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Distal-less and *dachshund* pattern both plesiomorphic and apomorphic structures in chelicerates: RNA interference in the harvestman *Phalangium opilio* (Opiliones)

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Abstract

The discovery of genetic mechanisms that can transform a morphological structure from a plesiomorphic (=primitive) state to an apomorphic (=derived) one is a cardinal objective of evolutionary developmental biology. However, this objective is often impeded for many lineages of interest by limitations in taxonomic sampling, genomic resources, or functional genetic methods. In order to investigate the evolution of appendage morphology within Chelicerata, the putative sister group of the remaining arthropods, we developed an RNA interference (RNAi) protocol for the harvestman *Phalangium opilio*. We silenced the leg gap genes *Distal-less (Dll)* and *dachshund (dac)* in the harvestman via zygotic injections of double-stranded RNA, and used *in situ* hybridization to confirm RNAi efficacy. Consistent with the conserved roles of these genes in patterning the proximo-distal axis of arthropod appendages, we observed that embryos injected with *Dll* dsRNA lacked distal parts of appendages and appendage-like structures, such as the labrum, the chelicerae, the pedipalps, and the walking legs, whereas embryos injected with *dac* dsRNA lacked the medial podomeres femur and patella in the pedipalps and walking legs. In addition, we detected a role for these genes in patterning structures that do not occur in well-established chelicerate models (spiders and mites). *Dll* RNAi additionally results in loss of the preoral chamber, which is formed from pedipalpal and leg coxapophyses, and the ocularium, a dorsal outgrowth bearing the eyes. In one case, we observed that an embryo injected with *dac* dsRNA lacked the proximal segment of the chelicera, a plesiomorphic podomere that expresses *dac* in wild type embryos. This may support the hypothesis that loss of the chelicerate *dac* domain underlies the transition to the two-segmented chelicera of derived arachnids.

Keywords: Gene silencing; RNAi; Arachnida; Chelicerata; arthropod leg; leg gap genes

Introduction

The diversity of arthropod appendages has prompted an historic, fascinating series of debates that span the homology of various appendage types (Snodgrass 1938; Boudreaux 1987; Boxshall 2004), the fate of Cambrian arthropods' "great appendages" (Weber 1952; Rempel 1975; Scholtz 1997, 2001; Budd 2002; Maxmen et al. 2005; Jager et al. 2006; Brenneis et al. 2008), the nature of the mandible (Anderson 1973; Manton 1977; Boudreaux 1987), and the implications of all of these for arthropod phylogeny. In complement to paleontology and morphology, gene expression and function data have become integral to deciphering arthropod evolution. Specifically, the suite of genes that patterns the proximo-distal axis of appendages (*Distal-less* [*Dll*], *dachshund* [*dac*], and the cofactors *homothorax* [*hth*] and *extradenticle* [*exd*]—commonly referred to as "leg gap" genes) has been utilized as an independent body of evidence for testing evolutionary hypotheses pertaining to appendages (reviewed by Angelini and Kaufman, 2005). In several cases, studies of leg gap gene expression and/or function have supported earlier hypotheses based on morphological evidence, such as the gnathobasic nature of the mandible (Panganiban et al. 1994, 1995; Scholtz et al. 1998; Prpic et al. 2001), the ancestral bipartite structure of the arthropod leg (González-Crespo and Morata 1996; Dong et al. 2001; Abzhanov and Kaufman 2000; reviewed by Angelini and Kaufman 2005; Janssen et al. 2010), and the serial homology of mandibulate head appendages (Rieckhof et al. 1997; Dong et al. 2001, 2002; Ronco et al. 2008). In other cases, typified by the dispute over the nature of the labrum, leg gap gene data do not unambiguously favor one hypothesis over another (Popadić et al. 1998; Scholtz 1997; reviewed by Scholtz and Edgecombe 2006).

The leg gap genes serve to regionalize the arthropod leg, conferring proximal (*hth* and *exd*), medial (*dac*), and distal (*Dll*) identities (Sunkel and Whittle 1987; Cohen and Jürgens 1989; Mardon et al. 1994; González-Crespo and Morata 1996; Lecuit and Cohen 1997; Rieckhof et al.

1997; Abu-Shaar and Mann 1998; Casares and Mann 1998; Abu-Shaar et al. 1999; Wu and Cohen 1999; Dong et al. 2001, 2002; Rauskolb 2001; reviewed by Angelini and Kaufman 2005). However, these genes also play a role beyond patterning the PD axis. For example, *Dll* is known to have additional roles in patterning sensory organs and bristles (Sunkel and Whittle 1987; Cohen and Jürgens 1989; Mittmann and Scholtz 2001; Williams et al. 2002), sexually dimorphic non-appendage outgrowths (Moczek and Nagy 2005; Moczek et al. 2006; Moczek and Rose 2009), and even antero-posterior gap gene function in spiders (Pechmann et al. 2011). Similarly, *dac* patterns elements of the central nervous system, particularly photoreceptors of insect compound eyes (Mardon et al. 1994). Nevertheless, barring *Dll*, evolutionary inference of leg gap gene activity remains limited by the concentration of functional analyses within hexapods. For example, while *dac* orthologues are inferred to pattern the medial regions of legs of all panarthropods based on expression domains (Janssen et al. 2010), *dac* knockdown phenotypes have only been observed in a handful of winged insects.

