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Metamorphic labral axis patterning in the beetle *Tribolium castaneum* requires multiple upstream, but few downstream, genes in the appendage patterning network

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Abstract

The arthropod labrum is an anterior appendage-like structure that forms the dorsal side of the preoral cavity. Conflicting interpretations of fossil, nervous system and developmental data have led to a proliferation of scenarios for labral evolution. The best supported hypothesis is that the labrum is a novel structure that shares development with appendages as a result of co-option. Here, we use RNA interference in the red flour beetle *Tribolium castaneum* to compare metamorphic patterning of the labrum to previously published data on ventral appendage patterning. As expected under the co-option hypothesis, depletion of several genes resulted in similar defects in the labrum and ventral appendages. These include proximal deletions and proximal-to-distal transformations resulting from depletion of the leg gap genes *homothorax* and *extradenticle*, large-scale deletions resulting from depletion of the leg gap gene *Distal-less*, and smaller distal deletions resulting from knockdown of the EGF ligand *Keren*. However, depletion of *dachshund* and many of the genes that function downstream of the leg gap genes in the ventral appendages had either subtle or no effects on labral axis patterning. This pattern of partial similarity suggests that upstream genes act through different downstream targets in the labrum. We also discovered that many appendage axis patterning genes have roles in patterning the epipharyngeal sensillum array, suggesting that they have become integrated into a novel regulatory network. These genes include *Notch*, *Delta*, and *decapentaplegic*, and the transcription factors *abrupt*, *bric à brac*, *homothorax*, *extradenticle* and the paralogs *apterous a* and *apterous b*.

Keywords

Tribolium castaneum; Coleoptera; co-option; labrum; epipharynx; sensilla; metamorphosis; RNA interference; Notch signaling; leg gap genes

INTRODUCTION

The arthropod labrum is an appendage-like structure that develops in the anterior head and functions as an upper lip, covering the dorsal side of the ventrally located preoral cavity. Such a structure occurs in all extant arthropods except sea spiders (pycnogonids), which bear their mouth terminally on a specialized rostrum (Brenneis et al. 2011). Extensive debate has surrounded most aspects of labrum evolution (e.g., Bitsch and Bitsch 2007; Budd 2002; Rempel 1975). Data from the fossil record (e.g., Budd 2002; Walossek and Müller 1990), innervation (Bitsch and Bitsch 2007, 2010; Boyan et al. 2002, 2003; Harzsch 2006) and

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development (e.g., Haas et al. 2001a, b; Kimm and Prpic 2006; Popadi et al. 1998; Posnien et al. 2009; Schmidt-Ott et al. 1994, Urbach and Technau 2003) have all been considered in evaluating hypotheses for the evolution of the labrum. The labrum has been proposed to be homologous across euarthropods (e.g., Budd and Telford 2009) or, alternatively, to have evolved independently in e crustaceans and chelicerates (e.g., Eriksson and Terfelt 2012; Liu et al. 2009; Walossek and Müller 1990). It has been proposed to be serially homologous to the ventral appendage pairs (Boyan et al. 2002, 2003; Eriksson et al. 2010; Kimm and Prpic 2006; Popadi et al. 1998) or to only the endites of the ventral appendages (Haas et al. 2001). Alternatively, the labrum may be a novel outgrowth of the body wall (Haug et al. 2012; Maas et al. 2003; Walossek and Müller 1990; Waloszek et al. 2007) or digestive tract (Bitsch and Bitsch 2010; Liu et al. 2009). The question of labral homology is closely connected to the question of how it originated—through transformation of an ancestral appendage pair or as a novel structure.

Developmentally, the labrum originates from the most anterior part of the head in insects, including the red flour beetle *Tribolium castaneum* (Posnien et al. 2009) and the fruit fly *Drosophila melanogaster* (Schmidt-Ott et al. 1994). Subsequent posterior-ventral migration leads to the ventral position of the labrum and mouth (Khila and Grbi 2007) and may also account for the tritocerebral connections of its stomatogastric innervation (Bitsch and Bitsch 2010; but see Harzsch 2006). Embryologically, in many species the labrum arises as two lobes that resemble embryonic appendage primordia, and the unpaired labrum forms by fusion of these lobes (e.g., Khila and Grbi 2007; Kimm and Prpic 2006; Liu et al. 2009; Posnien et al. 2009; reviewed in Scholtz and Edgecombe 2006). Embryonic gene expression patterns are similar in the developing labrum and ventral appendages, and these data have generally been interpreted as supporting the appendicular nature of the labrum and thus serial homology of the labrum and more posterior ventral appendages (Kimm and Prpic 2006; Popadi et al. 1998; Prpic et al. 2001). In particular, the leg gap genes *Distal-less* (*Dll*), *dachshund* (*dac*), *homothorax* (*hth*) and *extradenticle* (*exd*) are expressed in discrete domains along the proximodistal axis of the ventral appendages and labrum in insects (Angelini and Kaufman 2004; Jockusch et al. 2004; Mittmann and Scholtz 2001; Popadi et al. 1998; Posnien et al. 2009; Prpic et al. 2001), myriapods (Prpic and Tautz 2003) and chelicerates (except that labral *dac* expression is absent in some; Barnett and Thomas 2013; Khila and Grbi 2007; Pechmann and Prpic 2009; Prpic et al. 2003; Sharma et al. 2012).

An alternative to the appendicular hypothesis for the origin of the labrum is the hypothesis that the labrum is a novel non-appendicular outgrowth of the digestive tract or body wall. This hypothesis has received strong support from a recent comparison of embryonic development of the labrum and ventral appendages (Posnien et al. 2009). The position of the labral primordia differs fundamentally from that of the appendage primordia, violating a major criterion for serial homology. In the absence of serial homology, similarity of developmental patterning in the labrum and ventral appendages is explained by co-option of a gene regulatory network (Posnien et al. 2009). To date, most comparisons of labral and appendage development have focused on gene expression; functional data are much more limited. However, some studies of co-opted gene networks have revealed greater similarity in gene expression than in gene function (e.g., Moczek and Rose 2009).

