



When does the *spiny legs*¹ allele of the *prickle* gene cause extra joints?

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Wild-type flies have four ball-and-socket joints that divide each tarsus into five segments (Tajiri *et al.*, 2010). Mutations in genes of the planar cell polarity (PCP) pathway—*e.g.*, *frizzled* and *dishevelled*—can cause extra joints that, curiously, are upside-down (Held *et al.*, 1986). The most extreme phenotype occurs with the *spiny legs*¹ allele of the *prickle* gene (*pk*^{*sple*1}) (Tree *et al.*, 2002). This mutation evokes an extra, inverted joint in tarsal segments T2, T3, and T4, plus a partial extra joint in T1. Although much is known about how PCP genes enforce cell polarity (Goodrich and Strutt, 2011), little is known about how they induce extra joints (Bishop *et al.*, 1999; Held, 2005).

The present study investigates the timing of *prickle* gene action in joint formation. Does *pk*^{*sple*1} induce all of its extra joints simultaneously or sequentially? Does it affect bristle polarity at the same time that it affects joint formation? Does it act before or after puparium formation?

Materials and Methods

The *pk*^{*sple*1} allele (henceforth called *sple*¹) was experimentally suppressed at different times by expressing the wild-type *sple* isoform (here called *sple*^{WT}) of the *prickle* gene (Lin and Gubb, 2009) via the *Gal4-Gal80* technique (McGuire *et al.*, 2003). This isoform was linked to the upstream activating sequence (UAS) of the transcription factor Gal4, which was ubiquitously expressed via the *daughterless* locus (*da-Gal4*). Both constructs were inserted into a third chromosome (Gubb *et al.*, 1999) kindly supplied by David Gubb. Expression of the *Gal4* gene was manipulated by a temperature-sensitive allele of *Gal80ts*, which inhibits *Gal4* at 18°C but not at 30°C (McGuire *et al.*, 2004).

Because *sple*¹/*sple*¹; *da-Gal4 UAS-sple*^{WT}/*TM3, Ser* males proved to be infertile, experimental and control individuals were obtained as F₁ offspring by crossing males from a *sple*¹/*CyO*; *da-Gal4 UAS-sple*^{WT}/*TM3, Ser* stock (barely fertile) with females from a *sple*¹/*sple*¹; *Gal80ts/Gal80ts* stock (quite fertile). Curly-winged (*sple*¹/*CyO*; *da-Gal4 UAS-sple*^{WT}/*Gal80ts*) siblings were scrutinized for any artifactual side-effects of the *da-Gal4 UAS-sple*^{WT} constructs, but their legs looked wild-type (data not shown). Serrated-wing siblings whose legs had an *sple*¹ phenotype (*sple*¹/*sple*¹; *Gal80ts/TM3, Ser*) were examined for any extraneous effects of *Gal80ts*, but none were found at either temperature.

Flies were raised on Ward's *Drosophila* Instant Medium plus live yeast. Since females did not oviposit at 30°C, eggs were collected daily at room temperature (22°C), whereupon the food vials were placed at the starting temperature (18°C or 30°C). Nutrition was optimized (and overcrowding minimized) to prevent retardation that could affect staging. Legs were dissected in 70% ethanol, placed in gum arabic fluid (Faure's) between cover glasses (Lee and Gerhart, 1973), and examined at 200× in a Nikon microscope. Females were used unless stated otherwise. Sample sizes for each time point were 6 legs (left and right midlegs from 3 flies), except the cohorts upshifted at PF (N = 12 legs) and downshifted at -15 hBPF (N = 4 legs). Presence or absence of joints was scored based on the ball and socket components of the hinge alone, without regard to the completeness of any associated intersegmental membrane.

