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# Selection for abdominal tergite pigmentation and correlated responses in the trident: a case study in *Drosophila melanogaster*

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In *Drosophila melanogaster*, abdominal tergite pigmentation and the appearance of a trident-shaped thoracic pattern exhibit similar biogeographical variation and sensitivity to temperature. These pigmentation traits may be under common selection pressure in natural populations or may be genetically correlated. To investigate the nature of this interaction, replicated populations of *D. melanogaster* were selected for increased or decreased melanization of the abdominal tergites for 40 generations. Selection for abdominal tergite pigmentation leads to correlated changes in trident formation. Although selection was performed only on female flies, male pigmentation also responded to selection. © 2012 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2012, **106**, 287–294.

ADDITIONAL KEYWORDS: artificial selection – correlated responses – melanization.

## INTRODUCTION

Pigmentation in insects is extremely diverse (True, 2003; Gray & McKinnon, 2007) and *Drosophila* species have substantial variation in abdominal melanization (David *et al*., 1985; Munjal *et al*., 1997; Rajpurohit, Parkash & Ramniwas, 2008a; Wittkopp *et al*., 2011). Geneticists have suggested pigmentation variation is caused by adaptive changes in multiple genes (Wittkopp, Carroll & kopp, 2003). Melanin synthesis pathways are conserved in insects, making drosophilids an ideal model system for understanding the function of and genetic basis for differences in pigmentation. The ecological and genetic basis of abdominal pigmentation in *Drosophila* has been studied on many occasions (David *et al*., 1985; Munjal *et al*., 1997; Wittkopp, True & Carroll, 2002; Takahashi *et al*., 2007), although several important questions remain.

In *Drosophila melanogaster*, a dark pigmented area with a general triangular pattern (the 'trident') is often observed on the mesonotum of the thorax (Fig. 1). The trident has long been recognized as a highly polymorphic trait in natural populations of *D. melanogaster* (Morgan & Bridges, 1919; Plough & Ives, 1934; Jacobs, 1960; David *et al*., 1985; Moreteau *et al*., 1995; Munjal *et al*., 1997; Gibert, Moreteau & David, 2000; Gibert *et al*., 2004; Wittkopp *et al*., 2002). Recent developmental studies in *D. melanogaster* have shown significant amounts of Yellow and Ebony proteins in the epidermal cells below the trident (Wittkopp *et al*., 2002). The presence of the trident has been reported in temperate and tropical high altitude populations of *D. melanogaster* (David *et al*., 1985; Munjal *et al*., 1997; Gibert, Moreteau & David, 2009), as well as in the sibling species *Drosophila simulans* (Capy, David & Robertson, 1988) and cold adapted *Drospohila nepalensis* (S. Rajpurohit, unpubl. data).

Latitudinal and altitudinal clines for pigmentation in *Drosophila* species have been observed in several parts of the world. Darker populations are observed at higher latitudes and altitudes, where environmental conditions tend to be cooler (David *et al*., 1985; Munjal *et al*., 1997; Pool & Aquadro, 2007; Rajpurohit *et al*., 2007, 2008a, b; Parkash, Rajpurohit & Ramniwas, 2009). Darker morphs of

