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Induction of ectopic transverse rows by *Ubx* on fly legs.

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The four-winged "bithorax" fly is familiar to students of genetics. It epitomizes the dramatic phenomenon of homeosis—the conversion of one body part into another. In this case, loss-of-function (LOF) mutations in the gene *Ultrabithorax* (*Ubx*) transform a third thoracic segment (T3) to resemble a second thoracic segment (T2), and in so doing, replace the inconspicuous balancer organs (halteres) with a second pair of wings (Lewis, 1978).

Less well known are the effects of *Ubx*-LOF on the hindlegs. Those legs undergo a similar homeosis to mimic the midlegs—an evolutionary ground state (Casares and Mann, 2001). Thus, *Ubx* dictates T3 (*vs*. T2) identity both dorsally (haltere *vs*. wing) and ventrally (hindleg *vs*. midleg). *Ubx* resides in a cluster of other "*Hox*" genes that collectively control segmental identity in bilaterally symmetric phyla throughout the animal kingdom (Held, 2017).

The *Hox* gene *Sex combs reduced* (*Scr*) governs forelegs just as *Ubx* governs hindlegs. However, certain gain-of-function (GOF) *Scr* phenotypes uncovered recently suggest that *Scr* is playing a subtler role as well (Akam, 1998): when *Scr* is forcibly expressed in midlegs, they acquire transverse rows (t-rows) of bristles not only on their anterior (A) side like forelegs, but also on their posterior (P) side like hindlegs (Held, 2010; Held *et al.*, 2017). These P-side rows suggest that *Scr* is directly inducing t-rows, regardless of segmental identity.

The present study was undertaken to investigate whether *Ubx* has the same ability as *Scr* to elicit t-rows indiscriminately. Indeed, we found that *Ubx* can induce t-rows not only on the P side of forelegs and midlegs—in conformity with T1→T3 and T2→T3 homeosis—but also on the A side of midlegs and hindlegs, where the effect cannot be attributed to homeosis alone.

Materials and Methods

We expressed *Ubx* by the "TARGET" (temporal and regional gene expression targeting) method (McGuire *et al.*, 2004), which relies on the yeast transgenes *Gal4*, *UAS*, and $Gal80^{ts}$. *Gal4* encodes a transcription factor that binds the upstream activating sequence *UAS* (Leung and Waddell, 2004). When *Gal4* is inserted in the *cis*-regulatory region of a "driver" gene, it is expressed at the same time and place as the driver, and any desired "puppet" gene—*e.g*., the wild-type (WT) *Ubx* gene—can be turned ON congruently by linking it to *UAS*. We used *Distalless*(*Dll*)*-Gal4* to elicit *UbxWT* expression in the tarsus and distal tibia of all

six legs (Kojima, 2004) and *scabrous*(*sca)-Gal4* to elicit *UbxWT* expression in proneural clusters (Baker and Brown, 2018) all over the fly (Renaud and Simpson, 2001; Troost *et al.*, 2015).

Dll-Gal4:UAS-UbxWT (abbreviated "*Dll>UbxWT*") flies were obtained (as F_1 offspring) by crossing *Dll-Gal4/CyO; tub-Gal80^{ts}* females X *UAS-UbxWT(isoform-Ia)/TM3, Ser^1* (Bloomington Stock #911) males, and *sca-Gal4:UAS-UbxWT* ("*sca>UbxWT*") flies were created by crossing *sca-Gal4/CyO; tub-Gal80^{ts}* females X *UAS-UbxWT(isoform-Ia)/TM3, Ser^1* males.

Confusingly, *TM3*-bearing (*Dll-Gal4/+*; *Gal80^{ts}/TM3, Ser^1*) F_1 males exhibited some of the traits that we expected for *Dll*-driven expression of *Ubx—viz*., scalloped wings (expected for *Ser^1*), abnormal antennae (~100% penetrance), and missing apical bristles (3/10 midlegs)—but other aspects of their phenotype set them apart from *Dll>UbxWT* males—*viz*., survival to eclosion, normal sex combs, and—to our surprise—extra, inverted leg joints. We traced the joints to the *Ser^1* marker on *TM3* (Bishop *et al.*, 1999; Miller *et al.*, 2016): we found that they also occurred in non-heat-treated *UAS-UbxWT/TM3, Ser^1* (Stock #911) flies. Ectopic joints were mainly confined to tarsal segments 3 and 4, but we also found extra, inverted joints in the tibias of *Dll-Gal4/+*; *Gal80^{ts}/TM3, Ser^1* flies from our youngest heat-treated cohorts, presumably due to a quirky interaction between *Dll-Gal4* and *Ser^1* about halfway along the length of the tibia.