Here, we use RNA interference (RNAi) to analyze the function of appendage axis patterning genes during labral metamorphosis in the red flour beetle *Tribolium castaneum*. In holometabolous insects including *Tribolium*, appendage patterning is separated into two stages, embryogenesis and metamorphosis. We focus our comparisons on metamorphosis for two reasons. First, appendage patterning has been studied in detail in *D. melanogaster*, and many of the downstream components of the gene regulatory network governing this process are active in the last larval instar, immediately prior to metamorphosis. Second,

RNAi-based analyses of metamorphic gene function are available for the ventral appendages of *T. castaneum* (Angelini et al. 2009, 2012a, b; Aspiras et al. 2011).

Our functional analyses in *T. castaneum* identify a labral axis patterning role for several upstream components of the appendage axis patterning pathway, including Decapentaplegic (Dpp) signaling and the leg gap genes *Tc-Dll*, *Tc-hth* and *Tc-exd*. By contrast, we failed to detect an axis patterning role for the leg gap gene *dac* and multiple genes that function downstream of the leg gap genes in *D. melanogaster*, including several with roles that are broadly conserved across arthropod appendages. This pattern of partial similarity between the labrum and ventral appendages reveals both ways in which development of these non-homologous structures has been evolutionarily linked as a result of co-option and ways in which it has evolved independently. Finally, many of the genes examined were required for patterning of the sensory array on the ventral side of the labrum in *T. castaneum*. These include the neurogenic genes *Notch* (*Tc-N*) and *Delta* (*Tc-Dl*), *Tc-dpp* and multiple transcription factors, including *abrupt* (*Tc-ab*), the paralogs *apterous a* and *apterous b* (*Tc-ap a/Tc-ap b*), *bric à brac* (*Tc-bab*), *Tc-hth* and *Tc-exd*.

METHODS

Tribolium castaneum was obtained from Carolina Biological Supply Company. Methods for rearing beetles, RNA interference (RNAi), and clearing of specimens have been described previously (Angelini et al. 2009, 2012a, b). Table 1 lists the genes examined, size of dsRNA constructs, and penetrance in ventral appendages. Angelini et al. (2012b) quantified the magnitude of knockdown for most of these genes using qRT-PCR; it ranged from 24% (for *Tc-N*) to 75% (for *Tc-Dll*). For most genes, specimens with moderate to strong phenotypes in other appendages were selected for closer examination of labral phenotypes. For some genes, few specimens with specific appendage defects were available; for these, a subset of specimens was chosen haphazardly. A minimum of nine individuals per RNAi treatment were examined for defects in the labral and distal sclerites, proximal support rods, setal patterns, and epipharyngeal sensillum array. Normal labral morphology was characterized based on a combination of wild type, buffer-injected and *gfp* RNAi specimens.

The labrum was dissected off of cleared specimens and examined under Nomarski optics. Images were taken with an Optronics Magnafire digital microscope camera mounted on a Zeiss Axioscope Z plus microscope and edited in Adobe Photoshop. For some figure panels, multiple images were shot in different focal planes and then combined using the Auto-Align Layers and Auto-Blend Layers functions in Adobe Photoshop CS4. For most genes in which defects were observed, a small set of specimens (usually three) were prepared for scanning electron microscopy (SEM), as were six control specimens subjected to RNAi using *gfp* dsRNA. Whole heads or dissected labra were rinsed in 100% ethanol three times over a 24-hour period, then dehydrated with a hexamethyldisilazane treatment, mounted on pin stubs with carbon tape, and sputter coated with gold palladium. All SEM images were taken on a Zeiss DSM982 Gemini field emission scanning electron microscope.

RESULTS

Morphology of the Wild Type Labrum and Epipharynx

Morphology of the labrum has been described for *Tribolium confusum* (El-Kifl 1959); the *T. castaneum* labrum is similar. The labrum lacks joints and internal muscles. A large sclerite comprises the majority of the dorsal side of the labral region. Its proximal edge is fairly straight; the lateral edges are convexly rounded, and the distal edge is concave, resulting in two protruding lobes (Fig. 1A–B, 2A). The dorsal cuticle is scalloped and unpitted, with short setae regularly spaced across the distal half of the sclerite (Fig. 1A). The lateral and

distal edges of the sclerite bear long setae. Distally and ventral to this labral sclerite, a much smaller sclerite sits between the protruding lobes (Fig. 1B); it is covered by long, stout setae arranged in tightly packed rows.

The ventral side of the labrum forms the epipharynx, which, with the exception of the support structures, is unsclerotized. The proximal edge of the labral sclerite is attached to the sclerotized portion of the epipharynx from which four sclerotized rods project. Two rods extend posteriorly along the lateral edges of the epipharynx. Two other rods, the tormae, extend medially; these adjoin a V-shaped structure that is covered with a dense patch of sclerotized hairs (Fig. 1B). Laterally, the epipharynx is covered with setae: long, broad setae are densely packed near the distal end, while shorter, thinner more widely-spaced setae form paired lateral patches. The proximal edge of these setal patches is flanked by a narrow region of distinctly scaled cuticle.

Between the two setal patches on the epipharynx is an array of sensilla containing three distinct morphological types as visualized with Nomarski optics (Fig. 1C–D, 3A). Proximally, there is a regularly arranged row of 11–17 (14.5 ± 1.6 , average $\pm$ standard deviation; $n=27$ specimens) small, closely packed sensilla. The center of each sensillum contains a barely distinguishable peg. These sensilla are internal and not visible with SEM (Fig. 3B). Their arrangement, morphology and location suggest they function as pressure receptors. Morphologically similar sensilla also form the distal end of the array. These number 2–10; they are widely spaced and variably arranged. Between the proximal and distal sensilla is a set of 12–15 (13.7 ± 0.8 ; $n=12$ specimens) large, evenly spaced coeloconic sensilla. Each has a broad conical peg, which sits in a pit and terminates in an apical pore (Fig. 3B). The array also includes 9–15 (12.3 ± 1.7 ; $n=12$ specimens) small trichoid sensilla; most of these are located between the proximal sensillum row and the coeloconic sensilla. A small number (0–2) of small internal sensilla may also be intermixed among the coeloconic sensilla.

The adult labrum forms by metamorphosis of the larval labrum. The larval labrum is composed of only a single large sclerite that resembles the adult labral sclerite in shape, except that it lacks the terminal indentation (Fig. 1E–F). The sclerotized proximal supports are more medial and less extensive than in adults. Distally, the labrum is fringed with approximately 8–10 evenly spaced setae both dorsally and ventrally. There is also a single long seta located centrally on the dorsal surface of each half of the labrum. Ventrally, two types of sensilla are distinguishable. One type resembles the adult coeloconic sensilla. This type occurs in four discrete regions. The second type resembles the proximal row sensilla of adults; a small number (ca. 4) are arranged in a central row. Trichoid sensilla are absent. In *D. melanogaster*, some of the larval labral sensilla persist through metamorphosis to become adult sensilla (Gendre et al. 2004); this has not been studied in *T. castaneum*.