For ease of comparison, all times have been recalibrated to the standard staging scale at 25°C (Ashburner, 1989) as explained previously (Held, 2010). Specifically, actual times at 18°C were divided by 2.0 (Held, 1990) to yield the times reported here ("@25°"). For ease of viewing, the images of left legs (Figures 1a, 1c, 2d, 3a, 3b, and 3f) were flipped (in *Adobe Photoshop®*) so as to appear as right legs. Abbreviations include: PF (puparium formation), BPF (before PF), APF (after PF), h (hours), US (upshifted), DS (downshifted), TSP (temperature-sensitive period), fem (femur), tib (tibia), and bas (basitarsus), which is synonymous with tarsal segment T1. Because the maximal expression of the extra joint in T1 is only partial even in *sple¹* homozygotes (Held *et al.*, 1986), that joint was excluded from this analysis.

For shifts at PF, white prepupae were collected (synchrony = +/- 0.5 h). For shifts BPF, vials of larvae were shifted, and the resulting pupae were harvested at 12-h intervals (actual time), recalibrated as 6-h intervals @25°C for downshifts (ages varying +/- 3 h) or reported unaltered as 12-h intervals @25°C for upshifts (ages varying +/- 6 h). For shifts APF, pupae were harvested at 12-h intervals (actual time) at the starting temperature, kept in moist petri dishes, and transferred to the other temperature when the desired age was reached. Larvae and pupae were handled as described previously (Held, 2010).

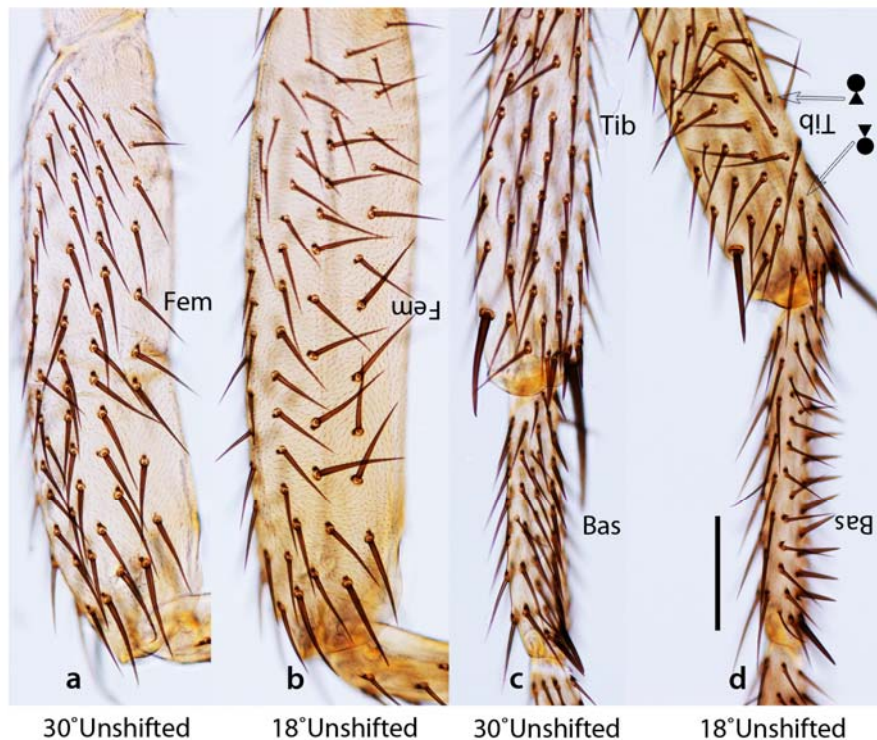


Figure 1. **a, c.** Femur (Fem), tibia (Tib) and basitarsus (Bas) from a control female raised at 30°C. **b, d.** Corresponding leg segments from a control female raised at 18°C. The 18°C leg displays a patch of reversed bristles on each segment. These misorientations are also evident (**d**) in the polarity of the bristle sockets (solid circles) relative to their associate "bracts" (Hannah-Alava, 1958) (solid triangles). Upside-down segment names (**b, d**) are used to denote reversed-bristle phenotypes (Figures 2, 3, and 4). In all panels here and elsewhere, the anterior face is depicted, with proximal at the top and distal at the bottom, dorsal to the left and ventral to the right (except Figure 4, where the leg is rotated 90° clockwise). All photos are at the same magnification (scale bar in **d** = 100 microns).