We turned *Ubx* ON by shifting F_1 larvae from 18°C (where Gal80^{ts} blocks *Gal4*) to 30°C (which disables Gal80^{ts} and lets *Gal4* activate *UAS*) at various times before puparium formation (BPF). We then collected pupae from those bottles at 12-h intervals and placed them in humidified petri dishes for the rest of development. Thus, the first batch included individuals aged 0-12 h BPF at the time of shift (average age = 6 h BPF), the second batch 12-24 h BPF (average age = 18 h BPF) and so on, up to 36-48 h BPF. This protocol allowed recovery of all dead pupae and eclosed F_1 adults, regardless of any leg defects that could mire flies in the food. After finding distinct degrees of homeosis ("mild" *vs*. "severe") in our 12-24 h BPF batch, we repeated the experiment using shorter (6-h) collection intervals and were thereby able to trace the "mild" pupal defects to 12-18 h BPF and the "severe" pupal defects to 18-24 h BPF (see text).

Operationally, we defined "t-row" bristles (normal or ectopic) as bristles whose sockets are aligned transversely and touching one another. By these criteria, as few as two adjacent bristles could be deemed a "t-row". Distinguishing A *vs*. P t-rows was difficult for basitarsi when t-rows merged. In those cases we used the medial sensillum campaniformia as a marker for the A/P boundary (Held, 2002). In accord with convention, we italicize gene names (*e.g*., *Ubx*) and use Roman typeface for protein products (*e.g*., Ubx). Abbreviations include "ta1-ta5" for tarsal segments 1 through 5, though ta1 is more commonly called the basitarsus.

Flies were raised on Ward's *Drosophila* Instant Medium plus live yeast. Experimental individuals were preserved in 70% ethanol, mounted in Faure's medium (Lee and Gerhart, 1973) between cover slips, and photographed with a Nikon microscope at 200× or 400× magnification.

Results and Discussion

Fly legs arise from imaginal discs that grow inside the larva (Schubiger *et al.*, 2012). *Ubx* is expressed in 3rd-leg discs (Brower, 1987), more strongly in the P compartment, where the t-rows reside, than in the A compartment (Held, 2002; Shroff *et al.*, 2007). *Ubx* is also expressed in the P compartment of wild-type 2nd-leg discs, where it plays a role in determining the distribution of small hairs (trichomes) on the back of the midleg femur (Stern, 1998; Kittelmann *et al.*, 2018). *Ubx* is not normally expressed in 1st-leg discs.

By placing *UAS-UbxWT* under the control of *Dll-Gal4* (in *Dll-Gal4/+; UAS-UbxWT/tub-Gal80^{ts}* flies) we were able to force *Ubx* to be expressed throughout the epidermis of the tarsus and distal tibia in 1st, 2nd, and 3rd leg discs for varying lengths of time before pupariation. The duration of exposure to Ubx was varied by shifting larvae from 18°C to 30°C and then collecting the maturing pupae at 12-h intervals. In this way we obtained cohorts whose age at the time of the shift was 0-12, 12-24, 24-36, or 36-48 h BPF (see Materials and Methods). The younger the larvae at the time of the shift, the longer they were exposed to exogenously imposed Ubx.

We mainly studied males because the sex comb offers a sensitive barometer of T1→T3 homeosis. This conspicuous row of dark bristles ("teeth") is found only on male forelegs (Hannah-Alava, 1958) (Figure 1). The greater the foreleg-to-hindleg conversion, the fewer the teeth, and the less rotated the comb was

relative to the transverse axis (Held *et al.*, 2004; Atallah *et al.*, 2009). Regardless of the time of the shift, all of the flies with reduced sex combs were found to have died as pupae at the pharate stage (just before eclosion). In the 0-12 h BPF cohort, for example, all 10 of the *Dll>UbxWT* males (among 80 F_1 total) died before eclosion and had unrotated or absent combs (4.1 $^{+}$/- 3.4 teeth, *vs*. 11.9 $^{+}$/- 0.9 in controls; n = 10 each). Suppression of sex combs by *Ubx-GOF* has been reported previously (Shroff *et al.*, 2007).