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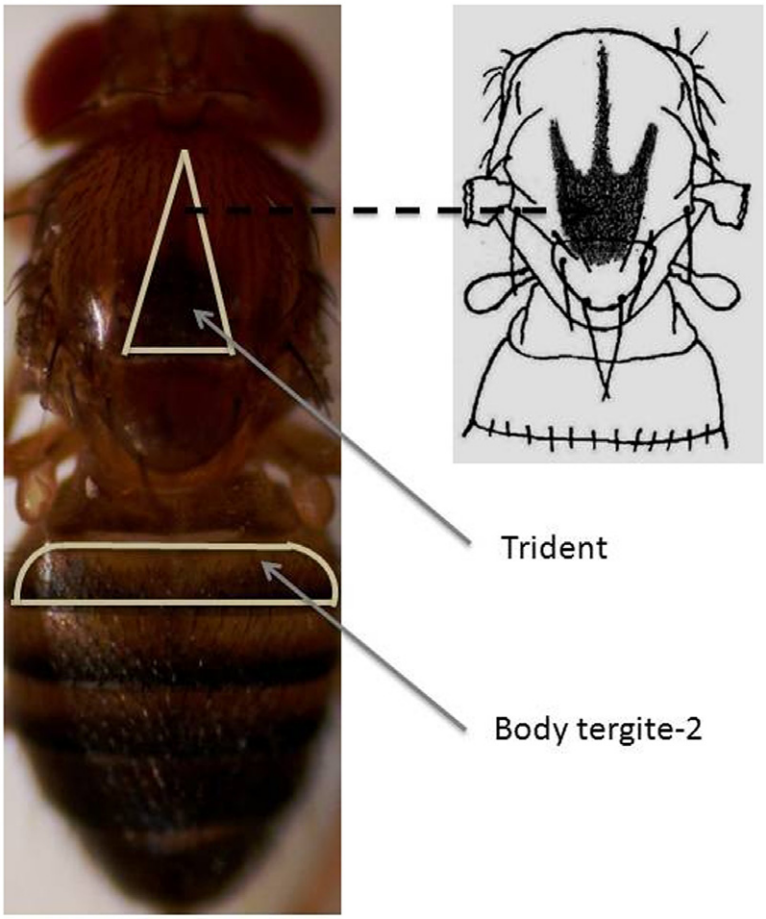


**Figure 1.** Schematic representation of trident and second body tergite areas selected for grey scores. The image shows a female *Drosophila melanogaster*.

*Drosophila polymorpha* are more abundant in dark, humid forests than open environments (Brisson *et al*., 2005). Physiological ecologists have therefore suggested a thermal budget hypothesis to explain pigmentation (Watt, 1969). Darker individuals could absorb solar radiation more efficiently in cold habitats, achieve higher body temperatures, and forage and reproduce more effectively (David *et al*., 1985; Capy *et al*., 1988; Gibert *et al*., 1996; Ottenheim, Volmer & Holloway, 1996). Similar geographical variation in the trident has also been described (David *et al*., 1985; Capy *et al*., 1988; Munjal *et al*., 1997).

Abdominal tergite pigmentation and the trident are affected by developmental temperature. Flies developing at lower temperatures are generally darker (David, Capy & Gauthier, 1990; Das, Mohanty & Parida, 1994; Gibert *et al*., 1996, 1999, 2000; Gibert, Peronnet & Schlotterer, 2007). At 17 °C, a prominent trident forms, whereas, at 25 °C, this pattern is much fainter (Munjal *et al*., 1997). The pattern has also been observed under natural conditions in wild populations. Darker phenotypes from higher latitudes and higher altitudes show a prominent trident presence (Munjal *et al*., 1997), although it is unknown whether this is caused by genetic or environmental factors, or both.

The parallel geographical and phenotypic patterns of trident pigmentation on the thorax and abdominal tergite pigmentation suggest that these pigmentation traits may be linked. To our knowledge, however, no studies have examined these traits simultaneously in wild or laboratory populations. To investigate the relationship between trident and abdominal tergite melanization, we performed an experimental evolution experiment in a recently-collected outbred population of *D. melanogaster*. We used artificial selection, where the degree of abdominal tergite melanization was imposed as the selection pressure and allowed to evolve over generations. If abdominal pigmentation and the trident are genetically linked, they should respond in a similar way under common selection

pressure. The present study also allowed us to investigate correlated responses to selection in a sexually dimorphic trait. We specifically selected for differences in female pigmentation only but, because males share almost all of their genome with females, their pigmentation may also be affected.

