How does *Scr* cause first legs to deviate from second legs?

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All six legs of *D. melanogaster* have longitudinal rows of bristles, but only the first and third pairs have transverse rows (Hannah-Alava, 1958). These "t-rows" serve as brushes for cleaning the eyes or wings during the grooming ritual (Szebenyi, 1969; Vandervorst and Ghysen, 1980), and the most distal t-row on the male foreleg basitarsus rotates during metamorphosis to form a sex comb (Held *et al.*, 2004; Atallah *et al.*, 2009a) that is used during the courtship ritual (Ng and Kopp, 2008). The t-rows and sex comb pose a number of tantalizing riddles at the levels of development, genetics, behavior, and evolution (Held, 2002b).

Evolution is thought to have inserted the t-rows and sex comb as modules into the more elementary midleg pattern (Stern, 1954; Hollingsworth, 1964). During development, the fore- and hindlegs are steered away from this midleg "ground state" (toward their distinctive anatomies) by the *Hox* genes *Sex combs reduced* (*Scr*) and *Ultrabithorax* (*Ubx*) (Struhl, 1982; Stern, 2003; Passalacqua *et al.*, 2010). If either *Scr* or *Ubx* malfunctions, then the fore- or hindlegs (respectively) revert to midleg identity as a default (Lawrence *et al.*, 1979; Shroff *et al.*, 2007; Sivanantharajah and Percival-Smith, 2009).

How *Ubx* acts in hindlegs has been studied previously (Rozowski and Akam, 2002), but how *Scr* works in forelegs is less well understood (Carroll *et al.*, 1995; Chesebro *et al.*, 2009). That is the subject of the present investigation.

The advantage of using fly legs to probe *Hox* gene action in general is the high resolution of their rich cuticular detail (Nottebohm *et al.*, 1994a). The sex comb in particular has become a popular tool for studying rapid evolution (Kopp and True, 2002; Randsholt and Santamaria, 2008; True, 2008), evo-devo mechanisms (Rogers *et al.*, 1997; Barmina and Kopp, 2007; Tanaka *et al.*, 2009), the dynamics of morphogenesis (Atallah *et al.*, 2009a; Atallah *et al.*, 2009b), and the genetics of dimorphisms (Graze *et al.*, 2007; Ahuja and Singh, 2008; Wasik *et al.*, 2010). The universal roles of *Hox* genes in bilaterian phyla means that whatever is learned here may be widely applicable elsewhere (Castelli-Gair, 1998; Pearson *et al.*, 2005).

If the above evolutionary scenario is correct, then the foreleg is actually a quilt of distinct pattern territories, with the more recent (foreleg-specific) modules (t-rows and comb) under *Scr* control and the more ancient (midleg) background (longitudinal rows) independent of *Scr* (Shroff *et al.*, 2007). One way to tease apart these components is via the time dimension. Ever since 1970 one of the most incisive methods for temporal dissection has been the usage of temperature-sensitive mutations (Suzuki, 1970). In 2003, this technology was made even more powerful by a yeast mutation that allows any desired fly gene to be turned ON or OFF at any desired time (McGuire *et al.*, 2003). This keen new "scalpel" (*Gal80ts*) is described in the next section.

Materials and Methods

Fly stocks were maintained on Ward's *Drosophila* Instant Medium plus Fleischmann's live baker's yeast. In all experiments nutrition was optimized and overcrowding was avoided in order to prevent any delays that could affect developmental staging. Flies were preserved and dissected in

70% ethanol. Legs were mounted in Faure's fluid (Lee and Gerhart, 1973) between cover glasses and examined at 200× magnification in an Olympus compound microscope.

Scr was artificially switched ON or OFF by using the *Gal80ts* method in combination with the *Gal4-UAS* system (Leung and Waddell, 2004). The rationale for this clever new technique (McGuire *et al.*, 2003) is as follows. *Gal80ts* prevents the transcription factor Gal4 from *trans*-activating its target "upstream activating sequence" *UAS* at 18˚C (permissive temperature) but not at 30˚C (restrictive temperature; McGuire *et al.*, 2004). Any gene attached to the *UAS* can be switched ON or OFF by simply raising or lowering the temperature whenever desired.

Data are reported relative to 25˚C, the conventional temperature for developmental staging (Ashburner, 1989). Actual times at 18˚C were converted to a normalized 25˚C scale by dividing them by 2.0 – the difference in developmental rate determined previously (Held, 1990). This rate was confirmed with a sample cohort (N = 29 prepupae) monitored for the time elapsed between pupariation and pupation (data not shown). No correction was made for the 12 h spent at 30˚C, since midpoints of pulses would have changed by only an hour (conversion factor ≈ 0.86 (Ashburner, 1989; Held, 1990)).

Abbreviations include: PF (puparium formation), BPF (before PF), APF (after PF), h (hours), @25˚C (time normalized to the developmental rate observed in wild-type flies at 25˚C), SC (sex comb), T1 (foreleg), T2 (midleg), T3 (hindleg), t1-t5 (tarsal segments 1-5), temp. (temperature), *ts* (temperature-sensitive allele), and TSP (temperature-sensitive period).

Experimental individuals were collected as white prepupae (WPP). This hour-long stage, termed "puparium formation," begins when the larva ceases moving, everts its anterior spiracles, and acquires a barrel shape to form the pupal case. It ends when the cuticle turns brown. A few examples of the pulsing protocol should suffice. For 12-h pulses starting before –12 h BPF, food bottles containing larvae raised at 18˚C were transferred to a 30˚C water bath so as to increase the food temperature as quickly as possible. The bath had to be covered, however, to prevent evaporative cooling, and the resulting humidity was too high for larvae to pupariate, so bottles were transferred to a dry shelf within the same 30˚C incubator after ~3 h. At 12 h after the initial transfer, bottles were returned to 18˚C, and cohorts of ~30 WPP were harvested periodically thereafter and kept in humid petri dishes at 18˚C until eclosion. For the "–6 to +6 h" pulse, WPP were collected 6 h after the initial transfer and kept in dishes at 30˚C for 6 more hours before putting them at 18˚C. For APF pulses, WPP were collected at 18˚C and kept in dishes at that temperature until the start of their pulse, whereupon the dishes were floated (sans lids) atop a 30˚C water bath (covered to prevent evaporation) for 12 h before being returned to 18˚C.