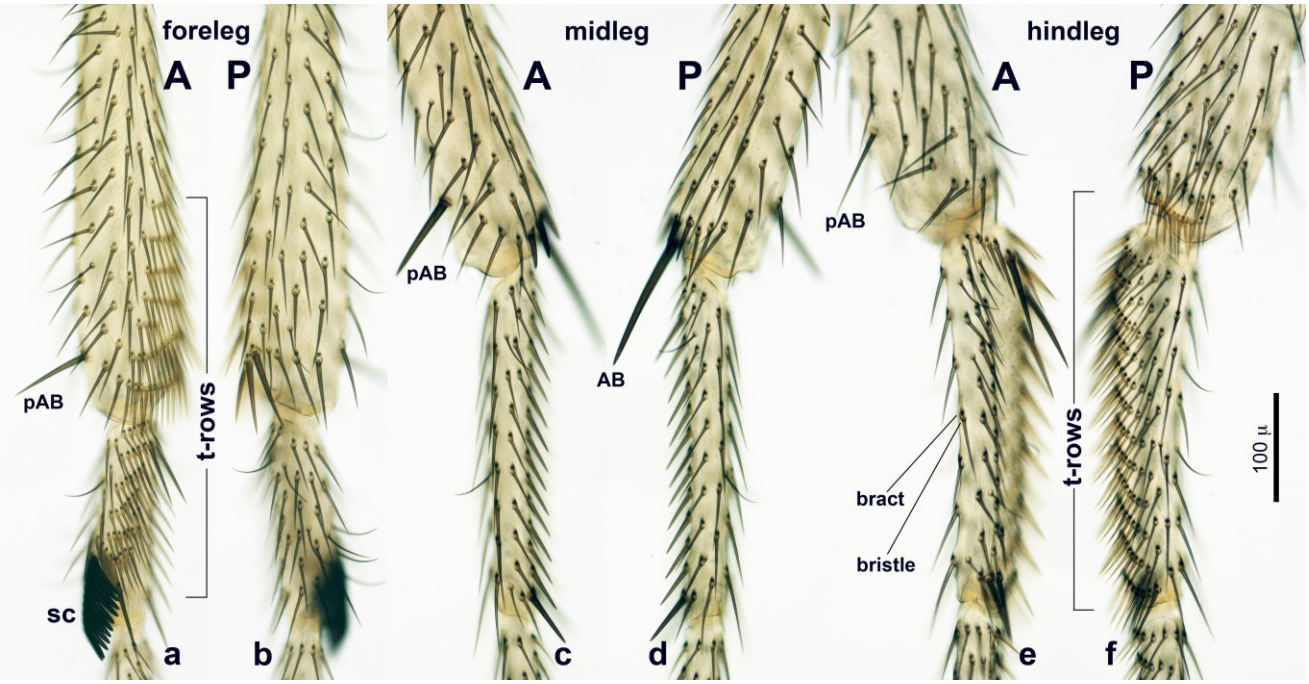

Figure 1. Foreleg (**a**, **b**), midleg, (**c**, **d**), and hindleg (**e**, **f**) anatomy in *D. melanogaster* (Hannah-Alava, 1958; Shroff *et al.*, 2007; Schubiger *et al.*, 2012). These legs are from control *Dll-Gal4/+; UAS-UbxWT/tub-Gal80^{ts}* males raised at 18°C—a temperature that lets *Gal80^{ts}* block *Gal4*'s activation of *UAS-UbxWT*, leading to a wild-type phenotype. Two segments are shown per leg: the basitarsus (below) and the distal portion of the tibia (above). Each tibia has a pre-apical bristle (pAB) on its dorsal side, but the midleg pAB is thicker, darker, and blunter. Only the midleg has an apical bristle (AB). Above the AB is an arc of ~6 "spur" bristles (**c** and **d**) that are shorter, blunter, and darker than nearby bristles. Parallel rows of transversely aligned (t-row) bristles decorate the anterior (A) side of the foreleg (**a**) and the posterior (P) side of the hindleg (**f**), but are lacking from the midleg. Flies use these rows as brushes to remove dust: forelegs bend forward to clean the eyes and hindlegs bend backwards to clean the wings (Szebenyi, 1969; Vandervorst and Ghysen, 1980). The sex comb (sc) develops from a t-row that rotates ~90˚ (Held *et al.*, 2004; Atallah *et al.*, 2009). Comb bristles are dark, thick, and blunt, while t-row bristles are yellow and tapered. Most tibial and tarsal bristles have a thorn-like protrusion ("bract") above their socket (**e**), though most t-row bristles on the tibia lack bracts. All photos are at the same magnification (scale bar in **f**). A and P images of the left foreleg were flipped horizontally for ease of comparison with the right midleg and hindleg. Some bristles were deflected due to being sandwiched between cover slips.