## MATERIAL AND METHODS

The founding population was established using 427 individuals of *D. melanogaster* that eclosed from rotten apples collected from Gilcrease Orchard (Las Vegas, Nevada, USA) in November 2008. Flies that eclosed from the original wild rots were considered generation 'zero'. Two selection treatments (dark or $D_{PIG}$ and light or $L_{PIG}$) and a control unselected treatment ($C_{PIG}$) were created with three-fold replication of each. $D_{PIG}$ and $L_{PIG}$ replicates were created by selecting 80 dark and 96 light females. Each group was randomly divided into three groups each to serve as replicate selection populations (Fig. 2). Control replicates were created by randomly sampling Generation-1 progeny reared from eggs laid before the selection lines were started. Dark and light females were selected (based on lateral body tergite melanization) as described previously (Capy *et al*., 1988; David *et al*., 1990).

We used artificial selection (Rose, Nusbaum & Chippindale, 1996) to select for dark or light pigmentation. Each generation, approximately 400 eggs per population were collected and allowed to develop. After eclosion adults were transferred to fresh food, and all pupae were allowed to eclose before selection was applied. From the resulting approximatey 200 females, the 20 darkest ($D_{PIG}$) or lightest ($L_{PIG}$) individuals were selected and allowed to lay eggs for the next generation. In the case of the control $C_{PIG}$ lines, 20 females were randomly selected. Selection was performed when females were at least 8 days old because melanin fixation takes 6–7 days in *D. melanogaster*. Females would have already mated at this age.

Fly populations were reared on cornmeal-sucrose-yeast medium at 24 ± 0.5 °C throughout selection. Constant lighting was used to minimize photoperiod-related effects on development and the expression of genes involved in pigmentation (Harker, 1965; Walter *et al*., 1991). For the present study, the 40th generation of selection was used. Flies were kept off selection for one generation before scoring them for trident and second abdominal tergite melanization. We chose tergite 2 as a representative tergite for abdominal pigmentation because this segment is the closest to trident, and because it did not produce any glare under the microscope light when taking digital images of trident and abdominal tergites (see below). A total of 29–49 adults per sex from each population were assayed.

To quantify trident and second abdominal tergite pigmentation, we used a digital imaging method. Wings were removed from adult flies (at least 8 days old), and the flies were placed dorsal side up in a glass depression slide, approximately 1 mm deep. Digital images were collected using ZoomBrowser EX v5.5 (Canon Inc.) software and a Canon PowerShot A 620 camera (7.1 megapixels) attached to an Axioplan2 microscope (Zeiss Instruments). All images were taken using ×2.5 objectives on the microscope and ×1.5 camera magnification. Calibrations of actual image size were made using a reference scale on a glass slide. The polygon selection option of IMAGEJ software (http://rsbweb.nih.gov/ij/) was used to select the trident and second abdominal tergite areas, and the grey score within the area was calculated (Fig. 1). IMAGEJ captures greyness based on the number of light pixels per unit area, such that higher values correspond to lighter phenotypes.

Statistical analysis was performed using STATISTICA, version 7 (StatSoft). We used a mixed-model analysis of variance (ANOVA) design where selection treatment and sex were treated as fixed effects and replicate was treated as a random effect nested within the selection regime (Sokal & Rohlf, 1981). For correlation analysis, a sequential Bonferroni correction was applied (Rice, 1989).

## RESULTS

After 40 generations of artificial selection based on female abdominal tergite pigmentation, $D_{PIG}$ and $L_{PIG}$ populations had evolved into distinctive colour morphs (Fig. 3). The mean values ($N = 29$–$49$) for trident and second abdominal tergite pigmentation of males and females are shown in Figure 4. For trident, as well as for abdominal tergite, higher pigmentation was observed in populations selected for higher abdominal pigmentation. The reverse was observed for populations selected for light phenotypes (Figs 3, 4). Males had lighter trident areas and second abdominal tergites than females in all selection treatments.