LOF Analysis: Results and Discussion

Two kinds of experiments were conducted that were reciprocal and complementary: (1) a LOF (loss of function) analysis where *Scr* was turned OFF using *UAS-Scr-RNAi* (interfering RNA) and (2) a GOF (gain of function) analysis where *Scr* was turned ON using *UAS-ScrWT* (wild-type allele). These *UAS* agents were targeted to legs using a *Gal4* insertion ("*Dll-Gal4*") in the enhancer region of the *Distal-less* (*Dll*) gene, which is expressed in the distal tibia and tarsus of all six legs (Held, 2002b). The LOF approach is described here; the GOF approach is described in the next section.

The *UAS-Scr-RNAi* construct (#46500) came from the Vienna *Drosophila* RNAi Center. It was pre-tested in *Dll-Gal4/UAS-Scr-RNAi* flies (lacking *Gal80ts*). The forelegs of those flies displayed a midleg pattern on the tibia and tarsus with 100% penetrance and expressivity, though the

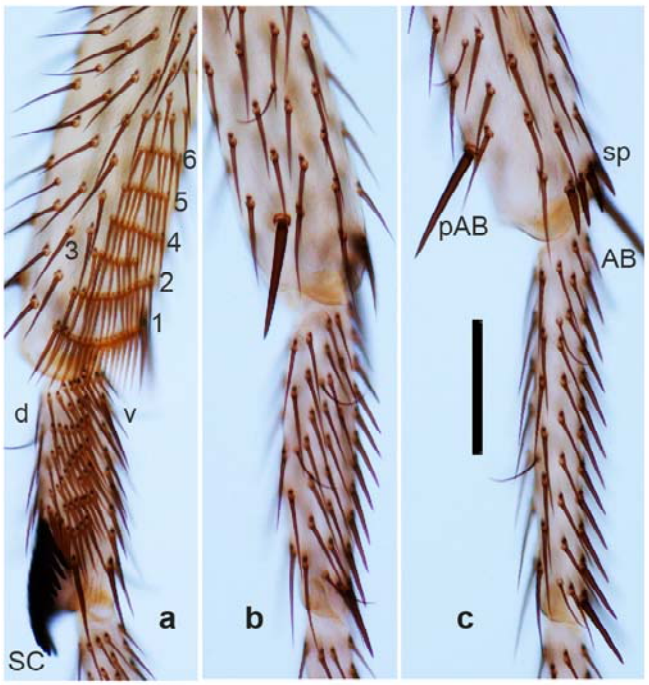

Figure 1. Basitarsus and distal tibia from forelegs (**a**, **b**) and a midleg (**c**). In all panels the anterior face is shown, proximal-distal is top to bottom, and dorsal-ventral is left to right, and the same orientation is used for all other legs depicted in this paper. All photos are at the same magnification (scale bar = 100 microns). **a**. Right foreleg from a control *Dll-Gal4/UAS-Scr-RNAi; tub-Gal80ts/+* male raised at 18˚C (permissive temp.). Tibial t-rows (1 to 6) occupy a triangular area. Basitarsal t-rows fill a rectangular area just ventral to the sex comb (SC). Tibial t-row bristles typically differ from ordinary mechanosensory bristles insofar as they lack bracts (tiny triangles above sockets), as well as being thinner and yellower. A longitudinal row runs along the ventral (v) edge of the basitarsus (d = dorsal). Except for bristles of the SC, which rotates 90˚, bristles point distally, though angles may be deflected due to sandwiching of the legs between cover glasses. **b**, **c**. Right foreleg (**b**) and midleg (**c**) from a *Dll-Gal4/UAS-Scr-RNAi* male raised at 25˚C. The foreleg has been homeotically transformed to resemble the midleg insofar as it lacks t-rows and has (out of focus) an Apical bristle (AB) and spur bristles (sp). However, the length of the foreleg basitarsus does not attain that of its midleg counterpart, perhaps due to the fact that size is controlled separately from pattern (Stern, 2003). The pre-Apical bristle (pAB) is not useful as a marker because it is present on forelegs (out of focus in **a**) as well as on midlegs. Curved bractless bristles are chemosensory.

foreleg basitarsus (Figure 1b) did not attain the full length of a midleg basitarsus (Figure 1c). The totality of the homeosis ensured high resolution for TSP assessments over the full range from 0 to 100% transformation, depending upon the timing of the pulses. Moreover, viability was comparable to balancer siblings (86:95), so the likelihood of tolerating heat pulses was high.

For pulse experiments, zygotes of the genotype *Dll-Gal4/UAS-Scr-RNAi; tub-Gal80ts/+* were obtained as F_1 offspring from a cross between *UAS-Scr-RNAi* males and *Dll-Gal4/CyO; tub-Gal80ts* females. Curly-winged (*CyO/UAS-Scr-RNAi; tub-Gal80ts/+*) siblings served as controls for any side-effects of the pulses not due to the *RNAi* agent. No such effects were seen.

To assess the potency of the *Gal80ts* allele, F_1 offspring were raised at 18˚C. Forelegs of such flies had wild-type chaetotaxy (100% penetrance; 100% expressivity), showing that *Gal80ts* completely suppresses *Gal4* at this temperature (Figure 1a). The anatomical parameters of these flies are given as horizontal lines marked "18 deg. Control" in the seven panels of Figure 2.

To assess the lability of the *Gal80ts* allele, F_1 offspring were raised at 30˚C, a stressful temperature that can kill larvae or pupae (Ashburner, 1989; his Figure 8.29), especially mutants whose health is compromised. Indeed, only a few non-curly adults were recovered (6 eclosed flies among 185 total = 3%). Their forelegs showed T1→T2 homeosis, but their tarsi were severely

stunted. This "dachshund" phenotype is typical of strong *Dll* mutants (Cohen and Jürgens, 1989) and may be a hypoplastic artifact of excessive Gal4 in *Dll*-targeted tissues. Similar defects were seen when F_1 larvae were shifted from 18˚C to 30˚C before –30 h BPF and kept at 30˚C for the rest of development. These deformities precluded any mapping of TSPs by 18˚C pulses against a 30˚C background, so flies were exposed to pulses of 30˚C against an 18˚C background instead.