In the 12-24 h BPF cohort, we recovered 15 *Dll>UbxWT* males (among 83 F_1 total) with unrotated or missing combs. These pharate males fell into two groups based on their tarsi: (1) "mild" pupae whose tarsi had all 5 segments (6 flies) and (2) "severe" pupae whose tarsi had only ~3 segments (9 flies), with the extent of T1→T3 and T2→T3 homeosis being greater in the severe subgroup (Figure 2). This mild/severe distinction

proved to be age-related: when we repeated the experiment using 6-h (*vs*. 12-h) collection intervals, all of the *Dll>UbxWT* males (8/8) in the older (12-18 h BPF) cohort displayed the mild syndrome, whereas the majority (9/16) of dead pupae in the younger (18-24 BPF) cohort displayed the severe one. The greater severity of the latter group makes sense because those larvae were exposed to Ubx for a longer period.

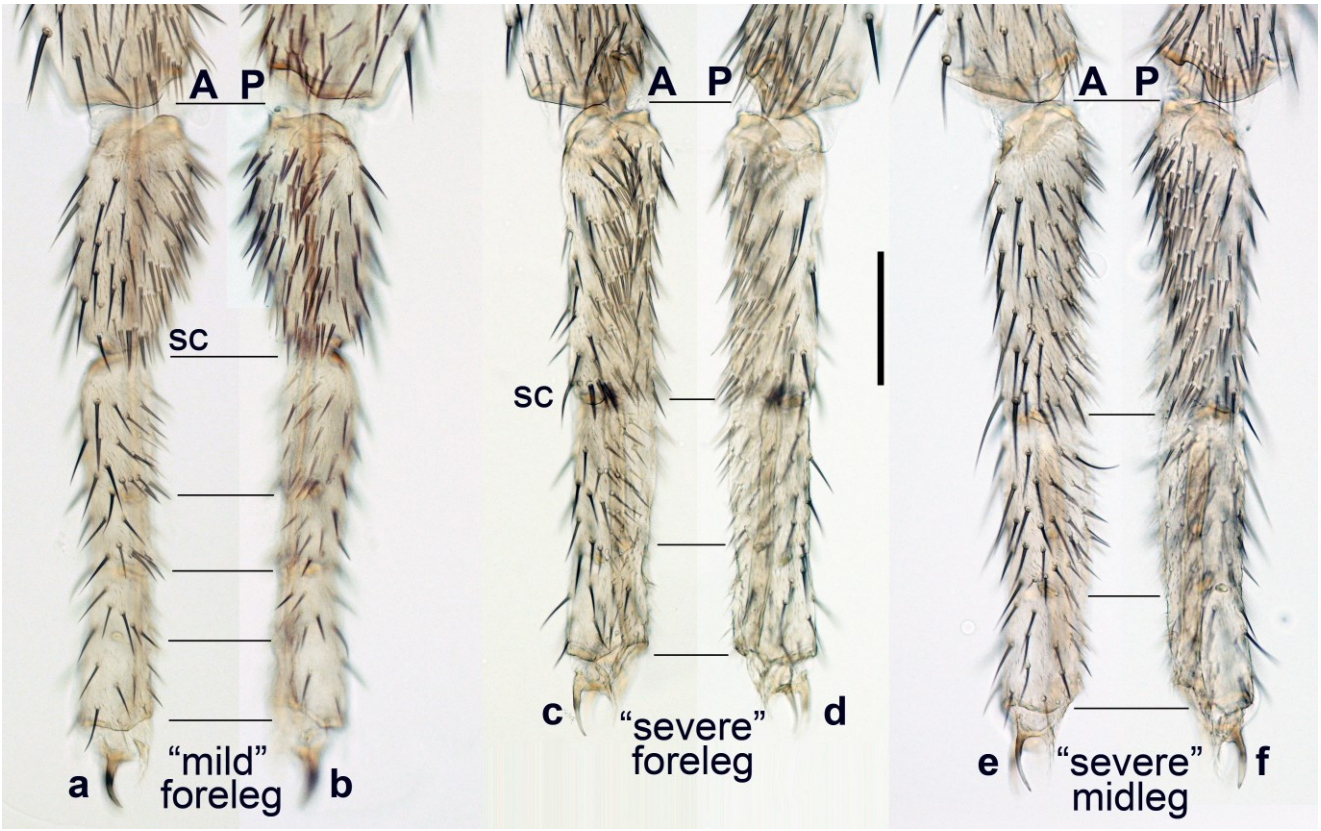

Figure 2. Alternative phenotypes among 15 *Dll-Gal4/+; UAS-UbxWT/tub-Gal80^{ts}* males recovered as dead pupae from temperature shifts at 12-24 h BPF (see Materials and Methods). **a**, **b**. "Mild" phenotype observed for 6 sex-comb-deficient males whose tarsi were stunted but still had 5 segments (= wild-type number). Horizontal lines mark segment boundaries. Flies in this subgroup displayed moderate T1→T3 homeosis, with vestigial (as here) or missing combs and fewer t-row bristles on the tibia and basitarsus. **c**, **d**. "Severe" phenotype for 9 sex-comb-deficient males whose tarsi had only ~3 segments. Flies in this subgroup had more t-row bristles on the P side of the foreleg on average than the mild subgroup (Figure 3), indicating stronger T1→T3 homeosis. **e**, **f**. Midleg tarsus of a "severe" fly. T-rows are visible on the P side of the basitarsus and, to a lesser extent, on the A side as well. Unlike *Scr-GOF* (Held *et al.*, 2017), *Ubx-GOF* does not elicit t-rows on ta2-ta5. Basitarsal shapes (**a-f**) are hindleg-like (*c.f.*, Figure 1). Other anomalies in the mild and severe subgroups included: (1) wider tarsi, (2) fused segments, (3) smaller bristles, (4) lighter pigmentation, (5) missing bracts, and (6) excess trichomes. The latter trait was surprising, given the ability of *Ubx* to suppress trichomes on the femur (Stern, 1998; Kittelmann *et al.*, 2018). Also, most pupae had a wider "apodeme" (Mirth and Akam, 2002; Soler *et al.