A mixed-model analysis of variance revealed that selection treatment and sex had highly significant effects on tergite 2 (Table 1). Similar results were obtained for trident, except that the interaction effect was statistically significant (Table 2). Tukey's post-hoc analysis revealed that all pairwise combinations of sex and selection treatment differed significantly. We also ran separate mixed-model ANOVAs for each sex. Within each sex, second abdominal tergites, and

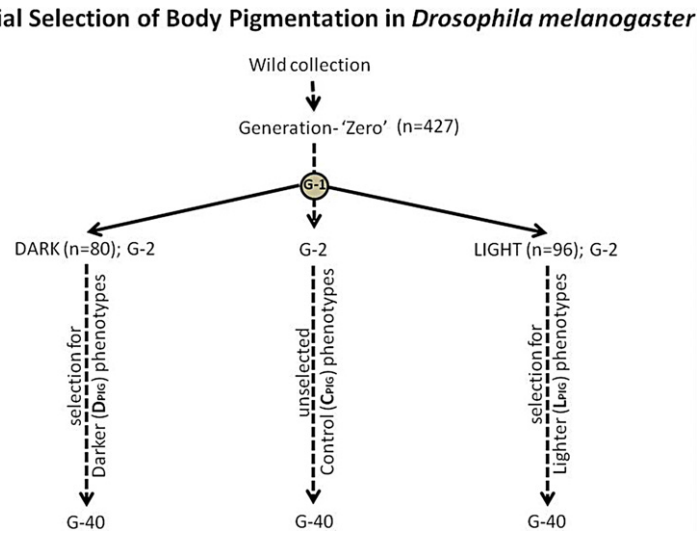


**Figure 2.** A flowchart explaining the artificial selection scheme for body pigmentation in *Drosophila melanogaster*. After egg lay for Control lines (between G-1 and G-2), females were sorted into two groups of darkest and lightest phenotypes, which were used to found dark $D_{PIG}$ and light $L_{PIG}$ selection lines.

**Table 1.** Results of mixed-model analysis of variance applied to the grey scale of the second abdominal tergite ($N = 28$–49 of each sex per population)

| Factor | d.f. | SS | MS | *F* | *P* |
|---|---|---|---|---|---|
| Selection | 2 | 30 595 | 15 297 | 131.45 | 0.000010 |
| Sex | 1 | 162 500 | 162 500 | 1 782.75 | < 0.000001 |
| Selection × Sex | 2 | 423 | 211 | 2.32 | 0.18 |
| Replicate (Selection) | 6 | 703 | 117 | 1.28 | 0.39 |
| Replicate (Selection × Sex) | 6 | 550 | 92 | 1.99 | 0.065 |
| Error | 611 | 28 161 | 46 | | |

**Table 2.** Results of mixed-model analysis of variance applied to the grey scale of trident ($N = 28$–49 of each sex per population)

| Factor | d.f. | SS | MS | *F* | *P* |
|---|---|---|---|---|---|
| Selection | 2 | 32 982 | 16 491 | 85.77 | 0.000038 |
| Sex | 1 | 118 266 | 118 266 | 934.77 | < 0.000001 |
| Selection × Sex | 2 | 2 135 | 1 067 | 8.43 | 0.018 |
| Replicate (Selection) | 6 | 1 166 | 194 | 1.52 | 0.31 |
| Replicate (Selection × Sex) | 6 | 767 | 128 | 5.55 | 0.000013 |
| Error | 611 | 14 072 | 23 | | |

tridents were significantly darker in $D_{PIG}$ flies than controls, which were significantly darker than $L_{PIG}$ flies (data not shown).

We calculated nonparametric (Spearman) and parametric (Pearson) correlation coefficients between trident and segment pigmentation. Both methods gave approximately the same results, which are summarized in Table 3. There were significant positive correlations ($P < 0.05$) in females from seven of the nine populations but only one in males. After sequential Bonferroni correction for multiple comparisons (Rice, 1989), five correlations in females remained statistically significant (Table 3). None of the negative correlations were statistically significant.