The precision with which TSPs can be defined is inversely related to pulse duration, but 6-h pulses yielded weaker phenotypes than 12-h pulses. For example, a 6-h pulse from –9 to –3 h BPF reduced the number of sex comb bristles by only 29% (mean = 8.1; N = 10) relative to the 18˚C control (mean = 11.4; N = 10), whereas a 12-h pulse from –12 to 0 h BPF lowered it by 86% (mean =1.6; N = 10). Hence, 12-h pulses were used (*cf.,* Shellenbarger and Mohler, 1978). To enhance resolution, successive pulses were staggered at 6-h intervals. Dependence on *Scr* was assayed from –48 h BPF (start of 3rd instar) to +36 h APF when bristle elongation is well underway (Graves and Schubiger, 1981) and cuticle deposition has begun (Reed *et al.*, 1975).

Figure 2 shows the temporal dependence of foreleg features on *Scr* function. Following convention (Held, 1990), pulse midpoints (6 h after initiation) are plotted along the *x* axis. Whereas some *Gal80ts* studies do show maximal responses of target genes in only 6 h (McGuire *et al.*, 2003), a recent investigation exhibited a much longer lag before full onset: when a wild-type allele of *Ubx* is ectopically expressed in the wing, Ubx protein is detectable by 6 h after an upshift, but its level continues to rise until a plateau is reached 16 h after initiation (A. Pavlopoulos and M. Akam, pers. communication). In the absence of comparable data for Scr protein expression, it is possible that all data points in Figure 2 should shift ≥ 10 h to the right.

Number of sex comb teeth (Figure 2a). The sex comb (named for its presence in only one sex and its resemblance to a hair comb) is homologous to the most distal basitarsal t-row in females (Tokunaga, 1962), but its bristles or "teeth" are darker, thicker, blunter, more curved, and more numerous than t-row bristles (Hannah-Alava, 1958). Using a *ts* allele of the sex-transforming gene *tra-2*, Belote and Baker (1982) found that bristle number becomes fixed at the male (~10) or female (~7) level between *ca* –8 h BPF and +8 h APF @25˚C. In the present study the *RNAi*-mediated transformation was not from male to female, but rather from T1 to T2, so it is not surprising that the maximal effect is a reduction to zero teeth (T2 state) at –12 h BPF. Nor is it surprising that recovery to a T1 state occurs over the same span (–8 to +8 h) as the *tra-2ts1* TSP. What is surprising is that *Scr*-LOF affects tooth number as early as two days BPF. As the number decreases to zero (Figure 3a-e), the missing teeth are usually not replaced by t-row bristles. This rule (an exception is shown in Figure 3c) implies that in wild-type flies *Scr* makes teeth directly from ordinary epidermal cells, rather than first inducing t-row bristles and then modifying them into teeth. Disabling *doublesex* via *UAS-dsx-RNAi* partly transforms teeth into t-row bristles (A. Kopp, pers. communication), so *Scr* is probably not acting via *dsx* (Randsholt and Santamaria, 2008). The sinusoidal shape of this curve and the ones in Figure 2b-d suggests a gradual (analog) process like tissue growth (leading to more bristles; Chesebro *et al.*, 2009) or movement (leading to sex comb rotation; Atallah *et al.*, 2009a), as opposed to the spike in Figure 2g, which implies a binary (digital) switch. If so, then *Scr* would be driving each process along its entire course, instead of just launching it. If evolution did indeed "shoehorn" t-rows and sex combs into a midleg background (Held, 2002b), then intercalary growth (and movement) may have been required to make room for the new modules within the old pattern.

Sex comb rotation (Figure 2b). Another salient difference between the sex comb and its female t-row counterpart is that the former rotates to a longitudinal orientation, whereas the latter remains transverse (Tokunaga, 1962). This 90˚ rotation occurs at 16-28 h APF (Held *et al.*, 2004; Atallah *et al.*, 2009a). Disabling *Scr* via *RNAi* inhibits rotation (Figure 3a-c) with a time course that parallels

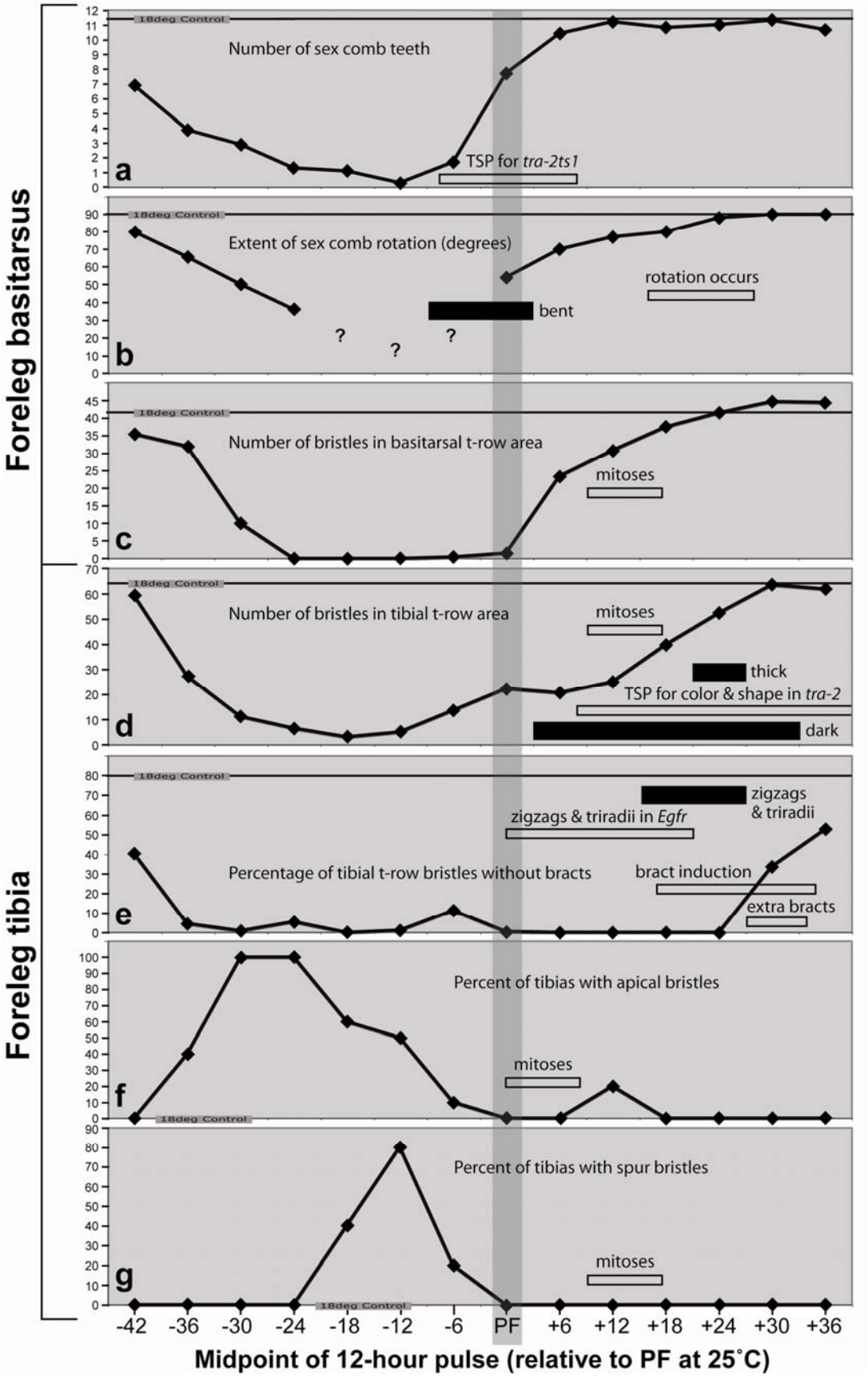
Foreleg basitarsus
Foreleg tibia
18deg Control
Number of sex comb teeth
TSP for tra-2ts1
a
Extent of sex comb rotation (degrees)
rotation occurs
bent
b
Number of bristles in basitarsal t-row area
mitoses
c
Number of bristles in tibial t-row area
mitoses
thick
TSP for color & shape in tra-2
dark
d
zigzags & triradii
zigzags & triradii in Egfr
Percentage of tibial t-row bristles without bracts
bract induction
extra bracts
e
Percent of tibias with apical bristles
mitoses
f
Percent of tibias with spur bristles
mitoses
g
-42 -36 -30 -24 -18 -12 -6 PF +6 +12 +18 +24 +30 +36
Midpoint of 12-hour pulse (relative to PF at 25˚C)