*, 2004), which appears here as a hollow internal tube, and apodemes were shorter in "severe" pupae—extending from the claws up to ta1 (8/30 legs), ta2 (10), ta3 (5), or absent (7)—*vs*. "mild" pupae—extending to tibia (6/30 legs), ta1 (8), ta2 (11), ta3 (2), ta5 (2), or absent (1). Images of the left leg in **c** and **d** were flipped for ease of comparison with right legs in other panels. All images are at the same magnification; scale bar in **d** = 100 microns.

With even longer exposure to Ubx (24-36 h BPF cohort), the majority (19/24) of *Dll>UbxWT* pupae had legs that were truncated at the level of the tibia—precluding any assessment of effects on sex combs or tarsal t-rows. Similar truncations also occurred when *Dll-Gal4* was combined with *UAS-ScrWT* instead of *UAS-UbxWT* (Held, 2010), so this stunting could be due to a "flooding" of leg cells with exogenous transcription factors during growth of the imaginal discs. Indeed, we found that *Dll-Gal4* alone (sans *UAS*) can curtail tarsal length by 30% when larvae are exposed to 30°C throughout the third instar, as well as reducing the number of tarsal segments to three. Transcription factor "pollution" of this kind might therefore explain why "severe" pupae have fewer tarsal segments than "mild" pupae (12-24 h BPF cohort; Figure 2).

Figure 3 plots the number of laterally adjacent ("t-row") bristles as a function of larval age at the time of the upshift. Neither wild-type flies nor *Dll>UbxWT* controls raised at 18°C have t-rows on the P side of their foreleg basitarsi (Figure 1), but *Dll>UbxWT* males that are shifted to 30°C as larvae do display t-rows there, and the number of t-row bristles increases from 16.7 (0-12 h BPF) to 24.0 (12-18 h BPF) to 49.0 (18-24 h BPF; n = 10 each) with the duration of Ubx exposure. Indeed, the maximum (49.0) exceeds the number of t-row bristles on the A side of the same legs (39.5), and it approaches the level on the P side of hindlegs (55.1) in the same cohort. This P-side phenotype was expected for T1→T3 homeosis based on previous reports (Shroff *et al.*, 2007), as was the A-side loss of comb teeth (see above), but the number of t-row bristles on the A side stayed constant instead of vanishing. The endurance of the foreleg's A-side t-rows may be due to persistence of Scr expression there despite the imposition of exogenous Ubx.