Results

Flies of the genotype *sple¹/sple¹; da-Gal4 UAS-spleWT/Gal80ts* were exposed to various temperature regimens. Control flies were raised entirely at 18°C or 30°C. At 18°C, *Gal80ts* disables *Gal4* so that the *spleWT* allele linked to *UAS* cannot suppress *sple¹*, leading to extra joints and misoriented bristles (Figures 1b, 1d, and 2f). At 30°C, *Gal80ts* malfunctions, so *Gal4* activates *UAS*, hence expressing *spleWT* and suppressing *sple¹*, yielding legs that look wild-type (Figures 1a, 1c, and 2a). The ability of *spleWT* to rescue *sple¹* to 100% penetrance and expressivity allows partial phenotypes to be assessed with high precision. Experimental flies of the same genotype were upshifted (18°C to 30°C) or downshifted (30°C to 18°C) at different times.

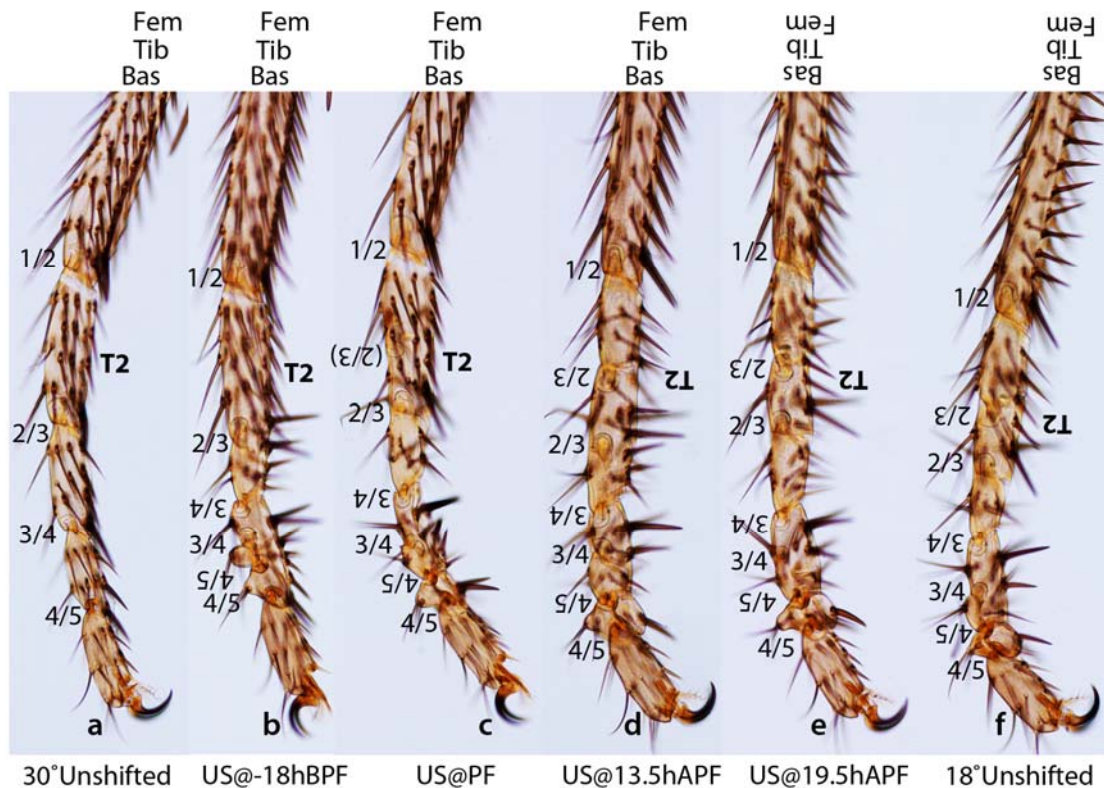


Figure 2. Upshift results. **a.** Tarsus from a control ("unshifted") fly raised entirely at 30°C. **b-e.** Tarsi from flies upshifted (from 18°C to 30°C) at various times (normalized to 25°C) before (BPF) or after (APF) pupariation (see Materials and Methods). **f.** Tarsus from a control ("unshifted") fly raised entirely at 18°C. Upside-down numbers denote inverted joints (2/3, 3/4, 4/5), while upside-down names indicate reversed bristles on particular segments (T2, Bas, Tib, Fem).