Figure 2 (facing page). TSPs for anomalies caused by exposing *Dll-Gal4/UAS-Scr-RNAi; tub-Gal80ts/+* larvae or pupae to a 12-h pulse of 30˚C (restrictive temp.). Midpoints of pulses are plotted @25˚C along the *x* axis. Horizontal lines give the mean (N = 10) for flies raised entirely at 18˚C (permissive temp.; "18 deg. Control"). Each data point is the average of N = 10 legs (forelegs of 5 males), except –42 to –30 (N = 8) and +30 to +42 (N = 6). Insets present data from this study (thick solid bars) or others (thin open rectangles). **a**. Number of teeth in the sex comb, ignoring gaps or zigzags. *Open rectangle:* TSP (–8 to +8 h @25˚C) when bristle number becomes fixed—*i.e.*, impervious to upshifts (that convert females into males) or downshifts—in *tra-2ts1* mutants (Belote and Baker, 1982). **b**. Angle of the sex comb relative to the distal end of the basitarsus, with zero denoting no rotation and 90˚ maximal rotation. Question marks are guesses based on points in **a**. For bent combs, only the distal portion was considered. *Solid bar:* TSP when pulses cause a sharp bend in the sex comb (Figure 3g). *Open rectangle:* period (16-28 h APF) when the comb rotates in wild-type pupae (Held *et al.*, 2004; Atallah *et al.*, 2009a). **c**. Number of bristles in the t-row region of the basitarsus (see text for definition). *Open rectangle:* period (~9-17 h APF) when bristle precursor cells undergo mitoses (Nottebohm *et al.*, 1994b). **d**. Number of bristles in the t-row region of the tibia. *Solid bars:* TSPs when t-row bristles are dark or thick—*e.g.*, Figure 3h and i. *Open rectangle (above):* period (~9-17 h APF) when bristle precursor cells undergo mitoses (Nottebohm *et al.*, 1994b). *Open rectangle (below):* TSP (8-48 h APF @25˚C) when the color and shape of sex comb teeth (not t-row bristles!) becomes fixed in *tra-2ts1* mutants (Belote and Baker, 1982). *N.B.:* the latter TSP starts after the bristle-number TSP (insert in **a**) ends. **e**. Percentage of bristles in the t-row area that lack bracts. The wild-type level (~80%) is not attained by any data points. *Solid bar:* TSP when t-rows exhibit zigzags (*e.g.*, Figure 3i) or Y-shaped "triradii" (Cummins and Midlo, 1943; *e.g.*, rows 3 and 4 in Figure 3h). *Open rectangles (top to bottom):* TSP (0-21 h APF @25˚C) when shifts with a *ts* allele (*Egfr-ts1a*) of the *Epidermal growth factor receptor* gene induce zigzags or triradii in tibial t-rows (Held, 2002c); TSP (17-35 h APF @25˚C) when bracts are induced on the midleg tibia, as surmised from shifts with *Egfr-ts1a* (Held, 2002a) and heat-shocks with *poxn* (Layalle *et al.*, 2004); and TSP (27-34 h APF @25˚C) when bracts can be induced on chemosensory bristles using *ts* alleles of *Notch* or *shibire* (Held, 1990; Layalle *et al.*, 2004). **f**. Percentage of foreleg tibias that have an Apical bristle. *Open rectangle:* period (0-8 h APF) when (1) the precursor cell of the Apical bristle divides (Nottebohm *et al.*, 1994b) and (2) loss of *Ubx* from that site on the hindleg (where *Ubx* suppresses an Apical bristle) can allow it to make an Apical bristle (Rozowski and Akam, 2002). **g**. Percentage of foreleg tibias with spur bristles. *Open rectangle:* period (~9-17 h APF) when precursor cells of the spur bristles divide (Nottebohm *et al.*, 1994b).