In contrast to the forelegs, the midleg and hindleg phenotypes that we observed defy a simple explanation based on homeosis alone, because *Ubx-GOF* induces t-rows on the A side. The numbers of t-row bristles evoked on the A side of midleg basitarsi were 20.2, 22.0, and 37.2 (for 0-12, 12-18, and 18-24 h BPF shift times, respectively), and the numbers on hindleg basitarsi were 21.4, 26.0, and 33.2 for the same cohorts. *Ubx-GOF* is evidently capable of initiating t-row development directly, rather than indirectly (via its orthodox role in enforcing leg identity).

Our previous analysis of *Scr-GOF* (Held *et al.*, 2017) led to the same conclusion about *Scr* as we reached here about *Ubx*—namely, that it can induce t-rows on either the A or P side of any basitarsus (fore-, mid-, or hindleg) with one exception. The ability of excess Scr to elicit t-rows on the P side of the foreleg is minimal. Conceivably, Scr might be suppressed there by *engrailed* (*en*)—the selector gene for P compartments (Morata and Lawrence, 1975; Lawrence, 1984)—though En's inhibition would probably be post-transcriptional, because neither the *Dll-Gal4* driver nor the *UAS-ScrWT* construct are likely to have *en*-dependent enhancers. *Ubx* is inhibited by *en* in the wing (Emerald and Shashidhara, 2000), but not in the hindleg where *Ubx* is heavily expressed on the P side. If *Ubx* (unlike *Scr*) can evade suppression by *en* on the foreleg as well, then that immunity could explain why *Ubx-GOF* induces four times more t-row bristles on the P side of the foreleg (49.0 at 18-24 h BPF) as *Scr-GOF* (12.9 at 20 h BPF; Figure 3).

As in our earlier *Scr-GOF* study, we used a second *Gal4* driver in addition to *Dll-Gal4*. The *scabrous* gene (*sca*) is expressed in proneural clusters, which are groups of epidermal cells from which bristle cell progenitors are selected. They precede bristles and cover a larger area (Held, 2002). Milder effects were expected for *sca>UbxWT* than for *Dll>UbxWT* because *sca* is expressed just before bristle differentiation, which leaves the epidermal cells only a few hours to switch their identities from T1 or T2 to T3. No *sca>UbxWT* flies were obtained from the 36-48 h BPF shift, so we focused on *sca>UbxWT* males from the 24-36 h BPF cohort.

All of the 13 *sca>UbxWT* males that we recovered (among 104 F_1 total) in the 24-36 h BPF cohort died as phrarate pupae, and their second legs all lacked apical bristles, which was consistent with a T2→T3 transformation, as has been reported before (Rozowski and Akam, 2002). Sex combs persisted on all of their forelegs (defying any T1→T3 conversion), but all of the sex comb teeth therein were yellow, thin, pointed, and shriveled. Indeed, all of the bristles on all six legs were yellow, thin, and reduced in length. Transverse rows also persisted on the foreleg basitarsus and tibia (also defying a T1→T3 conversion), but the alignment of the bristles therein was commonly disrupted, often resulting in clumping of bristle sockets along the rows.

Ectopic "t-row" bristles (*i.e.*, extra laterally-adjacent bristles) were commonly seen on *sca>UbxWT* forelegs, midlegs, and hindlegs—albeit far fewer than on the *Dll>UbxWT* legs described above. Foreleg basitarsi had an average of 3.3 ectopic t-row bristles on their P side (osculating with row-1 bristles; n = 20 legs), while hindleg basitarsi had an average of 1.5 ectopic t-row bristles on their A side (osculating with row-

8 bristles; n = 13 legs). Midleg basitarsi had an average of 3.1 ectopic t-row bristles on their P side (osculating with row-1 bristles; n = 20 legs) but only 0.3 ectopic t-row bristles on their A side (osculating with row-8 bristles; n = 20 legs). This 10-fold A/P asymmetry (0.3/3.1) was surprising given the symmetry (4.3/4.6) that we previously witnessed for ectopic t-row bristles on *sca>ScrWT* midlegs (Held *et al.*, 2017).