Sclerite Patterning

Appendage gap genes—We examined the effects on labral morphology of RNAi-mediated knockdown of the four leg gap genes, transcription factors that pattern large regions of the proximodistal appendage axis: *Tc-Dll*, *Tc-dac*, *Tc-exd* and *Tc-hth*. We did not detect alterations in labral morphology in response to RNAi targeting *Tc-dac* (data not shown), even in specimens that showed strong phenotypes in other appendages. By contrast, knockdown of *Tc-hth*, *Tc-exd*, or *Tc-Dll* resulted in extensive labral defects. Phenotypes resulting from *Tc-hth* or *Tc-exd* knockdown (Fig. 2B–E) were very similar, as expected given that in *Drosophila* these proteins form a heterodimer before translocation to the nucleus (Rieckhof et al. 1997; Ryoo et al. 1999). All regions except the distal sclerite were affected. The labral sclerite was smaller and altered in shape; it became narrower with a more convex proximal edge. From the placement of the epipharyngeal setal patches, it

appeared that this sclerite was greatly truncated laterally. In the strongest *Tc-hth* and *Tc-exd* phenotypes, the labral sclerite clearly resembled the distal sclerite in shape, although it remained larger. It also had a distal fringe of thick setae, like those on the distal sclerite, and had lost most short bristles (Fig. 2D). Proximal support rods were entirely deleted. In moderate phenotypes, distolateral extensions of the labral sclerite were present (Fig. 2B); it is unclear if these represent remnants of the reduced sclerite or fused proximal support rods. In both moderate and severe phenotypes, the V-shaped epipharyngeal rods were highly reduced or absent, but the sclerotized hairs remained. The extent of exposed epipharyngeal wall increased both distally and laterally, indicating that the epipharynx was not reduced to the same degree as the labral sclerite. The alignment of features on the dorsal and ventral sides of the labrum was also altered, with ventral (epipharyngeal) structures found more distal relative to dorsal structures than in wild type specimens.

A phenotypic series was also observed in response to *Tc-Dll* depletion, with phenotypes ranging from a single reduced sclerite (Fig. 2F, I) to paired sclerotized structures (which in some specimens appeared tubular rather than flat; Fig. 2G) to complete deletion of both sclerites and the proximal support structures, leaving a reduced, untanned region of the epipharynx (Fig. 2H). The tormae were most resistant to *Tc-Dll* knockdown, but were reduced in the most severely affected specimens. As in *Tc-hth* and *Tc-exd* RNAi specimens, in *Tc-Dll* RNAi specimens the sclerotized hairs associated with the V-shaped rods remained.

Other genes—RNAi-mediated knockdown of the single *T. castaneum* TGF- α type EGF ligand, which we call *Keren* (*Tc-Krn*) on the basis of highest sequence similarity to *D. melanogaster Krn*, also produced major alterations to overall labral morphology (Fig. 2J). In response to *Tc-Krn* RNAi, the labral sclerite was substantially narrower and the distal sclerite was deleted. As a result, a larger region of unsclerotized epipharynx was exposed dorsally. Loss of the distal sclerite was also observed in a small fraction (2 of 16) of *Tc-N* RNAi specimens (Fig. 4C).

Knockdown of *Tc-dpp*, which encodes a secreted signaling ligand, led to changes in the shape of the labral sclerite. In the most severe phenotypes, the labrum lacked proximal constrictions and formed a half circle in outline (Fig. 2K). There was substantial additional tissue centrally, producing more deeply indented terminal lobes. These alterations were accompanied by a change in the three-dimensional structure, such that the labrum of *Tc-dpp* RNAi specimens bowed out dorsally, rather than lying flat (Fig. 2L).

Relatively subtle effects were observed in response to knockdown of the *odd-skipped*-related transcription factor encoded by *drumstick* (*Tc-drm*, Fig. 2M). The labral sclerite had a shortened proximodistal axis, was relatively wider, and reached its maximum width more proximally. Shortening of the tormae was accompanied by wider separation at the base of the V-shaped rods. The lateral rods also extended at a different angle. Simultaneous knockdown of the paralogous transcription factors *Tc-ap a* and *Tc-ap b* led to alterations in or loss of the proximal V-shaped rods and their sclerotized hairs and to expansion of the medial ends of the tormae (Fig. 2N).

The labral sclerites and sclerotized proximal support regions appeared to retain their normal size and shape in response to knockdown of most other appendage patterning genes we examined, including the Notch ligands *Serrate* and *Tc-Dl* and the transcription factors *Tc-ab*, *aristaleless* (*Tc-al*), *Tc-bab*, *clawless* (*Tc-cll*), *Tc-dac*, *pdm/nubbin*, *rotund* (*Tc-rn*), *spalt*, and *spineless* (*Tc-ss*; data not shown). All of these genes caused defects elsewhere in the organisms (Table 1), and several also caused sensory defects in the labrum.

Sensory Patterning

Notch signaling pathway—Knockdown of the neurogenic genes *Tc-N* and *Tc-Dl* led to major changes in the number of sensilla and setae. In response to *Tc-Dl* knockdown, the most penetrant phenotype was an increase in the number of proximal row sensilla (Fig. 3C), observed in 14 of 21 specimens. Ten specimens had >30 proximal sensilla; the row also became disorganized and expanded laterally to form patches. The most extreme increase in number of proximal sensilla (to 50+ in two specimens) was accompanied by the most extreme increase in number of distal sensilla (to 12 and 23) and a substantial increase in number of trichoid sensilla (to 20 or more; Fig. 3C), indicating that regulation of these is coordinated. An increased number of these latter two sensillum types was also observed in additional specimens with a smaller increase in the number of proximal sensilla. In three of the six specimens with a wild type number of proximal sensilla, the sensilla had an abnormal, patchy arrangement. The number of coeloconic sensilla was never increased beyond the maximum observed in wild type specimens, but was often decreased (to as few as 8). Even when the number of coeloconic sensilla was within the wild type range, their arrangement was often more irregular, with some beginning to fuse together (Fig. 3D, box). Finally, in a single specimen, a severe reduction of all sensillum types was observed, to six proximal, three coeloconic, and no distal or trichoid sensilla. The epipharyngeal setae also became less regularly arrayed and more clumped in response to *Tc-Dl* knockdown.