Upshifts (Figure 2). Upshifts restore *spleWT* function. They rescue T2 to a wild-type state at -18 hBPF (Figure 2b) but not later: this rescuing ability wanes at PF (Figure 2c; extra joint in 75% of legs) and disappears by 13.5 hAPF (Figure 2d). Evidently, the extra joint in T2 is irreversibly induced by *sple¹* at or near PF. The extra joints in T3 and T4 must be induced much earlier (before -18 hBPF) because upshifts cannot rescue those segments even at -18 hBPF (Figure 2b). Bristle

polarity can be rescued in the basitarsus, tibia, and femur through 13.5 hAPF (Figure 2d) but no later (Figure 2e), implying that *sple*¹ firmly reorients bristles there between 13.5 and 19.5 hAPF.

Downshifts (Figure 3). Downshifts remove *sple*^{WT} function. The later the downshift, the more normal the leg, because *sple*^{WT} has had sufficient time to suppress *sple*¹ in the affected legs areas. The order in which segments lose their extra joint is: T2 (before -51 hBPF, where 2/6 legs showed an extra joint), T3 (-51 to -27 hBPF), and T4 (-21 to -15 hBPF). Subsequently, segments are cured of their reversed bristles: T2 and basitarsus (-15 hBPF to PF), then tibia and femur (PF to 18 hAPF). Although tibial bristle polarity *per se* is restored at PF, residual bristles in its "hot spot" still display bracts on the distal (abnormal) side of the socket (not shown).