Chelicerates, the putative sister group to the remaining arthropods (mandibulates), is a diverse lineage that includes Arachnida (e.g., spiders, mites, scorpions), Merostomata (horseshoe crabs and the extinct sea scorpions [Eurypterida]), and possibly Pycnogonida (sea spiders). Due to the phylogenetic significance of chelicerates, developmental processes in chelicerate model organisms are greatly relevant to evolutionary inference. For example, the retention of the chelicerate deutocerebral appendage, demonstrated in the mite *Archezogozetes longisetosus* and the spider *Cupiennius salei*, has supported a particular interpretation of arthropod segmental homologies (Damen and Tautz 1998; Telford and Thomas 1998; Hughes and Kaufman 2002; Scholtz and Edgecombe 2006). Investigation of the *Notch* pathway in the spider *Cupiennius salei* has similarly demonstrated conservation of pathways in segmentation processes of both the body and the appendages (Stollewerk et al. 2003; Prpic and Damen 2009).

Four of the 12 extant orders of euchelicerates (Xiphosura + Arachnida) have been studied with respect to leg gap genes: horseshoe crabs (Xiphosura), mites (Acari), spiders (Araneae), and harvestmen (Opiliones). Horseshoe crabs are the least studied in this regard: only protein expression of *Dll* is known in *Limulus polyphemus* (Mittmann and Scholtz 2001). In mites, leg gap gene data are limited to expression and function of *Dll* (Thomas and Telford 1999; Khila and Grbic 2007). *Dll* acts as a typical leg gap gene in *Tetranychus urticae*: a parental knockdown of *Dll* results in the truncation of appendages due to the loss of distal leg structures, as in insects (Dong et al. 2001; Rauskolb 2001; Khila and Grbic 2007). The expression of all four leg gap genes has been profiled in multiple spider species (Abzhanov and Kaufman 2000; Schoppmeier and Damen 2001; Prpic et al. 2003; Prpic and Damen 2004; Pechmann and Prpic 2009; reviewed by Pechmann et al. 2010). As with mites, functional data in the spider *Cupiennius salei* (obtained via zygotic RNAi) have focused on the activity of *Dll*, demonstrating the conserved role of this gene in appendage axis formation (Schoppmeier and Damen 2001). Additionally, parental RNAi in another spider species, *Parasteatoda tepidariorum*, has demonstrated an intriguing novel role for *Dll* as an anterior-posterior axis gap gene that regulates the segmentation of the body (Pechmann et al. 2011). Specifically, a parental knockdown of *Dll* results in the loss of one to two body segments (bearing the first walking leg pair or the first and second walking leg pairs). In contrast to these multiple studies of the role of *Dll*, no functional data are available for *dac*, *hth*, or *exd* in any chelicerate.

The most recent addition to the suite of chelicerate study organisms is the harvestman *Phalangium opilio*, for which we previously profiled the expression domains of the leg gap genes (Sharma et al. 2012b). These data demonstrated that leg gap gene expression in the harvestman is largely comparable to that of the spider. Harvestmen may be considered to represent a lineage of “primitive” arachnids (like scorpions), as they bear a number of plesiomorphic structures not observed in spiders or mites. Their early appearance in the fossil record and placement in most

chelicerate phylogenies enables phylogenetic polarization of developmental data observed in the other chelicerate models (Dunlop 2010; reviewed in Giribet and Edgecombe 2012; Sharma et al. 2012a) (Fig. 1A). Several harvestman structures that express leg gap genes do not occur in spiders or mites. First, the preoral chamber is formed from the pedipalpal and first leg pair endites, and is exclusive to harvestmen and scorpions (although the preoral chambers of scorpions also include the endites of the second walking legs). This structure facilitates manipulation of food particles. In contrast to derived arachnids, scorpions and harvestmen do not have suctorial mouthparts; they feed using the proximal-most parts of their limbs, the coxapophyses (coxal endites) of the pedipalps and first pair of walking legs (Fig. 1B, 1C). Previously, we reported that these two pairs of endites express *Dll* in *P. opilio* prior to outgrowth (Sharma et al. 2012b), in a manner comparable to the spider “maxilla” (a structure formed from the pedipalpal coxal endites, which also express *Dll* and are retained in adults [Schoppmeier and Damen 2001; Pechmann et al. 2010]), the gnathendites of crustacean mandibles (which are also used in feeding; Panganiban et al. 1995; Scholtz et al. 1998), and the labial and maxillary endites of insects (Giorgianni and Patel 2004; Jockusch et al. 2004; Angelini et al. 2012a). These data suggest that *Dll* is involved in coxal endite outgrowth in both chelicerates and pancrustaceans.