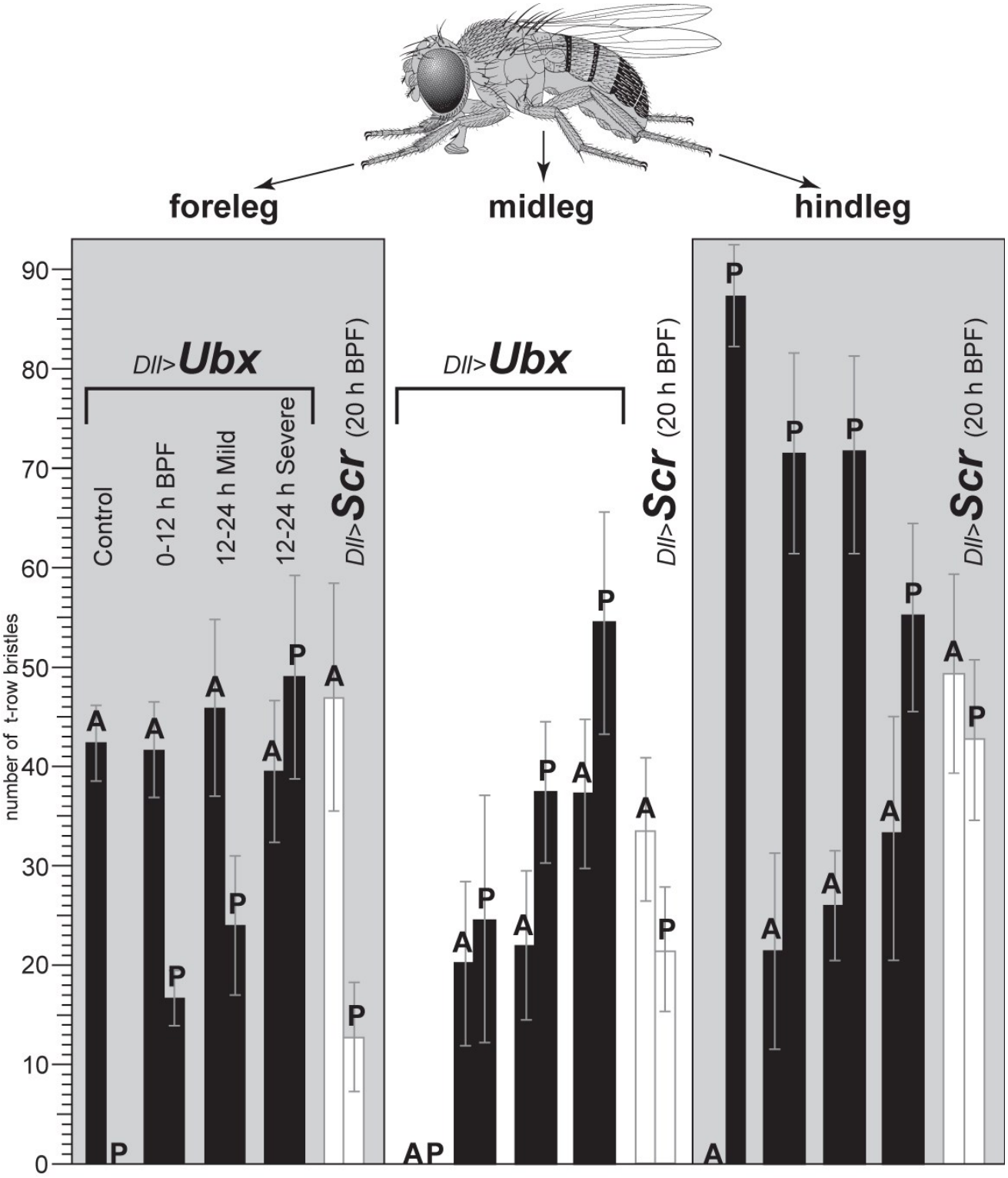

Figure 3. Mean numbers of laterally adjacent ("t-row") bristles (+/- standard deviation) on the anterior (A) and posterior (P) sides of foreleg, midleg, and hindleg basitarsi from *Dll>UbxWT* males (N = 10 legs/bar) shifted to 30°C at different times before puparium formation (0-12 h BPF or 12-24 h BPF). All of the *Ubx*-GOF individuals analyzed here died as pharate pupae before eclosion. Pupae with all 5 tarsal segments were assigned to a "mild" group, while those with truncated tarsi (only 2 or 3 segments remaining) were pooled into a separate "severe" group. The mild group was traced to 12-18 h BPF, while the severe group was traced to 18-24 h BPF (see text). The left-most pair of histograms for each leg type pertain to control flies (same genotype as experimental flies) raised entirely at the permissive temperature for $Gal80^{ts}$ (18°C). The rightmost pair of histograms for each leg type (white bars) gives comparative data for *Dll>Scr* males shifted to 30°C at 20 h BPF (Held *et al.*, 2017). When legs are sandwiched between cover slips, they tend to orient their A and P sides facing up or down, but sometimes they rotate, and the merging of t-rows can obscure the A/P boundary. In those cases we used the medial sensillum campaniformia as a marker for the ventral midline (see Materials and Methods).