The most striking phenotype observed in response to RNAi targeting *Tc-N* was the complete loss of sensilla on the epipharynx, which was observed in 2 of 16 individuals (the same two in which the distal sclerite was deleted; Fig. 3G). Additionally, epipharyngeal (Fig. 4G), labral (Fig. 4C) and distal sclerite setae were greatly reduced or eliminated. Thus, the only sensory structures that were unaffected were the stout distal setae on the distal edge of the epipharynx (Fig. 4G). Four *Tc-N* RNAi specimens showed less extreme loss of sensory structures (Fig. 3F). In these cases, the remaining proximal sensilla were irregularly arranged and widely spaced. The number of coeloconic sensilla was also greatly reduced, and the few remaining sensilla were sometimes joined into a compound structure. Finally, six *Tc-N* RNAi specimens resembled *Dl* RNAi specimens in having an increased number (up to 26) and disordered arrangement of proximal sensilla. The increase was generally smaller than was observed in *Tc-Dl* RNAi specimens, and was accompanied by reduction in the number of other sensilla, as well as by fusions and changes in their morphology (Fig. 3E).

Other genes—The number and arrangement of epipharyngeal sensilla was sensitive to the knockdown of several other genes. Depletion of either *Tc-Krn* or *Tc-Dll* led to a reduction in the number of all sensillum types. The most severely affected *Tc-Dll* RNAi specimens retained at most a few epipharyngeal sensilla, and also lacked most setae (Fig. 2H). In *Tc-Krn* RNAi specimens, loss of medial tissue was accompanied by a decrease in number of sensilla of each type; there were 5–8 sensilla in the proximal row (Fig. 3H). In some specimens, the proximal sensilla remained closely abutting, while in others their spacing was increased or irregular. The coeloconic sensilla were also closer together than in wild type.

The effects of six other dsRNA treatments (*Tc-exd*, *Tc-hth*, *Tc-dpp*, *Tc-ab*, *Tc-bab*, *Tc-ap a/ Tc-ap b*) on the number or arrangement of sensilla in the proximal row could not be attributed to larger-scale changes in the labrum or epipharynx. In all of these RNAi phenotypes, the coeloconic sensilla appeared normal in size, number and arrangement. In *Tc-exd* RNAi specimens, the proximal sensilla increased somewhat in number (to a maximum of 26; it exceeded the wild type range in 75% of specimens; Fig. 3I). The row appeared more disordered than in wild type, but the sensilla remained closely abutting. *Tc-*

hth RNAi produced a smaller, but statistically significant ($t=4.14$, $p=0.0002$), increase in the number of proximal row sensilla to a maximum of 18 (mean 16.5 ± 1.3 , $n=15$ specimens).

Knockdown of four other genes caused reductions in the number of proximal row sensilla. In *Tc-dpp* RNAi specimens, the proximal row sensilla were reduced in number (Fig. 3J) to as few as 7, and the remaining proximal sensilla were widely spaced; the number of trichoid sensilla was also reduced, and the coeloconic sensilla were less evenly distributed. Knockdown of *Tc-ab* also led to a reduced number (as few as 8) of widely spaced proximal sensilla (Fig. 3K). This was accompanied by a slight increase in the number of trichoid sensilla (Fig. 3L). Additionally, there was a reduction in number of bristles on the labral sclerite (Fig. 4D). RNAi targeting *Tc-bab* also perturbed multiple aspects of sensillum patterning. The proximal sensilla were more widely spaced, less aligned, and reduced in number compared to wild type (Fig. 3M); the edges of the row often incorporated trichoid sensilla, in contrast to the wild type pattern. The number of distal sensilla was reduced (to a maximum of 3). Finally, some sensilla displayed intermediate or novel morphologies (3M,N).

Sensory patterning was altered in multiple ways in response to simultaneous knockdown of the paralogous transcription factors *Tc-ap a* and *Tc-ap b*. The number of sensilla in the proximal row was reduced to as few as 8; these sensilla remained closely apposed to each other, unlike in the other reduction phenotypes (Fig. 3O). Trichoid sensilla were reduced to an even greater degree, and were almost completely absent in the most severe phenotypes (Fig. 3O,P). Additionally, the lateral epipharyngeal setae (Fig. 4I,J) and dorsal labral bristles (not shown) were reduced in number. The area covered by scaly cuticle was increased and portions of the epipharyngeal wall were inappropriately sclerotized (Fig. 4I,J).

DISCUSSION

It has long been appreciated that morphological diversity can be generated by tinkering with a conserved “toolkit” of genes (Jacob 1977; Knoll and Carroll 1999). One form of tinkering is co-option, the deployment of a gene in a novel context. Co-option of genes or gene regulatory networks has emerged as a general explanation for developmental similarity of non-homologous structures and for the origin of novelty (e.g., Moczek and Rose 2009; Monteiro 2012; Oliver et al. 2012). Co-option of an ancestral axis patterning network used in ventral appendage patterning is currently the best-supported hypothesis for the origin of the labrum (Posnien et al. 2009). Below, we compare labral patterning to ventral appendage patterning in *T. castaneum*, and interpret the similarities and differences in light of the co-option hypothesis. We conclude by comparing labral patterning across species in order to infer which aspects of labral patterning are ancestral.

Multiple Upstream Components of the Ventral Appendage Axis Patterning Network are Required for Labral Axis Patterning at Metamorphosis in *T. castaneum*

A model for appendage axis patterning comes from detailed studies in the leg (Fig. 5; reviewed in Kojima 2004; Estella et al. 2012) and antennal (Dong et al. 2000, 2001, 2002; Emerald et al. 2003; Emmons et al. 2007) imaginal discs of *D. melanogaster*. Comparative data suggest that this axis patterning network is relatively conserved across both arthropods and appendage types (reviewed in Angelini and Kaufman 2005), including at metamorphosis in *T. castaneum* (Fig. 5; Angelini et al. 2009, 2012a,b). The secreted signaling factors Wingless (Wg) and Dpp initiate development of the proximodistal axis through regulation of the leg gap genes. Distally, high levels of Wg and Dpp activate *Dll* expression and repress *dac* and *hth* expression (Diaz-Benjumea et al. 1994; Giorgianni and Mann 2011; Lecuit and Cohen 1997; Wu and Cohen 1999). *Dll* activates *dac* in an intermediate domain (Giorgianni and Mann 2011) and interactions between the leg gap

genes define discrete domains along the proximodistal axis (Abu-Shaar and Mann 1998; Giorgianni and Mann 2011; Wu and Cohen 1999; reviewed in Estella et al. 2012). Dpp (jointly with Wg) also activates EGF signaling distally (Campbell 2002; Estella et al. 2012; Galindo et al. 2002), which plays a key role in further subdivision of the appendage axis (Fig. 5).