Second, the ocularium is a dorsal mound that bears the single pair of eyes in Phalangida (the non-cyphophthalmid harvestmen; Giribet et al. 2010) (Fig. 1B). This structure is postulated to result from coalescence of a pair of *Dll* domains in the eye fields (specifically, the semilunar grooves; Sharma et al. 2012b). Eye mounds have evolved independently in several chelicerate lineages, such as pycnogonids and harvestmen. The outgrown nature of the ocularium suggests cooption of *Dll* to the development of this structure, given that in other arthropods, *Dll* patterns non-appendage outgrowths of the body wall, such as beetle horns (Moczek and Nagy 2005; Moczek et al. 2006; Moczek and Rose 2009).

Third, harvestmen bear a three-segmented chelicera, which occurs in a grade of chelicerate orders leading to Arachnida (e.g., Xiphosura, Eurypterida, Pycnogonida), as well as some extant arachnid orders (e.g., Scorpiones, Opiliones; reviewed by Sharma et al. 2012b) (Fig. 1B). This disposition of cheliceral types has resulted in the reconstruction of the three-segmented chelicera as a symplesiomorphy (Dunlop 1996; Wheeler and Hayashi 1998; Giribet et al. 2002; Shultz 2007). The proximal segment of this appendage strongly expresses *dac* in the harvestman. In contrast, *dac* is not expressed in the chelicerae of spiders, which are two-segmented (as in most derived arachnids). Based on these observations, we previously postulated that a loss of the *dac* domain could represent the genetic mechanism for the evolutionary transition from the three- to the two-segmented cheliceral type (Sharma et al. 2012b).

To date, the functional significance of the expression domains of *Dll* and *dac* in chelicerate orders with the plesiomorphic cheliceral type has not been tested. Functional genetic testing is infeasible in many of these groups, which may either lack structures of interest (e.g., horseshoe crabs do not have a preoral chamber) or are intractable for reasons pertaining to life history (e.g., scorpions give live birth after a gestation period lasting several weeks to months). However, the recently established harvestman model organism *P. opilio* possesses all aforementioned structures of interest, and is tractable for laboratory studies (Sharma et al. 2012a, b). Here we describe methods for gene silencing in *Phalangium opilio* via zygotic injection of double stranded RNA (dsRNA). We report the conserved functions of *Dll* and *dac* as canonical leg gap genes in the pedipalps and legs. We additionally observe that knockdown of *Dll* interferes with the development of the preoral chamber's coxal endites and the ocularium. In a single embryo, we observe that a knockdown of *dac* results in a two-segmented chelicera lacking the proximal segment, suggesting that the evolutionary transition from the three- to the two-segmented chelicera is achieved by the loss of the *dac* domain, and thus, of the proximal segment.

Materials and Methods

Embryo collection and preparation

Adults of *Phalangium opilio* (Arachnida, Opiliones, Eupnoi, Phalangidae) were hand collected between 9:00 PM and 3:00 AM from various sites in Weston, Massachusetts, and Storrs, Connecticut, USA in June through August of 2012. Adults were maintained and embryos collected as previously described (Sharma et al. 2012a).

Embryo preparation for RNAi followed a modified protocol for the spider *C. salei* (Prpic et al. 2008). After 3-4 days of development, a clutch of embryos was dechorionated in 50% bleach solution and arranged into 8 × 8 grids on an agar dish. Glass cover slips (22 mm × 22 mm), previously coated with “heptane glue” (made by dissolving double-sided Scotch brand adhesive tape into heptane) and dried to create a layer of adhesive, were lowered onto each grid. In this manner, each clutch was approximately divided into three groups: 60% of the clutch was designated for injection with dsRNA for the gene of interest, 20% for injection with dsRNA for *DsRed* (exogenous dsRNA), and 20% for uninjected controls. This experimental design loosely follows that of Schoppmeier and Damen (2001).

Like spider embryos, harvestman embryos contract 3-7 days after egg laying, forming a peri-vitelline space filled with peri-vitelline fluid (Juberthie 1964). Embryos were allowed to develop in deionized water until the peri-vitelline space was observed. Embryos with a clear peri-vitelline space were immediately prepared further for injection (see below).

