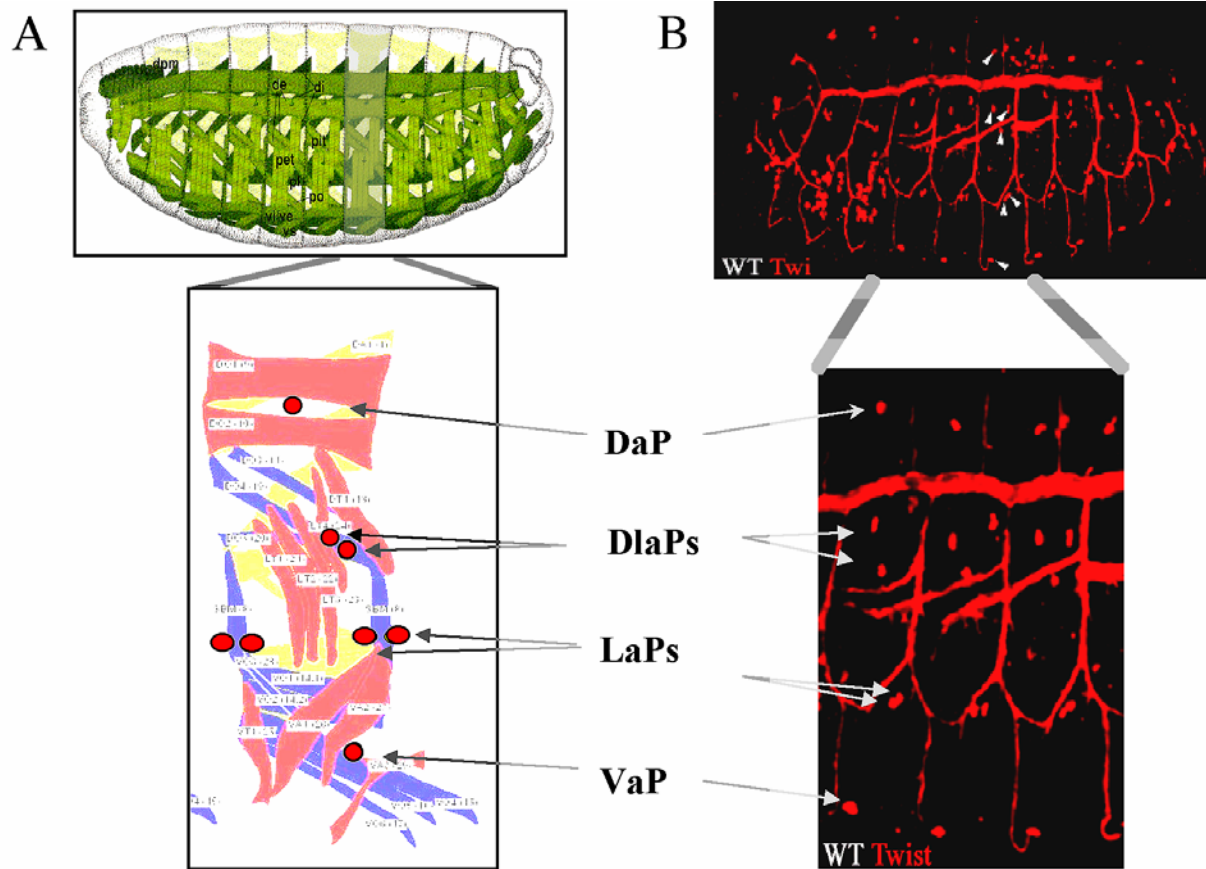


Figure 1. Adult muscle precursors in a *Drosophila* embryo. A, Schematic representation of aP cells in embryonic abdominal segment. In the abdomen, six aP cells have been identified in each hemisegment. A single ventral cell (VaP), a pair of lateral cells (LaPs), one dorsal (DaP) and two or three dorso-lateral cells (DLaPs). B, Lateral view of stage 15 embryo stained with antibodies against Twist protein to reveal the positions of adult muscle precursors (aP). By the end of embryogenesis, twist expression persists in aP cells that have segment-specific arrangements. A non-specific staining is seen in the tracheal system.