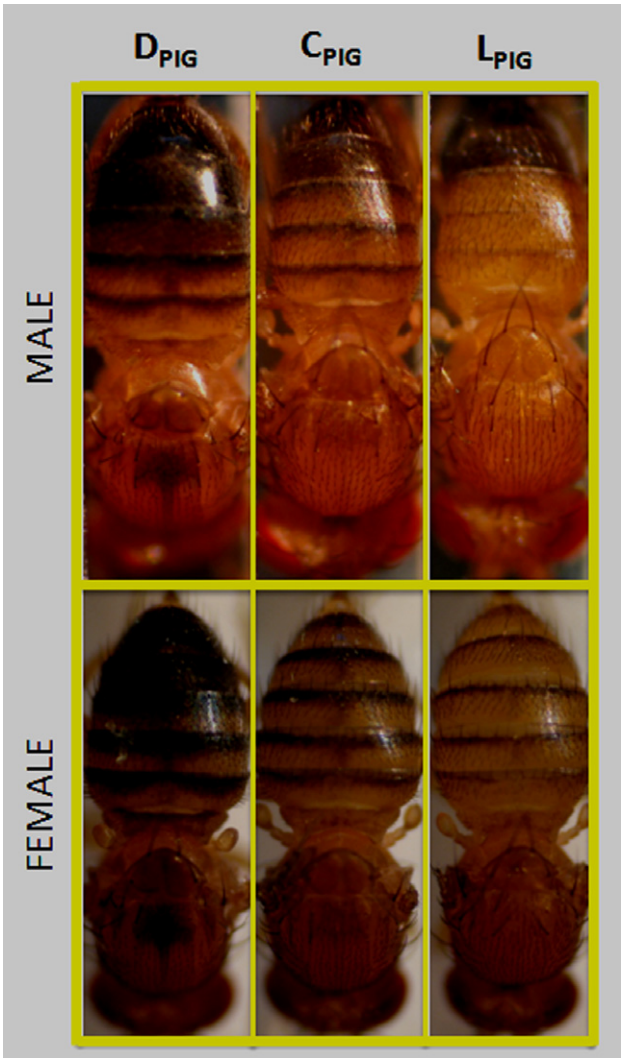


**Figure 3.** Representative phenotypes after 40 generations of selection based on pigmentation of female abdominal tergites. Note the prominent trident marking on the dark $D_{PIG}$ flies.

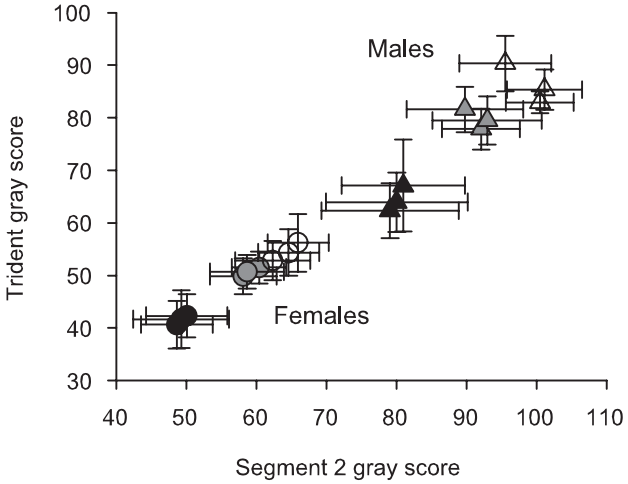


**Figure 4.** Correlation between second tergite and trident pigmentation in selected populations of *Drosophila melanogaster*. Higher grey scores correspond to lighter phenotypes. Black symbols, $D_{PIG}$ (dark) populations; grey symbols, $C_{PIG}$ (control) populations; white symbols, $L_{PIG}$ (light) populations. Circles, females; triangles, males. Data are the mean ± SD.