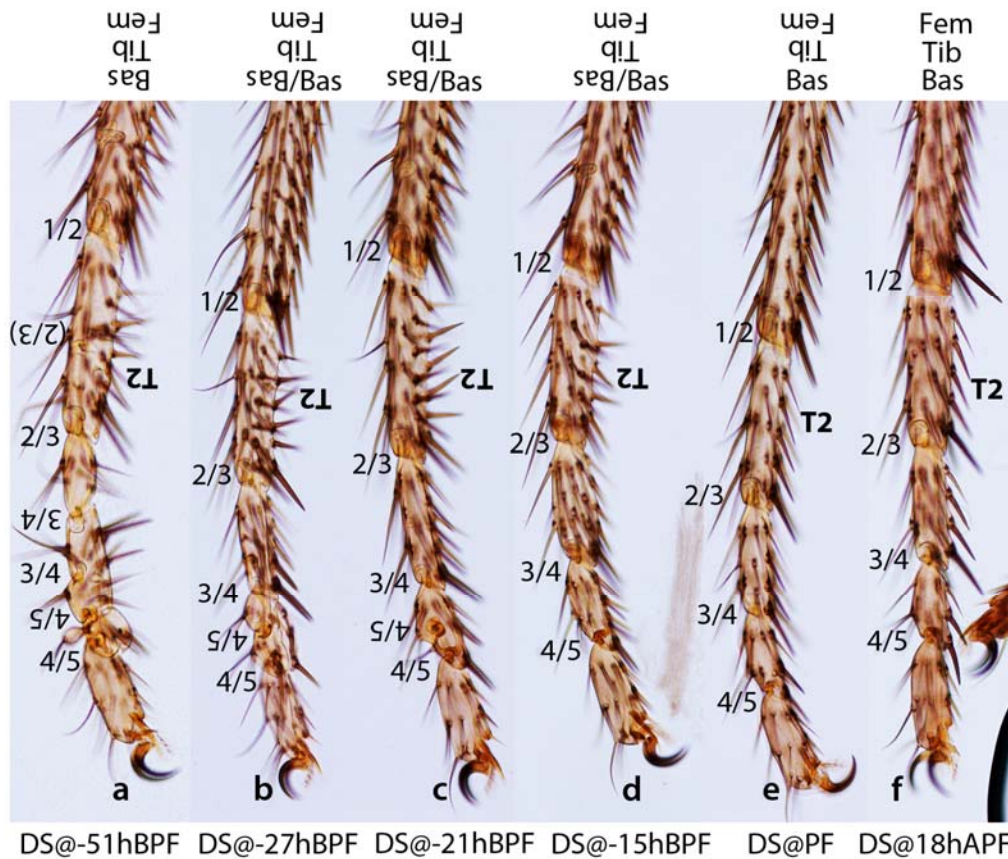


Figure 3. Downshift results. Tarsi from flies downshifted (from 30°C to 18°C) at various times (normalized to 25°C) before (BPF) or after (APF) pupariation (see Materials and Methods). Upside-down numbers denote inverted joints (2/3, 3/4, 4/5), while upside-down names indicate reversed bristles on particular segments (T2, Bas, Tib, Fem). Heterogeneous basitarsal phenotypes (some tarsi normal, others abnormal) are denoted by "inverted Bas/right-side-up Bas".

Discussion

Figure 4 plots these results along a time line. One conclusion to be drawn is that bristles and joints are affected differently in time. Downshifts cure the extra-joint defect (-51 to -15 hBPF) before bristle polarity (-15 hBPF to 18 hAPF). Extra joints are induced more than a day and a half before

overt histological differentiation of normal joints, which occurs at 24-38 hAPF (Mirth and Akam, 2002), but bristle polarity is affected shortly before elongation of bristle shafts (Graves and Schubiger, 1981) and cuticle deposition (Reed *et al.*, 1975) at 36 hAPF. That the extra joint in T2 is initiated before the 3rd instar, which begins circa -48 hBPF, is startling.

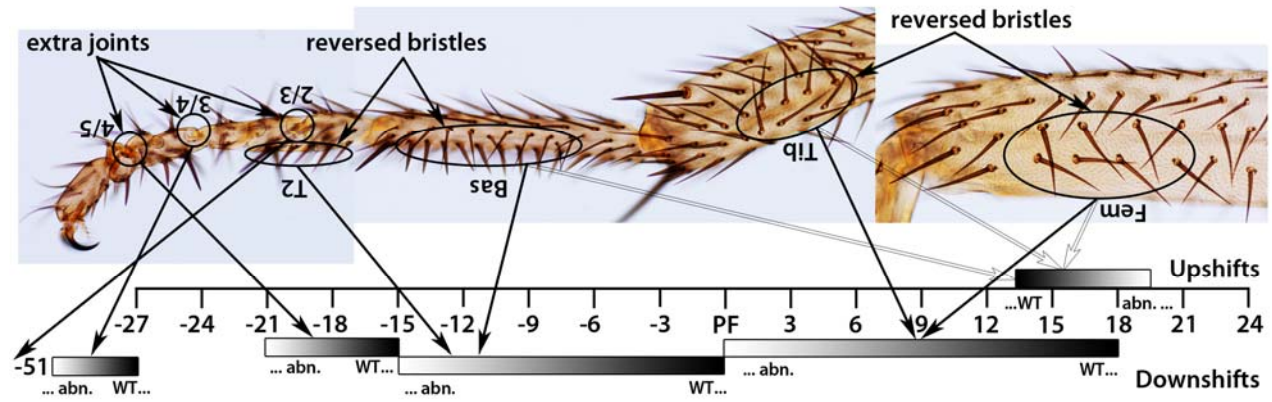


Figure 4. Stages (bars) when traits (circled) start to manifest mutant phenotypes (upshifts) or return to normal (downshifts) plotted along a time axis. Gradient shading shows the transition from abnormal (abn.) to wild-type (WT) or vice versa. Arrows point to bars (*i.e.*, time spans), not to specific times within these ranges. Outlined arrows for upshifts and solid arrows for downshifts. Upside-down numbers denote inverted joints (2/3, 3/4, 4/5), while upside-down names indicate reversed bristles on particular segments (T2, Bas, Tib, Fem). Omitted is the transition from normality to abnormality (upshift) for the extra joint phenotype in T2 (-18 hBPF to PF; see text).