## Results and Discussion

The performed gain-of-function screen has led to the identification of a number of candidate genes whose overexpression influences specification of the *Drosophila* aP cells (Bidet *et al.*, 2003, and data not shown). Among candidates are genes implicated in the Receptor Tyrosine Kinase (RTK) pathway, which appears to play an instructive role during specification of aP cell progenitors. Here we focus on two RTK components identified by our screen and encoded by *tramtrack* (*ttk*) and *rhomboid* (*rho*). *Ttk* is a BTB/POZ domain transcription factor known to function as a negative effector of RTK signalling pathway (Lai, 1997). *rho* encodes a seven-transmembrane domain protease essential for the maturation of EGF/TGF $\alpha$  ligands (Urban *et al.*, 2001) and acting as a regulator of the EGFR signalling pathway. Although both genes are known to be required during

embryonic development, their myogenic functions and in particular their roles in specification of adult muscle precursors have not yet been analysed.

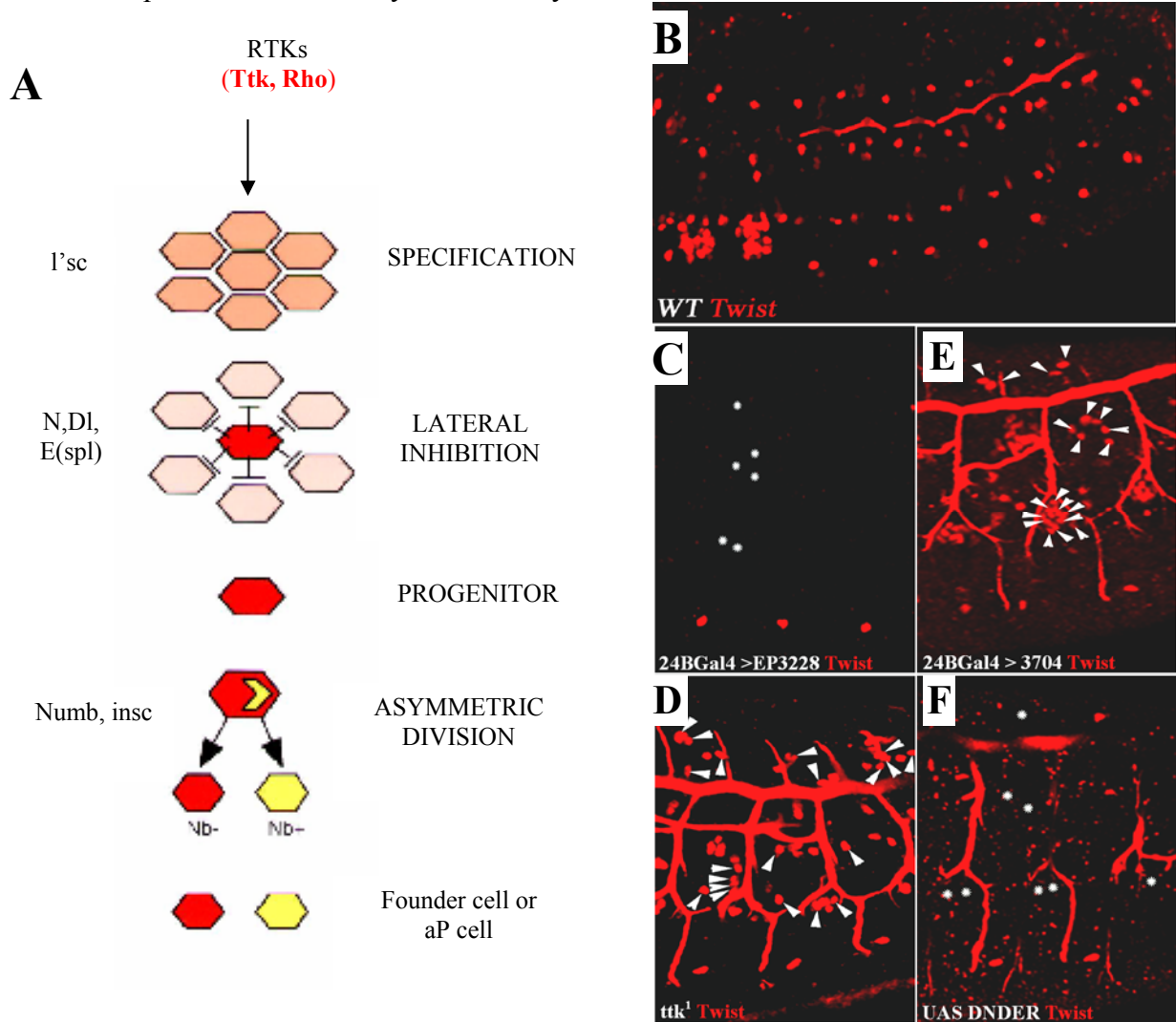


Figure 2. Tramtrack and Rhomboid are required for the specification of aP cells. A, Schematic representation of founder and aP cells specification. RTKs (FGFR and EGFR) are involved in setting up promuscular clusters from which the progenitor cells segregate. Progenitors segregation is mediated by a lateral inhibition mechanism involving the neurogenic genes (N, DL, E(spl)). Once progenitors are specified, they undergo asymmetric cell division to produce different muscle founder and aP cells. Intrinsic cellular factors like Numb and Inscuteable are crucial for asymmetric cell division during myogenesis. B, General lateral view of wild type embryo and (C-F) lateral views of three abdominal segments from different RTK mutants showing aP cells revealed by anti-Twist antibody. C, Ubiquitous expression of Tramtrack (EP3228) results in a loss of dorsal, dorso-lateral and lateral (asterisks) but not ventral aP cells (compare to the wild type B). In contrast, (D) loss of Tramtrack function (*Ttk*<sup>1</sup>) leads to the specification of supernumerary aP cells (arrows); (E) Over-expression of Rhomboid (EP3704) leads to a dramatic increase in twist expressing aP cells. In contrast, (D) the over-expression of a dominant negative form of EGFR results in a reduced number of aP cells (sterisks).