Figure 3. Forelegs from *Dll-Gal4/UAS-Scr-RNAi; tub-Gal80ts/+* males exposed to a 12-h pulse of 30˚C (restrictive temp.). For ease of comparison, the images of left legs in **b**, **c**, **g**, and **h** were flipped horizontally to appear as right legs; all others are right legs. Ages during pulses (banner at top) increase from **a** to **j** and are normalized to 25˚C (see Materials and Methods). Minus signs are BPF; plus signs are APF. T1→T2 homeosis (loss of t-rows, loss of SC teeth, failure of SC rotation, acquisition of Apical and spur bristles) increases from **a** to **e** and wanes from **e** to **j**. Other anomalies include presence of bracts on tibial t-row bristles (all except **j**), disruption of t-row alignment

Figure 3 (continued) (**g-i**), and bent sex comb (**g**). All photos are at the same magnification. **a**. The number of teeth is reduced even at this early time. **b**. Gaps are occasionally seen in the sex comb of pulsed flies. **c**. An exceptional case where two residual teeth are aligned with two ordinary t-row bristles. In most other legs from pulsed cohorts, remnant teeth contact one another but are collectively isolated. **d**. Note the Apical bristle (AB). Only one tooth is present, which precludes assessment of SC rotation. **e**. T1→T2 homeosis is nearly complete, except for the distal tibial t-row. **f**. Three t-rows have reappeared on this tibia, but none yet on the basitarsus. **g**. Tibial t-row bristles are as dark as surrounding bristles, as well as being misaligned (row 2), and the sex comb is bent. **h**. Tibial t-row bristles are not only darker but also thicker than surrounding bristles, and their alignment is even more disrupted. Rows 3 and 4 merge ventrally (toward right) at a Y-shaped "triradial" juncture. **i**. (Same pulse period as **h**.) Tibial t-row bristles resemble SC teeth and are jumbled midway along rows 2 and 3. **j**. The number of t-rows has returned to a foreleg state, as have t-row alignment, bristle shape, and bristle color. Most tibial t-row bristles have also resumed a bractless condition.

the effects of *Scr*-LOF on the number of teeth (Figure 2a), though the maximal effect is difficult to gauge when fewer than 3 teeth are present (pulse midpoints –18, –12, and –6 h BPF; *e.g.*, Figure 3d). This parallelism is perplexing because rotation occurs a day after the number of sex comb teeth is fixed. Why should *Scr* be needed so much earlier than the overt process it controls? Another surprise is a "bent-comb" anomaly seen in 40% of legs from pulses at 0-12 or 6-18 h APF (or 6-h pulses at 0-6 or 3-9 h APF) and in 10% of legs from flanking 12-h periods. In most cases the bend is midway along the comb, with the proximal and distal halves joined at a right angle (Figure 3g). Such combs seem to be "caught in the act," with *Scr* having been already used by the distal half to license its rotation but not yet used by the proximal half, which has been prevented from doing so by the pulse. The remaining combs from the affected pulse periods tend to be curved rather than bent, and their arc is likewise concave distally. A similar arc is seen in proximal portions of wild-type combs during the normal rotation process (Atallah *et al.*, 2009a), so the arc anomaly might represent a natural phase that has been frozen in time due to deprivation of *Scr* action.

Number of bristles in t-row areas of the basitarsus (Figure 2c) *and tibia* (Figure 2d). The t-row area is operationally defined here as the area subtended by the most proximal and distal rows containing at least three adjacent bristles with osculating sockets. All bristles in the area thus defined were counted, regardless of whether they were organized in rows. The t-row area normally is rectangular on the basitarsus but triangular on the tibia (Figure 1a). On both segments t-row bristles decline at about the same rate (midpoints –42 to –24 h; Figure 3a-e) as the number of sex comb teeth (Figure 2a), but they both recover from their minima more slowly, with the tibia recovering even more slowly than the basitarsus. Distal-to-proximal gradients of this sort (*e.g.*, basitarsus preceding tibia) have been uncovered along the leg for bristles (Graves and Schubiger, 1981; Nottebohm *et al.*, 1994b) and bracts (Held, 2002a). When the basitarsus lacks recognizable t-rows, the last t-row on the tibia is still present (Figure 3e), which explains why the tibial curve does not fall to zero like the basitarsal one. Both curves return to normal shortly after the bristle precursor cells undergo their differentiative mitoses at ~9-17 h APF (Nottebohm *et al.*, 1994b).

Color of tibial t-row bristles (*lower solid bar in* Figure 2d). Bristles of the basitarsal t-rows are normally yellower than the surrounding (brown) bristles, and tibial t-rows are even lighter still (Figure 1a). Removal of *Scr* function at any time from +6 to +30 h APF turns t-row bristles as brown as their neighbors (Figure 3g), if not browner (Figure 3h-i). The yellow color only returns with pulses at +36 h APF (Figure 3j). Despite how late this stage may seem relative to TSPs discussed above, it is still two days before overt melanization, which only commences in the tarsus at +77 h APF (Walter *et al.*, 1991).

Shape of tibial t-row bristles (*upper solid bar in* Figure 2d). In addition to being yellower, the tibial t-row bristles in wild-type flies are noticeably thinner than nearby bristles. The pulse period +18 to +30 h (midpoint +24) makes t-row bristles thicker than nearby bristles (Figure 3h, i). Indeed, these fatter t-row bristles vaguely resemble sex comb teeth in both shape and color. Similar effects have been reported previously for flies carrying inserts of a male-specific *doublesex* (*dsx*) gene linked to the *hsp70* heat-shock promoter (Jursnich and Burtis, 1993), regardless of whether heat shocks were administered (their Figure 1C). In wild-type forelegs *dsx* is expressed mainly in the sex comb area (Robinett *et al.*, 2010), so this partial conversion of t-row bristles into teeth implies a possible expansion of *dsx* activity into the t-row area (where *Scr* is normally expressed). Why disabling *Scr* (via *RNAi*) should enhance *dsx* (rather than suppress it) is unclear since (1) *dsx* is thought to regulate *Scr* rather than the other way around (Barmina and Kopp, 2007), and the interaction is thought to be positive rather than negative.

Alignment of tibial t-row bristles (*solid bar in* Figure 2e). At about the same time as t-row bristles thicken, t-row alignment is disrupted (midpoints +18 and +24). Anomalies include Y-shaped intersections of adjacent t-rows (Figure 3h─a juncture termed a "triradius" in fingerprints (Cummins and Midlo, 1943). More commonly, there are jumbled clumps of bristles (Figure 3i). Both types of defects are also seen when the EGFR pathway is disabled (Held, 2002c), and their respective TSPs overlap. These flaws are intriguing because they offer clues to how bristle cells "self assemble" into rows (Atallah *et al.*, 2009a). *Scr* could be acting via the EGFR pathway.