Under the hypothesis that the labrum originated by co-option of the appendage patterning network (Posnien et al. 2009), we would predict that appendage axis patterning genes have axis patterning functions in labral development. Consistent with this prediction, we detected labral axis patterning roles for the Dpp and EGF signaling pathways and for three of the leg gap genes, *Tc-Dll* and the cofactors *Tc-hth* and *Tc-exd* (Fig. 5). At metamorphosis, depletion of *Tc-hth* or *Tc-exd* caused proximal labral defects, including loss or reduction of the V-shaped rods and other proximal support elements, as well as alteration of the labral sclerite (Figs. 2B–E). We interpret the latter as transformations of the labral sclerite towards distal sclerite identity on the basis of shape and setal patterns. Homeotic transformation of proximal regions towards more distal appendage identity in response to *Tc-hth/Tc-exd* RNAi were also observed in multiple ventral appendage types (Angelini et al. 2012a, b; Smith et al. in review). This resemblance is interesting because aspects of the phenotype are known only from *T. castaneum* and relatives within the Tenebrionidae (Smith et al. in review) and have not been observed in other insects (*Gryllus bimaculatus*, Ronco et al. 2008; *Oncopeltus fasciatus*, Angelini and Kaufman 2004; *D. melanogaster*, Azpiazu and Morata 2002; Casares and Mann 2001; González-Crespo and Morata 1995; Rauskolb et al. 1995). This suggests that a gene function shared between the labrum and ventral appendages arose in the lineage leading to *Tribolium*. The derived similarity across non-homologous structures within a lineage provides evidence that pleiotropy generated by co-option can lead to linked patterns of evolution like those that are characteristic of serially homologous structures (Angelini et al. 2012b; Monteiro and Podlaha 2009).

At metamorphosis, depletion of *Tc-Dll* leads to loss of distal components of both the labrum and most ventral appendages. Additionally, proximal regions of the labrum-epipharynx complex are also lost. By contrast, the ventral appendages always retained proximal structures, although sometimes in altered form (Angelini et al. 2009, 2012a, b). Thus, the labral phenotypes are even more severe than would be predicted based on the ventral appendage phenotypes. We concur with Schoppmeier and Damen (2001) who suggested that complete labral deletion reflects a substantially different requirement for *Dll* in labral patterning than in ventral appendage patterning.

Signaling through the Dpp and EGF pathways is required for axis patterning in both the labrum and ventral appendages (Fig. 5). The labral *Tc-dpp* RNAi phenotypes resembled mild leg phenotypes; both structures showed relative expansion along the dorsoventral axis. More severe leg phenotypes showed distal truncations (Angelini et al. 2012b); truncation phenotypes were not observed in the labrum. In the labrum, the distal sclerite was deleted in response to RNAi targeting the EGF ligand *Tc-Krn* (Fig. 2J). Distal patterning defects, consisting of deletions, fusions or reductions, were also observed in all ventral appendage types, including the mandibles (Angelini et al. 2009; 2012a, b).

dac* and Most Downstream Components of the Ventral Appendage Axis Patterning Network are Not Required for Labral Axis Patterning at Metamorphosis in *T. castaneum

While three of the leg gap genes were required for normal labral metamorphosis, the labrum of *Tc-dac* RNAi specimens appeared normal. This result contrasts with the requirement for *dac* in development of an intermediate domain in all ventral appendages except the mandibles in *T. castaneum* (Angelini et al. 2009, 2012a, b). The failure to detect a role for *Tc-dac* in labral metamorphosis was also surprising in light of its role in the metamorphic

development of proper labral proportions in the dung beetle *O. taurus* (Simonnet and Moczek 2011). Labral morphology differs greatly between *Tribolium* and *Onthophagus*, with the latter showing more morphologically discrete regions along the proximodistal axis. To test whether the lack of proximodistal landmarks led us to overlook more subtle effects of RNAi targeting *Tc-dac*, we compared the length and width of the labrum in *Tc-dac* and *gfp* RNAi specimens, but found no significant differences (one-sided t-test; $p = 0.28$ for length; $p = 0.18$ for width). Another possible explanation for the apparent difference between species is different degrees of gene knockdown. However, the *dac* RNAi leg phenotypes were very similar between these two species, and penetrance of the leg phenotype is high (Angelini et al. 2012b; Moczek and Rose 2009). This suggests that the difference in labral effects may reflect a genuine difference in the metamorphic requirement for *dac* in these two beetle species. A reduction or loss of *dac* functionality in appendage development has been observed in multiple evolutionary contexts, including in the antennae, maxillae and labium of *D. melanogaster* (Abzhanov et al. 2001; Dong et al. 2002) and the labium of the milkweed bug *O. fasciatus* (Angelini and Kaufman 2004). These appendage types have a reduced or highly modified proximodistal axis, paralleling the difference between the labra of the beetles *O. taurus* and *T. castaneum*.

During appendage development, the leg gap genes and EGF signaling regulate, directly or indirectly, multiple downstream targets required for further growth and subdivision of the proximodistal axis in *D. melanogaster*. These include the transcription factors *al*, *bab*, *cll*, *odd-skipped* paralogs, *pdm*, *rn*, *ss*, and *ap* (Fig. 5; Natori et al. 2012; reviewed in Kojima 2004). Notch signaling, which regulates joint formation and segment growth (Bishop et al. 1999; de Celis et al. 1998; Rauskolb and Irvine 1999), is activated by different combinations of transcription factors at each segment boundary. With the exception of *Tc-cll*, RNAi targeting these genes in *T. castaneum* all caused metamorphic defects in at least one ventral appendage type at moderate to high frequencies (Table 1; Angelini et al. 2009; 2012a, b). However, we failed to detect labral axis patterning phenotypes for most of them. The main exceptions were depletion of *Tc-N*, which led, at low frequency, to loss of the distal sclerite (Fig. 4C), and depletion of the *odd-skipped* family member *Tc-drm*, which led to alterations in the proximal support rods and labral sclerite (Fig. 2M). The absence of axis patterning effects for other RNAi treatments suggest that much of the downstream appendage axis patterning network is not active during metamorphic labral axis patterning and thus that upstream components of the network bring about their axis patterning effects via different downstream mechanisms in the labrum and ventral appendages.