We have identified the EP insertion EP3228 upstream of *ttk*. Embryos issued from the cross between the 24B-GAL4 driver and the EP3228 line reveal a dramatically reduced number of aP cells (Figure 2B). Most precisely, except the ventrally located VaPs, all other adult muscle precursors are absent (asterisks in Figure 2B). Importantly, a similar influence on aP cells can be observed after targeted overexpression of another negative RTK effector Yan (data not shown) strongly suggesting that *ttk* regulates number of *twist*-positive cells via the RTK pathway. This possibility is confirmed by the opposite phenotype observed in *ttk* mutants, in which the number of dorsal, dorso-lateral, and lateral aP cells is significantly increased (Figure 2B).

According to the previous reports (Buff *et al.*, 1998; Carmena *et al.*, 1998), the two RTKs (FGFR and EGFR) are involved in setting up promuscular clusters from which the progenitor cells segregate. Thus, *ttk* acting as negative RTK effector appears to control the number of progenitors from which the aP cells will arise (see Figure 1A). The inability of *ttk* to control VaP cells specification remains to be elucidated.

The increased number of aP cells observed after targeted overexpression of *rho* (Figure 2E) is fully consistent with the promoting function of RTK and in particular of EGFR pathway in aP cell specification. The key role of EGFR is confirmed by the reduction of aP cell number in embryos expressing a dominant negative variant of EGFR (Figure 2F).

Considering the presented above similarities between the *Drosophila* aP cells and the vertebrate satellite cells it appears interesting to test whether the influence of RTK pathway on muscle stem cell specification has been conserved in evolution.

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