Bracts on tibial t-row bristles (Figure 2e). Tibial t-rows differ from basitarsal ones insofar as they lack bracts, except at the edges. Bracts are tiny pigmented cuticular protrusions (of no known function) associated with mechanosensory bristles on distal leg segments (Hannah-Alava, 1958). They are normally absent from chemosensory bristles (Layalle *et al.*, 2004) and tibial t-row bristles. Bracts arise from ordinary epidermal cells by induction from adjacent bristle cells via an EGFR signal emitted around +17 to +35 h APF (del Álamo *et al.*, 2002; Held, 2002a). In the present investigation, tibial t-row bristles display bracts with pulses from –36 h BPF to +24 h APF. This TSP is remarkable for its length (2.5 days), as well as for its ~100% penetrance and expressivity. Tibial t-rows begin to recover their bractless state at +30 h APF, when bract induction is thought to occur.

Apical bristle (Figure 2f). Midlegs have two large bristles (macrochaetes) on the distal tibia. The pre-Apical bristle also exists on forelegs, so it is not a useful marker for homeosis, but the Apical bristle is distinctively large, dark, and bractless, so it can serve that function. The precursor cell of the Apical bristle is first detectable at puparium formation (Nottebohm *et al.*, 1994b) and undergoes two mitoses over the next ~8 h (Rozowski and Akam, 2002). The TSP for T1→T2 homeosis to an Apical bristle (Figure 3d) lasts a day (–36 to –12 h BPF) and reaches its peak about one day (27 h) before the precursor cell arises.

Spur bristles (Figure 2g). Just proximal to the midleg's Apical bristle are ~5 peg-shaped "spur" bristles arranged in a transverse row that differs from a foreleg t-row insofar as the sockets of its bristles are not always in contact (Hannah-Alava, 1958). Its precursor cells presumably arise along with other tibial microchaetes at ~9 h APF and undergo differentiative mitoses over the next ~8 h (Nottebohm *et al.*, 1994b). The peak of the TSP for T1→T2 homeosis to spur bristles occurs at –12 h BPF, which is about a day (21 h) before the precursor cells arise. This hiatus is comparable to the analogous period for Apical bristle homeosis described above. *Scr* is, therefore, apparently needed to suppress midleg-specific bristle development at a fixed time interval before precursor initiation, regardless of the size or location of the bristles.

Shift experiments. Given the disparity between the Apical and spur bristle peaks (Figure 2f *vs.* 2g), it should be possible to elicit spur bristles alone (without an Apical bristle) by shifting larvae from 18˚C to 30˚C at –18 or –9 h BPF (with no subsequent downshift). When these upshifts were performed, the forelegs fulfilled this prediction (N = 6 each). Other upshifts at later times (0, 4, 8, 12, and 16 h APF) conform to expectations based on remaining TSPs in Figure 2 (data not shown). For example, each of these cohorts displays dark, thick, zigzag t-row bristles, so these traits can be induced by keeping *Scr* OFF for periods much longer than the charted TSPs. Interestingly, females show the same t-row anomalies as males, so the dark, thick bristle trait described above is unlikely to be imitating a (male-limited) sex comb tooth morphology per se.

GOF Analysis: Results and Discussion

To investigate a gene's action fully, LOF studies must be complemented by GOF ones. Here, that means ascertaining whether─and, if so, when─ectopic misexpression of the wild-type *Scr* allele can cause midlegs to adopt foreleg traits. For this purpose, the *UAS-ScrWT* (wild-type) construct (#7302) was obtained from the Bloomington Stock Center. Attempts to pre-test this construct failed because no *Dll-Gal4/UAS-ScrWT* flies (lacking *Gal80ts*) were seen among 239 F_1 adult offspring (all had curly wings) from a cross of *Dll-Gal4/CyO* X *UAS-ScrWT*.

Exposing *Dll-Gal4/UAS-ScrWT; tub-Gal80ts/+* larvae or pupae to 12-h pulses of 30˚C over the time span studied above produced only minimal homeoses (*e.g.*, one sex comb tooth per midleg). Interestingly, a comparable disparity in LOF *vs.* GOF thresholds was found for *Ubx* action in the hindleg *vs.* midleg (Rozowski and Akam, 2002): T3→T2 homeoses are easily achieved by disabling *Ubx* in T3, but T2→T3 transformations require that *Ubx* be expressed in midlegs at higher levels for longer periods, and even the strongest constructs were unable to eliminate spur bristles.

One option to overcome this threshold limitation was to use a more powerful *Gal4* driver, such as *rotund-Gal4* (*vs. Dll-Gal4*). Indeed, *rotund-Gal4:UAS-ScWT* males do make sex combs on midlegs (Barmina *et al.*, 2005), but unlike *Dll*, *rotund* is not expressed more proximally than the distal end of the basitarsus (Shroff *et al.*, 2007), so it is useless for studying t-rows. Another option was to use longer pulses. Pulses lasting 24 h (*vs.* 12 h) did achieve T2→T1 homeoses—*e.g.*, t-rows at –48 to –24 h, –36 to –12 h, and –24 to 0 h BPF (data not shown). Such durations, however, were too long to permit precise delineation of TSPs, and the earlier pulses stymied tarsal growth, hence precluding meaningful measurements.

In the end, upshifts were used instead of pulses to circumvent the problem. *Dll-Gal4/UAS-ScrWT; tub-Gal80ts/+* larvae and pupae were shifted from 18˚C to 30˚C at various times and allowed to complete development (in the absence of any subsequent downshift). Flies of this genotype did not eclose, so they had to be removed from their pupal cases. Forelegs and midlegs (N = 8 of each

per timepoint) were dissected, mounted, and examined. Representative cases are depicted in Figure 4.