## DISCUSSION

Pigmentation patterns of *D. melanogaster* responded rapidly to selection, with clear differences between $D_{PIG}$ and $L_{PIG}$ populations apparent within five generations (S. Rajpurohit, pers. observ.). Our results are consistent with those reported by Gibert *et al*. (2009), who found significant heritability (based on intraclass correlations) for abdominal pigmentation in European and Indian populations of *D. melanogaster*. Pigmentation correlations have previously been studied with respect to developmental temperature, comparing *D. melanogaster* from France and India (Gibert *et al*., 2000), although variation within a single population at a constant temperature has not been investigated previously. Gibert *et al*. (2009) also did not examine

**Table 3.** Nonparametric (Spearman's rank correlation coefficient) and parametric (Pearson product-moment coefficient) correlations between pigmentation scores of tergite 2 and the trident across selection replicates

| Population | Sex | Replicate | Spearman *R* | Pearson *R* |
|---|---|---|---|---|
| $C_{Pig}$ | F | a | 0.57*† | 0.64*† |
| $C_{Pig}$ | F | b | 0.50* | 0.52* |
| $C_{Pig}$ | F | c | 0.55*† | 0.58*† |
| $D_{Pig}$ | F | a | 0.65*† | 0.69*† |
| $D_{Pig}$ | F | b | 0.51* | 0.47* |
| $D_{Pig}$ | F | c | 0.64*† | 0.73*† |
| $L_{Pig}$ | F | a | 0.67*† | 0.63*† |
| $L_{Pig}$ | F | b | 0.35 | 0.31 |
| $L_{Pig}$ | F | c | 0.31 | 0.24 |
| $C_{Pig}$ | M | a | –0.14 | –0.12 |
| $C_{Pig}$ | M | b | 0.19 | 0.09 |
| $C_{Pig}$ | M | c | –0.11 | –0.06 |
| $D_{Pig}$ | M | a | –0.03 | 0.11 |
| $D_{Pig}$ | M | b | –0.06 | 0.01 |
| $D_{Pig}$ | M | c | –0.13 | 0.09 |
| $L_{Pig}$ | M | a | 0.46* | 0.53* |
| $L_{Pig}$ | M | b | 0.01 | 0.32 |
| $L_{Pig}$ | M | c | –0.17 | –0.16 |

*Statistically significant ($P < 0.05$).
†Statistically significant after sequential Bonferroni correction.
$C_{pig}$, control; $D_{pig}$, dark; $L_{pig}$, light; F, female; M male.

correlations between pigmentation traits, which can vary independently of one another, depending upon which genes are involved (Wittkopp *et al*., 2003).

We found that thoracic pigmentation (trident) also evolved when we selected for lighter or darker pigmentation of the abdominal tergites. As shown in Figure 4, these traits were highly correlated across all populations. Within populations, trident and tergite 2 pigmentation also tended to be correlated, especially in females (Table 3). This may reflect the fact that selection was performed on females, and not males, although alleles associated with pigmentation will be transmitted to both sexes in the next generation. It is interesting, however, that the percentage differences in trident intensity between $L_{PIG}$ and $D_{PIG}$ selection treatments were greater in males than in females (22.45% and 12.93% for males and females, respectively), as were differences in second body tergite pigmentation (18.76% and 15.02% for males and females, respectively).

It could be argued that the scoring method used to select dark and light females each generation introduced a bias for flies also differing in trident. We do not think this is the case because one has to keep the flies in a lateral position when scoring abdominal tergite pigmentation using the method employed (Capy *et al*., 1988; David *et al*., 1990). The thoracic trident is not visible and so should not subconsciously influence the experimenter's assessment of the overall darkness of the fly. The digital method used to quantify pigmentation would have provided a more objective selection method, although selected females would have been damaged by wing removal and mounting before being allowed to lay eggs.

We found that selection on abdominal tergite pigmentation in females impacted the male phenotype. Gibert *et al*. (2009) found strong genetic correlations between male and female trident pigmentation, indicating that selection on one sex should affect both. In the present study, the difference in grey score between $D_{PIG}$ and $L_{PIG}$ phenotypes (second abdominal tergite and the trident) was actually greater in males than females. The second abdominal tergite is darker in females than in males and so may not respond as strongly to selection, whereas pigmentation of the second tergite exhibits greater thermal plasticity in males (Gibert *et al*., 2009). Small variations in incubator temperature could have caused the relatively high variation in males compared to females (Fig. 4). Our results are consistent with the conclusions of Poissant, Wilson & Coltman (2010) suggesting that physiological and developmental traits are generally positively correlated between the sexes.