Patterning of the Epipharyngeal Sensillum Array Requires Many Components of the Appendage Patterning Network

Although candidate genes were not chosen based on an expectation of sensory patterning roles, defects in the epipharyngeal sensillum array were observed in a surprisingly high proportion of RNAi treatments. In addition to *Tc-N*, *Tc-Dl* and *Tc-Dll*, for all of which a role in sensory development was predicted *a priori* (see below), seven other genes were required for development of the wild type number or arrangement of sensilla (Fig. 5): two components of signaling pathways (*Tc-Krn* and *Tc-dpp*) and five transcription factors (*Tc-ab*, *Tc-ap a/Tc-ap b*, *Tc-bab*, *Tc-exd* and *Tc-hth*). The phenotypes resulting from *Tc-dpp*, *Tc-ab* and *Tc-bab* RNAi were similar: a reduction in the number of proximal row sensilla accompanied by wider spacing between them. RNAi targeting *Tc-ap a* and *Tc-ap b* also caused a reduction in the number of proximal sensilla, but the remaining sensilla were closely packed, as in wild type specimens. Loss of *Tc-exd* or *Tc-hth* had the opposite effect: an increase in number of proximal sensilla. While most of these genes are known to regulate the position of sensory structures in *D. melanogaster* (Hu et al. 1995; Kopp et al. 2000; Kurant et al. 1998; Romain et al. 2000; Tomoyasu et al. 1998), they do so in widely varying

contexts, rather than as part of a common network. These genes also function in different domains during appendage axis patterning, with *exd* and *hth* active proximally, and the other genes primarily active distally (Fig. 5). This suggests that their integration into a network regulating development of the proximal sensillum row may be a novel feature in the labrum.

A general requirement for *Dll* in the development of arthropod sensory structures has been hypothesized based on comparative analyses of gene expression and function (Mittmann and Scholtz 2001; Panganiban 2000; Williams et al. 2002). Labral sensory structures are almost completely deleted in *Tc-Dll* RNAi specimens; however, because this loss is accompanied by almost complete loss of the labrum (Fig. 2F–I), it is difficult to evaluate any specific role for *Tc-Dll* in labral sensory patterning. The *Tc-Krn* RNAi phenotype may also be non-specific: both the decrease in number of proximal sensilla and the tighter packing of coeloconic sensilla may result from the overall reduction in the size of the central sensory region (Fig. 4H), rather than from a specific requirement for *Tc-Krn* in patterning of this sensillum array.

The development of sensilla is well characterized in *D. melanogaster* and highly conserved across sensillum types (reviewed in Hartenstein 2005; Lai and Orgogozo 2004). A lateral inhibition mechanism involving the neurogenic genes *N* and *Dl* leads to the selection of one cell within each proneural cluster as the sensory organ precursor; division of this cell generates all of the cells of a single sensillum. In *D. melanogaster*, loss of *Dl* or *N* can lead to either an increase or decrease in the number of sensilla, depending on timing of the perturbation (Heitzler and Simpson 1991; Parks and Muskavitch 1993; Parody and Muskavitch 1993). Depletion of *Tc-N* or *Tc-Dl* also had complex effects in *T. castaneum*. However, there was no clear correlation between the type of defect and developmental stage at the time of dsRNA injection. Specimens showing increases and decreases in the number of proximal sensilla underwent their pupal molt 3–5 days after injection of *Tc-N* dsRNA and the single *Tc-Dl* RNAi specimen showing a reduction molted 4 days after injection, as did the majority of *Tc-Dl* RNAi specimens showing increases. A complex requirement for Notch signaling in the development of peripheral sense organs was also observed in a spider (Gold et al. 2009).

Comparisons across Taxa Suggest that All of the Leg Gap Genes were Required Ancestrally for Labrum Development

Comparisons of gene function across taxa can be used to infer which aspects of labral patterning are ancestral. Comparative functional data are limited primarily to gap genes. One complication is that data are available at different stages in different taxa. How to homologize developmental stages between hemi- and holometabolous insects is controversial (Truman and Riddiford 2002). Metamorphic data are available for one other beetle (Simmonet and Moczek 2011). Embryonic data are available for *T. castaneum* and several insect species that undergo hemimetabolous development. Comparative data are not available for *D. melanogaster*, as results from the tiny clypeolabral discs are not often reported.

In *T. castaneum*, *Dll* depletion leads to nearly complete deletion of the labrum at metamorphosis. Simonnet and Moczek (2011) found that *Dll* was required for distal labral development in the dung beetle *O. taurus*, but they did not observe deletions of more proximal regions. The metamorphic *Tc-Dll* deletion phenotype closely resembles embryonic phenotypes in a diversity of arthropods, including *T. castaneum* (Beermann et al. 2001). Complete deletion of the labrum in response to *Dll* down-regulation during embryogenesis was also observed in other orders of insects (Angelini and Kaufman 2004; Yoshiyama et al. 2013), including the primitively wingless firebrat *Thermobia domestica* (Ohde et al. 2009),

and in chelicerates (Khila and Grbi 2007; Schoppmeier and Damen 2001; Sharma et al. 2013).

A requirement for *dac* in labral metamorphosis was not detected in *T. castaneum* either by this study or by Yang et al. (2009). By contrast, *dac* was required for proper proportioning of labral regions at metamorphosis in *O. taurus* (Simonnet and Moczek 2011). Although labral metamorphosis was not perturbed by *Tc-dac* knockdown, embryos with reduced *Tc-dac* expression developed a smaller, divided labrum (Yang et al. 2009). An embryonic requirement for *dac* was also found in *O. fasciatus*: the labrum was deleted in strong *Of-dac* RNAi phenotypes, although it was unaffected in milder phenotypes (Angelini and Kaufman 2004). *dac* was not expressed in the labrum of *P. opilio*, the only chelicerate species for which *dac* RNAi data are available (Sharma et al. 2012; 2013). *dac* expression varies across chelicerates: it is not expressed in the labrum of a mite (Barnett and Thomas 2013) or one spider (Pechmann and Prpic 2009), but is expressed in the labrum of another spider (Prpic et al. 2003).