GOF kinetics matched LOF curves in some cases. For example, basitarsal t-rows disappear from GOF midlegs at about the time (0-8 h APF; Figure 4h-i) that they reappear on LOF forelegs (0-12 h APF; Figure 2c), though 6 h should be added for parity with pulse midpoints. However, Apical and spur bristles do not reappear until shifts at +8 h APF (5/8 midlegs) and +24 h APF (8/8 midlegs)

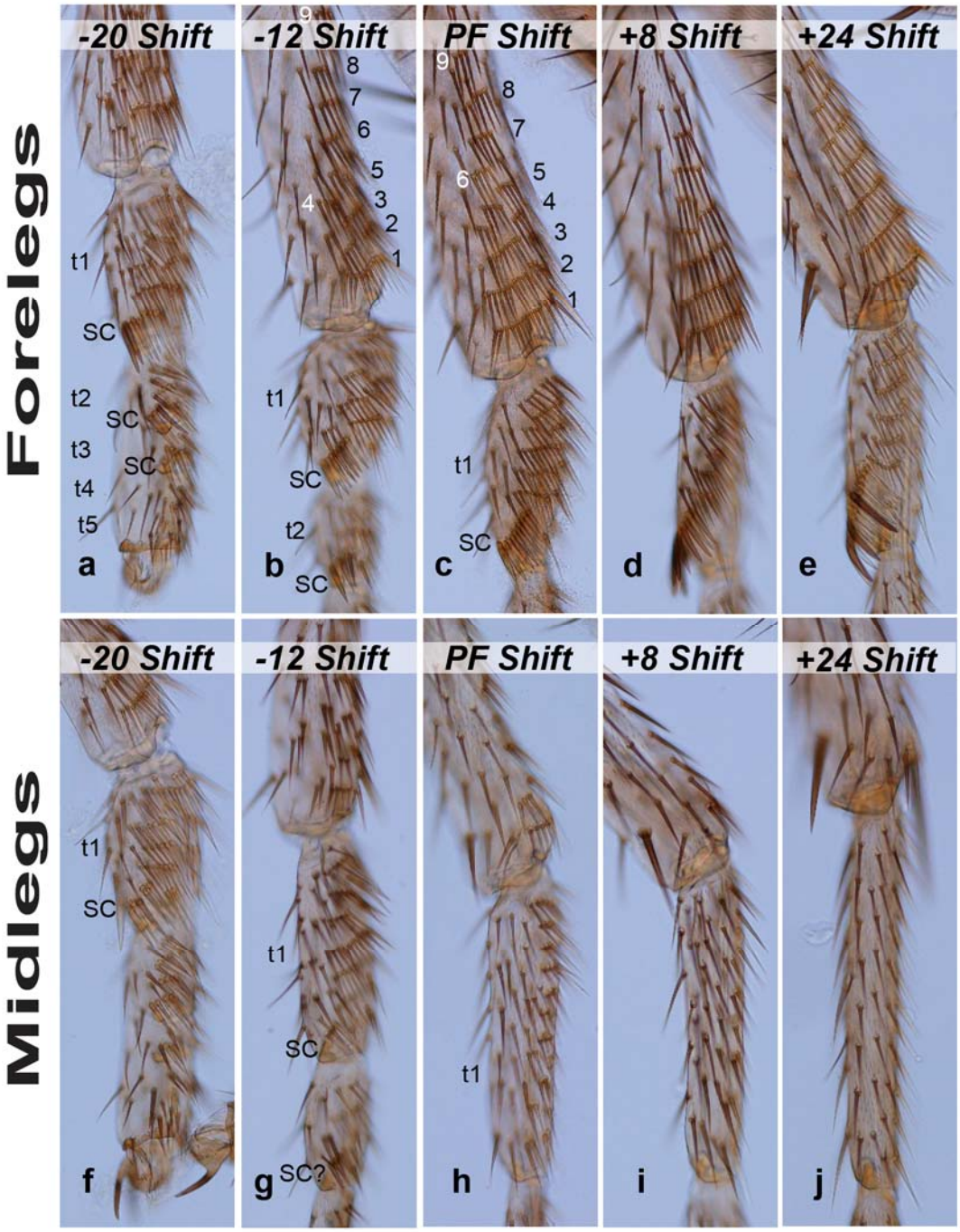

Figure 4 (facing page). Forelegs (above) and midlegs (below) from *Dll-Gal4/UAS-ScrWT; tub-Gal80ts/+* males (pharate adults) upshifted to 30˚C (restrictive temp.) but never subsequently downshifted (as opposed to the pulse series for *Scr*-LOF in Figure 3). For ease of comparison, the images of left legs in **a** and **h** were flipped horizontally to appear as right legs; all others are right legs. Ages at the time of upshift (banner at top) increase from **a** to **e** and from **f** to **j** and are normalized to 25˚C (see Materials and Methods). Minus signs are BPF; plus signs are APF. The foreleg and midleg from each cohort are from the same individual fly. T2→T1 homeosis (gain of t-rows and SC, SC rotation, and loss of Apical and spur bristles) decreases from **f** to **j**. In all legs the bristles on the tarsus and distal tibia (regions of strong *Dll-Gal4* expression) are as yellow and bractless as wild-type foreleg t-rows. In legs from early shifts (**a**, **b**, **f**, **g**), basitarsal t-rows are as wide as tibial t-rows, and segment girth expands proportionally. Bona fide (albeit shorter) t-rows are present (not shown) on the posterior (*sic*) face of 6/8 midlegs in the –20 h BPF cohort and 5/8 midlegs in the –12 h BPF cohort—revealing a surprising ability of *Scr* to induce t-rows at a site typical of hindlegs (a task normally performed by *Ubx*). All legs in Figure 4 were photographed at higher contrast (lower aperture) than those in Figs. 1 and 3 in order to delineate yellow bristles, and brightness was reduced for the same reason. All photos are at the same magnification. **a**. The tarsus is stunted, likely due to overexpression of *Dll-Gal4* at 30˚C (see text). The sex comb (SC) on the basitarsus (t1) has 9 "teeth," though these bristles are thinner and straighter than wild-type teeth. The 2 dorsal bristles are darker than the 7 ventral ones—a polarity seen in this cohort and others. Ectopic sex combs are on distal segments (t2 and t3). Rotation is minimal. Claws are missing. **b**. Excess tibial t-rows (9 total). (Control forelegs have 6.2 rows; N = 10.) The sex comb on t1 has rotated ~45˚; the ectopic comb on t2 by less. **c**. Excess tibial t-rows (9 total). The sex comb has rotated ~60˚. **d**. The curved comb has nearly fully rotated. Distal teeth are darker (*cf.* trend discussed in **a**). **e**. Three of the 13 teeth are brown (but still lighter than wild-type teeth). **f**. The sex comb (SC) on the basitarsus (t1) has 7 "teeth" (see comments about bristle shape in **a**) with a gap in the middle. The maximum number per comb reported for mutant *Scr*-GOF midlegs is 6 teeth (Pattatucci *et al.*, 1991), but it can reach ~16 with a *rotund-Gal4* driver (Barmina *et al.*, 2005). **g**. The basitarsal sex comb has only 3 teeth and has rotated ~55˚. There may be a secondary comb on t2, though the "teeth" are indistict. **h**. Basitarsal t-rows are meager, and only two small t-rows reside on the tibia. **i**. No t-rows are evident on the basitarsus or tibia. **j**. Except for its yellow hue and lack of bracts, this midleg looks wild-type (AB out of focus), but tiny hairs cover its anterior (not posterior) surface. (In wild-type basitarsi hairs only grow near the ventral midline.) This hair anomaly affected 7/8 midlegs in this cohort, but was less obvious in midlegs from earlier shifts. Another oddity seen here is a distinctly lighter color for bristles in the two most ventral longitudinal rows (8/8 midlegs). Three bristles belonging to one of those less pigmented rows are in focus along the right edge at the distal end.