Conclusions

Hox genes are famous for subdividing the bilaterian head-tail axis into metameres (Angelini and Kaufman, 2005; Held, 2017), but within insects they have, over the eons, insinuated themselves into the circuitry of segmental patterning at lower echelons as well (Weatherbee *et al.*, 1998; Pavlopoulos and Akam, 2011), all the way down to the level of bristles (Rozowski and Akam, 2002) and hairs (Stern, 1998; Kittelmann *et al.*, 2018). The data presented here (based upon two different *Gal4* drivers) show that *Ubx*, like *Scr* (Held *et al.*, 2017), can create t-rows in regions beyond its normal jurisdiction. Our results therefore affirm that *Hox* genes are micromanagers, in addition to serving as chief executive officers (Akam, 1998). However, we cannot fully decipher the nature of the link to t-rows until we know much more about how t-rows arise in normal development (Held, 2002).

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Mating latency and mating duration in *Drosophila melanogaster* strains maintained over 400 generations on four types of food.

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Sexual behaviour of *Drosophila* represents series of behavioral steps, expressed by both sexes that culminate in copulation: data concerning their description up to complex genetic backgrounds are widely available in literature (Hall, 1994; Greenspan and Ferveur, 2000; Beaver and Giebultowicz, 2004; Edward *et al*., 2014). Variation in male nutrition could be important for reproductive behavior, since the nutritional value of food has effects on the properties mediated by the accessory gland proteins (Fricke *et al*., 2008). In females, protein/sugar ratio in food affects fecundity and lifespan (Lee *et al*., 2008; Fanson *et al*., 2009; Rodrigues *et al*., 2015). Nutrition is also related with morphological traits, such as body size, as well as with physiological abilities that could be linked with mating. In this work, we have examined two components of *Drosophila* mating behavior and fitness, mating latency (ML) and mating duration (MD), in strains that have been cultivated over the years in various nutritive conditions. Mating latency is related to male age (Eastwood and Burnet, 1977), body size (Debelle *et al*., 2016) and vigor, and represents an important component of male competitive success (Bacigalupe *et al*., 2007). It is also referred to as a trait correlated with fecundity, fertility, and longevity (Hegde and Krishna, 1999). In females, ML is influenced by physiological state (Eastwood and Burnet, 1977) and related with their mate preference (Bacigalupe *et al*., 2007). Mating duration is correlated with female remating (Gilchrist and Partridge, 2000; Bretman *et al*., 2013) and sperm transfer (Yamamoto *et al*., 1997). It is determined by both sexes and tested in various experimental designs investigating complex genetic background (Mackay *et al*., 2005), sexual conflict (Edward *et al*., 2014), the effects of previous mating experience (Pavković-Lučić *et al*., 2014), social environments (Taylor *et al*., 2013), and so forth.

Previous studies that manipulated with environmental factors have revealed considerable plasticity in courtship/mating traits in *Drosophila* (see, for example, Bretman *et al*., 2009). Since the influence of environmental (nutritional) variation on ML and MD is insufficiently known, the aim of this study was to explore aforementioned behavioral traits in four *Drosophila melanogaster* strains after long-term laboratory growing on different diets. Previously, we have observed that these diets differ in protein content and C/N ratio, which was reflected on mating success and several fitness components (Trajković *et al*., 2017a; Trajković *et al*., 2017b).

D. melanogaster strains used in this experiment were maintained for more than 400 generations on four different diets (for recipes see Kekić and Pavković-Lučić, 2003): standard cornmeal diet ("St" strain), and diets that contain tomato ("T" strain), banana ("B" strain), and carrot ("C" strain) under conditions optimal for the species (temperature of 25°C, relative humidity of 60%, 300 lux of illumination, 12 h:12 h light/dark cycle). Once the hatching starts, virgin flies were separated by sex and strain every 8 hours. Females were kept in groups (5 *per* group), while males were housed separately, in order to prevent homosexual behavior (Napolitano and Tompkins, 1989). Separated flies of both sexes and strains were kept under optimal laboratory conditions until they were 4-5 days old, when mating assays were performed.