A requirement for *hth* in proximal labrum development at metamorphosis was found in both *T. castaneum* and the horned beetle *O. taurus* (Simonnet and Moczek 2011); in *T. castaneum*, the observed requirement for *hth* extends more distally than in *O. taurus*. A reduction in labrum size has been reported in two hemimetabolous species for which embryonic *hth* RNAi data are available, *O. fasciatus* (Angelini and Kaufman 2004) and *G. bimaculatus* (Ronco et al. 2008). In stronger embryonic *hth* RNAi phenotypes, large head deletions occur in both species, resulting in loss of the region from which the labrum develops. Labral expression of *hth/exd* is also observed in chelicerates (Barnett and Thomas 2013; Pechmann and Prpic 2009; Sharma et al. 2012), but its functional role has not been tested.

These comparative data suggest that *Dll* was required for embryonic development of the entire labrum in the common ancestor of arthropods, and that this requirement is retained through metamorphosis in holometabolous insects. These data additionally suggest that *hth* and *exd* also had an ancestral role in labral axis patterning, although functional data are only available for insects. The differences even within this limited sample of insects preclude more definitive statements about how *hth* and *exd* functioned ancestrally in the labrum. Finally, the comparative data suggest that *dac* had an ancestral role in embryonic development of the labrum in insects, but it is unclear whether this role was retained through metamorphosis in the common ancestor of holometabolous insects. The co-option model for the origin of the labrum predicts that the requirement for *dac* in labral development is ancestral for all arthropods, not just insects. However, based on the absence of *dac* expression in the labrum of most chelicerates, it is equally parsimonious to conclude that a labral patterning role was acquired after the divergence of chelicerates from other arthropods.

CONCLUSION

Comparisons of metamorphic RNAi phenotypes in the labrum and ventral appendages of *T. castaneum* reveal that multiple upstream, but few downstream, components of the appendage axis patterning network are required for labral axis patterning. The similarities included one that was unexpected: an aspect of the *hth* and *exd* phenotypes that is shared between the labrum and ventral appendages of *T. castaneum*, that appears to have evolved in the lineage leading to *Tribolium*. Sharing of a derived effect suggests that similarity of developmental patterning can be generated long after co-option, and that the evolution of non-homologous structures may be linked in ways resembling that of serial homologues. The mixture of similarities and differences is consistent with the pattern of partial similarity

emerging from other comparisons of development of traits hypothesized to share a developmental network due to co-option (e.g., Bowsher and Nijhout 2009; Moczek and Rose 2009; Oliver et al. 2012; Shirai et al. 2012). Differences in patterning of the labrum and ventral appendages may occur either because only a portion of the co-opted developmental network was activated in the new context or because initially more extensive similarity decayed with evolutionary time. Distinguishing between these evolutionary scenarios requires inferring the structure of the ancestral labral patterning network. At present, comparative data are limited to a few genes, in a few species, primarily during embryogenesis. Data from additional taxa are needed to evaluate the extent of conservation of labral patterning across taxa and to robustly infer its ancestral state.

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Figure 1.

Wild type morphology of the labrum in *T. castaneum*. A–D) adult labrum; E–F) larval labrum; A) dorsal focal plane showing labral sclerite; B) intermediate focal plane showing distal sclerite and proximal support structures; C) ventral focal plane showing epipharyngeal sensillum array flanked by lateral setal patches; D) same image as C, with sensilla color-coded by type, red: proximal/distal sensilla; green: trichoid sensilla; blue: coeloconic sensilla; E) dorsal focal plane, showing setae; F) ventral focal plane; arrowheads mark the four sets of coeloconic sensilla. Scale bars are 50 μm.

Figure 2.

RNAi phenotypes in the labral sclerites and proximal supports of *T. castaneum*. A) *gfp* RNAi; B–D) *Tc-exd* RNAi showing moderate (B; arrow marks extension of labral sclerite; arrowhead indicates reduced patch of sclerotized hairs) and severe (C–D) phenotypes; in D, black arrow indicates normal setae on the distal sclerite, while red arrow indicates labral sclerite setae that resemble the distal sclerite setae; E) *Tc-hth* RNAi phenotypes resemble *Tc-exd* phenotypes; F–H) phenotypic series for *Tc-Dll* RNAi showing mild reduction (F), substantial deletion, with tubular remnants arrows in G) and nearly complete deletion (H) of the labrum; I) SEM of mild *Tc-Dll* RNAi phenotype; arrow marks abnormal setae on membranous region; J) *Tc-Krm* RNAi phenotype showing loss of distal sclerite (arrowhead), reduction in size of the labral sclerite, and larger unsclerotized region exposed dorsally (arrow); K–L) *Tc-dpp* RNAi phenotypes showing increased tissue along the midline; this results in a more bowed labral sclerite (arrow in K) and altered relationship between the labral and distal sclerites (arrow in L); M) *Tc-drm* RNAi phenotypes showing subtle shape changes in the labral sclerite and alterations to the proximal support structures (arrows): the tormae are shorter, the angle between the V-shaped rods is altered, and the lateral rods

project from a more medial position, and greater angle; N) *Tc-ap a/Tc-ap b* RNAi specimen showing altered distal end of the tormae (arrow) and reduction in sclerotized hairs (arrowhead). Red is used to indicate abnormal structures in the online version of this figure. Scale bars are 50 μm .

Figure 3.