respectively—a day-or-so after they decline on LOF forelegs (Figure 2f-g). To refine the latter GOF data, a +16 h APF shift was also performed: only a single spur bristle was observed among 8 midlegs, whereas every such leg had a well-formed Apical bristle.

Amazingly, virtually all bristles on the tarsus and distal tibia were yellow and bractless on all six legs in males and females, regardless of the time of upshift (–20, –12, 0, +8, or +24 h). Because these traits are diagnostic of tibial t-rows on wild-type forelegs, the latter t-rows must adopt them because of *Scr* alone, not a combination of *Scr* with "area code" genes like *wingless* as proposed by Shroff *et al.* (2007). If *Scr* is sufficient to elicit these properties, however, then why aren't basitarsal t-rows (which are darker than tibial t-rows) and sex comb teeth also yellow and bractless in wild-type flies? Might other genes be modifying *Scr*'s action at those sites?

Perhaps, but there may be a simpler explanation. The thresholds of Scr protein that are needed for the yellow and bractless traits might simply exceed those of all other foreleg features. This "Dosage Hypothesis" makes several testable predictions:

1. *Scr* expression should be greater on the tibia than the tarsus during the relevant critical periods in wild-type flies. Published data cannot decide this issue at present (Barmina and Kopp, 2007).
2. Higher levels of *Scr* should convert basitarsal t-rows into tibial ones in every way, not just in color and bractlessness. Indeed, a total transformation is seen in *Dll-Gal4/UAS-ScrWT; tub-Gal80ts/+* forelegs (and midlegs) upshifted at –20 or –12 h BPF (Figure 4a, b, f, g). Basitarsal t-rows double their width to attain the dimensions of tibial t-rows.
3. Higher levels of Scr should also convert sex combs into tibia-like t-rows. Here the data are equivocal. Foreleg sex combs from the most affected cohorts (–20 and –12 h BPF) are odd in several respects: (1) they have fewer teeth (mean = 7.0 for –20 h BPF, N = 8; and 8.1 for –12 h BPF, N = 8; *vs.* ~11 in the wild type); (2) they rotate less (mean angle = 7˚ and range = 0-45˚ for –20 h BPF, N = 8; and mean = 29˚ and range = 10-50˚ for –12 h BPF, N = 8; *vs.* a constant 90˚ in the wild type); (3) bristles are thinner and straighter than canonical teeth (more common for –20 h BPF than –12 h BPF); and (4) shafts are sometimes deformed (*i.e.*, doubled, missing, or split at their tips: 5% of bristles for –20 h BPF and 11% for –12 h BPF; N = 8 each). The reduced rotation and t-row-like bristle shape are consistent with the hypothesis, whereas the reduced width is not.

Finally, the Dosage Hypothesis might explain why *Scr* appears to be needed over such long periods to prevent bract induction (2.5 days) and melanization (1 day). To wit, recovery from RNAi knockdown might be too slow to permit a full restoration of *Scr* expression to the requisite thresholds by the time that a high dose of Scr protein is needed to elicit these traits.

How *Scr* actually intervenes in the circuitry of bract induction (Layalle *et al.*, 2004) or pigment synthesis (Kopp, 2009) remains unknown. *Hox* genes have insinuated themselves willy-nilly into various levels of gene hierarchies over the eons (Akam, 1998; Stern, 1998; Weatherbee *et al.*, 1998; Castelli-Gair Hombría and Lovegrove, 2003), so it is hard to guess how *Scr* might be operating here. If *Ubx*, which acts via *spineless*, is any guide (Tsubota *et al.*, 2008), then *Scr* might be licensing at least some of its subordinate processes indirectly—*e.g.*, by priming (or blocking) its target genes at an early time t_1 for overt actions at later times t_2, t_3, etc., resulting in various time lags (t_1 to t_2, t_1 to t_3, etc.) for different traits as reflected in the TSPs uncovered here.

One of the most intriguing loci in the fly genome is *yellow* (*y*)—the headquarters for cuticle color (Geyer and Corces, 1987). The Yellow protein mediates melanization (Walter *et al.*, 1991; Wittkopp *et al.*, 2003), though how it acts is still being worked out (True, 2003). Long ago, William Nash (1976) charted a spectrum of TSPs for *ts* alleles of *yellow*, analogous to the graphs for *Scr* here (Figure 2), and he was equally baffled by lags between TSPs and melanization: "In some cases the time of *y* sensitivity for a cuticle structure precedes pigmentation by as much as 24 hr (wing) and in

other cases the time for *y* sensitivity partly overlaps the pigmentation process (sex combs)." Clearly, the relationship between time and space remains as enigmatic in fly genetics as it used to be in astrophysics. Someday *Scr* may solve this colorful conundrum as well as the much deeper mystery of how *Hox* genes tinker with anatomy.

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Availability of the University of Texas Publications Dealing with *Drosophila*

Marshall R. Wheeler

From 1940 to 1972 many research articles were published by the University Press in the series, "Studies in the Genetics of *Drosophila*" with J.T. Patterson as editor and later (from 1957-1972) with M.R. Wheeler as editor. In 1960 the series title was changed to "Studies in Genetics." There were also a few special issues. Many of these are now out of print (OOP); all known copies of the remaining issues have been made available by Dr. Wheeler. The copies are available from the office of the Editor, *Drosophila Information Service*; contact Dr. James N. Thompson, jr., (jthompson@ou.edu) for details.

Some issues were given titles and subtitles, but the Publication Number (*e.g.*, UTP 4213) is the best reference. This is the complete list of all the publications:

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