Overall pigmentation and the trident show similar latitudinal and altitudinal clines in several *Drosophila* species (David *et al*., 1985; Munjal *et al*., 1997; Rajpurohit *et al*., 2007; 2008a, b). Darker populations with a more pronounced trident are observed at higher latitudes and elevations. Darker abdominal and thoracic (trident) pigmentation will both contribute a darker body overall. This may increase absorption of solar radiation so that darker flies can remain active at lower environmental temperatures (True, 2003). In several insects, a significant effect of pigmentation on thermoregulation has been reported (Watt, 1969; Brakefield & Willmer, 1985). However, the importance of pigmentation for temperature regulation in drosophilids and other small insects needs to be established experimentally. Willmer & Unwin (1981) reported that insects the size of *Drosophila* do not attain temperatures even 1 °C above ambient, whereas large (> 100 mg), dark insects with poorly reflective bodies can achieve body temperatures > 10 °C above ambient. Thus, the ecological significance of melanization may be size-dependent. In contrast, Hirai & Kimura (1997) found that black morphs of *D. elegans* were slightly (0.26 °C) warmer than brown morphs under irradiation, and this difference was not affected by body size.

Pigmentation may have adaptive significance related to other physiological processes, such as desiccation resistance, protection against ultraviolet radiation (True, 2003) or resisting infection (Dombeck & Jaenike, 2004). At the population level, darker pigmentation is correlated with reduced water loss and increased desiccation resistance in several *Drosophila* species (Kalmus, 1941; Brisson *et al*., 2005; Rajpurohit *et al*., 2007, 2008a; Parkash *et al*., 2008). Within populations, darker pigmentation is also associated with increased desiccation resistance (Parkash *et al*., 2009). Laboratory studies in *D. melanogaster* and *D. polymorpha* show differences in desiccation resistance between colour morphs (Kalmus, 1941; Brisson *et al*., 2005). We are currently testing the hypothesis that the $D_{PIG}$ populations are more desiccation resistant than the $L_{PIG}$ populations. By contrast, natural populations of *Drosophila americana* from a longitudinal cline in North America are darker in more humid areas (Wittkopp *et al*., 2011), suggesting that selection promoting the pigmentation cline in *D. americana* might be different from that in other *Drosophila* species.

Parallel clines in pigmentation and the trident could result from parallel independent selection on each trait, phenotypic plasticity, or genetic correlations. The correlated response to artificial selection on abdominal tergite melanization and the trident in $D_{PIG}$ and $L_{PIG}$ lines indicates a shared genetic basis for these traits. Several other studies of interactions between body pigmentation and secondary sexual characteristics such as sex comb have reported genetic correlations among them (Kopp *et al*., 2003;

Gibert *et al*., 2007). Telonis-Scott, Hoffmann & Sgro (2011) concluded that differences in *ebony* expression could explain two-thirds of the variation in trident pigmentation along a cline in Australia, although only at 25 °C, and *ebony* expression appears to be subject to natural selection in sub-Saharan Africa (Pool & Aquadro, 2007). By contrast, Rebeiz *et al*. (2009) found that high elevation African populations of *D. melanogaster* had been selected for abdominal tergite pigmentation but not for trident pigmentation, which corresponded to molecular evolution of an abdominal specific enhancer of the *ebony* gene as the major underlying factor. Local adaptation and genetic variation can affect responses to selection, and comparison of the findings obtained in the present study with those of Rebeiz *et al*. (2009) provides a clear example of this fact. Other molecular candidates include known structural and regulatory genes involved in melanin synthesis (Wittkopp *et al*., 2003). It should be possible to identify additional candidates through genome sequencing (Turner *et al*., 2011).

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