Secondly, bristle polarity is restored in a distal-to-proximal sequence (T2 & Bas before Tib & Fem) that is consistent with the order in which bristles develop along the leg axis (Graves and Schubiger, 1981), whereas extra joints disappear in an opposite, proximal-to-distal, sequence (T2, then T3, then T4). Previous studies failed to establish a clear order for normal joint formation (de Celis *et al.*, 1998; Rauskolb, 2001; Mirth and Akam, 2002), so we cannot know whether this extra-joint sequence conforms to the normal joint sequence.

The temporal uncoupling of bristle polarity from joint formation is further evident in the three time points (-27, -21, and -15 hBPF) when reversed bristles persist in T2 despite a lack of even the slightest remnant of an extra joint in that segment (Figure 3b-d). Of course, a comparable genetic dissociation was already evident in the ability of *sple*¹ to cause bristle reversals on the femur and tibia in the absence of any extra joints at those locations (Held *et al.*, 1986).

Note that the time spans delineated by bars in Figure 4 are not temperature-sensitive periods (TSPs). Rather, they represent the starts or ends of TSPs. The start of a TSP is classically defined as the first shift that yields the mutant trait, with the endpoint defined as the first shift that restores the wild-type phenotype (Suzuki, 1970). Rephrasing this formula in terms of the *Gal80ts* conditions used here, the start would be the first upshift that yields the *sple*¹ trait, with the endpoint being the first downshift that restores the wild-type phenotype.

Paradoxically, trying to apply these criteria to the extra joint in T2 yields a start time (-18 hBPF to PF) later than the endpoint (before -51 hBPF). Likewise, trying to define a TSP for bristle polarity on the tibia and femur yields an onset (13.5 to 19.5 hAPF) later than, albeit overlapping with, the terminus (PF to 18 hAPF).

In an effort to resolve this dilemma, pulse experiments were performed. Flies of the usual genotype (*sple¹/sple¹*; *da-Gal4 UAS-spleWT/Gal80ts*) were exposed to a 24-h pulse of 30° (upshift, then downshift back to 18°C) at one of three consecutive times (N = 6 legs each). With a pulse in mid-third instar (-36 to -12 hBPF), flies lost their extra joints in T2 and T3 but retained the extra joint in T4. With a pulse that straddled PF (-12 hBPF to 12 hAPF) they lost the extra joint in T2 but retained extra joints in T3 and T4. And with a later pulse (12 to 36 hAPF), all extra joints were present. The first pulse failed to cure any bristle polarity defects, the second rescued T2 and the basitarsus, and the third cured T2, the basitarsus, the tibia and femur.

The bristle-polarity effects of the pulses were reassuring. They conform to the expected sequence based on the downshift bars in Figure 4, though the third pulse (12 to 36 hAPF) should not have cured T2 and the basitarsus if their TSP ends at PF. The main problem is the joint data. They imply that the TSP for T3 ends before that for T2, which contradicts the downshift order (T2 before T3). Moreover, they show that the extra joint in T2 can be eliminated by a pulse (-36 to -12 hBPF) that begins after -51 hBPF, when the TSP for that trait was supposed to have ended.

Part of the explanation for these inconsistencies may be artifactual peculiarities of the *Gal4-Gal80* method itself. When a wild-type allele of *Ubx* is ectopically expressed in the wing using this technique, *Ubx* protein is detectable by 6 h after an upshift, but its level continues to rise to a plateau at 16 h and does not peak until 24 h (Pavlopoulos and Akam, 2011). If the same is true for upshifts that activate *spleWT*, then the times in Figure 4 must be recalibrated substantially. For downshifts a complicating factor is the unknown perdurance of *spleWT* mRNA and protein. Finally, certain joints may need higher thresholds of the *spleWT* isoform than others in order to be suppressed. Further work will be required to disentangle these confounding variables.

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