RNAi phenotypes in the epipharyngeal sensillum array of *T. castaneum*. Light (A, C, E–K, M, O) and scanning electron (B, D, L, N, P) micrographs. A–B) *gfp* RNAi specimen showing sensillum array: proximal sensillum row (boxed in A; the same sensillum type is present distally; this type is not visible in SEM), trichoid sensilla (arrows), and coeloconic sensillum (arrowheads); scale bar in B is 20 μ m; *Tc-Dl* RNAi specimens showing increases in the number of proximal and distal sensilla (C) and reduction and fusion of sensilla (box and inset in D); *Tc-N* RNAi specimens showing increase (E) and decrease (F) in number of proximal sensilla, along with reduction of other sensillum types; arrowheads indicate partial fusion of coeloconic sensilla; G) Extreme *Tc-N* RNAi reduction phenotype; H) *Tc-Krn* RNAi specimen, with reduction in sensillum array resulting from loss of medial region; as a result, the lateral setal patches obscure the array; I) *Tc-exd* RNAi specimen showing increased number of proximal sensilla; note also the changed alignment between dorsal and ventral sides; J) *Tc-dpp* and K) *Tc-ab* RNAi specimens both have a reduced number of proximal sensilla which are widely spaced; K–L) *Tc-ab* RNAi specimens also have an increased number of trichoid sensilla; M–N) *Tc-bab* RNAi specimens have a reduced number of proximal sensilla which are widely spaced and some sensilla with novel morphologies (arrowheads in M, N); small trichoid sensilla may be present distally (arrow in N); O–P) *Tc-ap a/Tc-ap b* RNAi specimens showing abnormal sclerotization of the epipharynx (boxed) and a reduced number of proximal and trichoid sensilla.

Figure 4.

RNAi phenotypes affecting setae and bristles in *T. castaneum*. Light (A, C–E, G–I) and scanning electron (B, F, J) micrographs of the labrum; A–D show dorsal surface; E–J show epipharyngeal (ventral) surface; A–B) *gfp* RNAi specimen showing regular array of bristles on the labral sclerite and distal setae; scale bar in B is 100 μ m; C) *Tc-N* RNAi specimen (same as in Fig. 3F) lacking bristles on the labral sclerite and showing reduction in number of distal setae; D) *Tc-ab* RNAi specimen with a reduced number of dorsal bristles; E–F) *gfp* RNAi specimens; the setae in the lateral patches show a graded size distribution; arrowhead indicates scaled cuticle; G) *Tc-N* RNAi specimen (same as in Fig. 3G and 4C) lacking lateral setae and medial distal setae; H) *Tc-Krn* RNAi specimen showing that setal patches nearly meet at the midline; I) (same specimens as in 3N) and J) *Tc-ap a/ap b* RNAi specimens

showing reduced number of lateral setae and expanded region of scaled cuticle (box and inset).

Figure 5.

Comparisons of labrum and leg patterning. A) *D. melanogaster* leg patterning network; arrows and bars indicate positive and negative regulation; reviewed in Kojima (2004) and Estella et al. (2012). Regions of the leg (B; based on Angelini et al. 2012b) and labrum (C) affected by RNAi in *T. castaneum*. The presence (+) and absence (-) of effects on labral sensilla are indicated in the right-hand (sr) column. Gene names without colored bars in B and C indicate genes for which no axis patterning defects were detected in the leg and labrum, respectively. *Bar* is included in (A) because of its key role in the regulatory network, but this gene was not studied in *Tribolium*. *ab* is omitted from (A) because neither its expression domain nor interactions have been described, although mutants do have abnormal legs (Hu et al. 1995). Abbreviations: cx, coxa; ds, distal sclerite; fe, femur; ls, labral sclerite; pr, proximal supporting rods; pt, pretarsus; sr, sensory; Ti, tibia; tr, trochanter; t1–t5, tarsal subsegments 1–5.

Genes, dsRNA constructs, and appendage penetrance

Appendage penetrance is calculated as the percent of specimens examined by Angelini et al. (2009, 2012a, b) that showed any defect in the ventral appendages (antennae, mouthparts, legs and genitalia). Phenotypic specimens were selected for labral dissections.

Table 1

Gene	abbreviation	Protein class	GenBank Accession Number	dsRNA size (bp)	appendage penetrance
<i>gfp</i>	<i>gfp</i>			600	6% ¹
<i>abrupt</i>	<i>ab</i>	BTB/Zn-finger TXF	XM_969854	198	80%
<i>apterous a/ apterous b</i>	<i>Tc-ap a/ Tc-ap b</i>	Homeobox TXFs	NM_001145869/ NM_001145916	508/ 514	14%
<i>aristalless</i>	<i>Tc-ql</i>	Homeobox TXF	NM_001114366	172	39%
<i>bric à brac</i>	<i>Tc-bab</i>	BTB/Psq TXF	XM_001812888	774	82%
<i>clawless</i>	<i>Tc-cll</i>	Homeobox TXF	XM_969890	171	0% ²
<i>dachshund</i>	<i>Tc-dac</i>	Novel TXF	XM_964678	359	76%
<i>decapentaplegic</i>	<i>Tc-dpp</i>	TGF-β ligand	NM_001039451	812	69%
<i>Delta</i>	<i>Tc-Dl</i>	DI/Ser-type EGF	XM_964994	180	37%
<i>Distal-less</i>	<i>Tc-Dll</i>	Homeobox TXF	NM_001039439	462	97%
<i>drumstick</i>	<i>Tc-drm</i>	Zn-finger TXF	XM_966887	225	100%
<i>extradenticle</i>	<i>Tc-exd</i>	Homeobox TXF	NM_001039412	236	100%
<i>homothorax</i>	<i>Tc-hth</i>	Homeobox TXF	NM_001039400	335	90%
<i>Keren</i>	<i>Tc-Krn</i>	EGF ligand	XM_001813564	188	89%
<i>Notch</i>	<i>Tc-N</i>	Notch receptor	NM_001114381	409	90%
<i>pdm/nubbin</i>	<i>Tc-pdm</i>	Homeobox TXF	XM_963346	332	56%
<i>rotund</i>	<i>Tc-m</i>	Zn-finger TXF	XM_966094	127	35%
<i>Serrate</i>	<i>Tc-Ser</i>	DI/Ser-type EGF	XM_964393	329	97%
<i>spalt</i>	<i>Tc-sal</i>	Zn-finger TXF	XM_968136	200	29%
<i>spineless</i>	<i>Tc-ss</i>	bHLH/PAS	XM_962783	355	92%

Abbreviations: bHLH, basic helix-loop-helix domain; BTB, Bric à brac/Tramtrack/Broad-complex domain; EGF, epidermal growth factor; PAS, PER-ARNT-SIM domain; Psq, Pipsqueak; TGF, transforming growth factor; TXF, transcription factor

¹ *gfp* RNAi specimens showed a low frequency of non-specific defects; similar defects are also observed at low frequencies in wild type specimens.

² Although appendages were normal, a high percentage of *Tc-cll* RNAi specimens demonstrated other phenotypic defects.