Gene identification, cloning, and synthesis of dsRNA

Identification of *Po-Dll* and *Po-dac* using a developmental transcriptome of *P. opilio* was previously described (Sharma et al. 2012b). Templates for dsRNA synthesis were generated from cDNA as follows: Both genes were amplified by PCR using gene-specific primers (GSP) with an added linker sequence (these primers were previously used for riboprobe synthesis; sequences published in Sharma et al. 2012b). PCR products were cloned using the TOPO® TA Cloning® Kit with One Shot® Top10 chemically competent *E. coli* (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol, and their identities verified by sequencing.

dsRNA synthesis was conducted with the MEGAscript® T7 kit (Ambion/Life Technologies, Grand Island, NY, USA) from amplified PCR product (above), following the manufacturer's protocol. The synthesis was conducted for 4h, followed by a 5 min cool-down step to room temperature. A LiCl precipitation step was conducted, following the manufacturer's protocol. dsRNA quality and concentration were checked using a Nanodrop-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and the concentration of the dsRNA was subsequently adjusted to 3.85-4.00 µg/µL.

Embryo injection

Upon the appearance of the perivitelline space, embryos were dehydrated at room temperature for 30 min. Subsequently, embryos were immersed in halocarbon oil 700 (Sigma-Aldrich, St. Louis, MO, USA). For injection, food-grade green dye was added to the dsRNA at a 1:20 dilution in order to visualize insertion of dsRNA. Microinjection needles were prepared from glass capillaries (1/0.58mm, 1B100F-4, World Precision Instruments, Sarasota, FL) using a micropipette puller (P-97, Sutter Instruments Co., Novato, CA, USA), and were backloaded with tinted dsRNA solution. Microinjections were performed on a Lumar stereomicroscope (Zeiss,

Oberkochen, Germany) equipped with a micromanipulator (MMO-202ND, Narishige, Tokyo, Japan) coupled to a microinjection unit (IM 300, Narishige, Tokyo, Japan). dsRNA was injected into the peri-vitelline space. Optimal pressure and injection time were empirically determined.

Because harvestman embryos are under high internal pressure, injected solution is frequently ejected from the embryos upon the retraction of the capillary. However, pre-injection dehydration greater than 30 min often leads to embryonic death. Consequently, our injection technique exploits a particular feature of eupnoid harvestman embryos: the eggs of *P. opilio* grow in size during development, requiring contact with a hydrated surface to do so (Gnaspini 2007).

Therefore, injections were performed so as to leave a droplet of dsRNA solution immediately outside the embryo, in contact with the injection site. In 1-2 days, the embryos were observed to absorb more of the solution than could initially be injected into the peri-vitelline space, as inferred from the increasing intensity of green color in the yolk of the embryos and the decreased size of the droplets.

Embryo hatching and imaging

Following injection, embryos were allowed to develop at 28 °C. To remove oil and glue, embryos were washed with heptane. To investigate gene expression, embryos were fixed and *in situ* hybridization was performed as previously described (Sharma et al. 2012a). Embryos were counterstained with Hoechst 33342 1 µg/ml (Sigma-Aldrich, St. Louis, MO, USA). Images were captured using an AxioCam HrC and an AxioZoom V16 stereomicroscope driven by Zen 2011 (Zeiss, Oberkochen, Germany). Leg mounts were imaged using an AxioImager compound microscope driven by AxioVision v 4.8.2 (Zeiss, Oberkochen, Germany).

Embryos were induced to hatch by washing briefly in heptane and subsequently with deionized water. Embryos injected with *DsRed*-dsRNA were allowed to hatch normally. Embryos with severe *Dll* knockdown phenotypes were artificially hatched by manual dissection, using the position of the eyes and the degree of sclerotization (in comparison with control embryos) as indicators of completion of embryonic development. Live hatched embryos were immediately fixed in 96% EtOH. Images were captured using an HrC AxioCam and a Lumar stereomicroscope driven by AxioVision v. 4.8.2 Zeiss (Oberkochen, Germany).

Results

RNAi in *Phalangium opilio*

Eight clutches (n = 710 viable embryos) were subjected to the procedures described above and injected with tinted *DsRed*-dsRNA in order to optimize the injection protocol. Embryos injected with tinted dsRNA solution were observed to absorb the color, which concentrated in the yolk. Those embryos that survived the injection procedure (see below) developed normally until hatching (data not shown), showing that neither the injection procedure, nor exogenous nucleic acids, nor the buffer used for the solution impeded normal development.

Subsequent to protocol optimization, an additional five clutches (n = 454 viable embryos) were subsequently used in RNAi experiments (Table S1). Zygotic injections induced significant mortality in *P. opilio* embryos, with survivorship ranging from 45-59% in various experimental treatments (Fig. 2; Table S1). However, survivorship of uninjected embryos glued to cover slips under oil was also variable (46% in *Dll* experiments; 87% in *dac* experiments). This may be attributable to intrinsic variability in clutch quality (some clutches of wild caught *P. opilio* have smaller eggs and high mortality rates even without dechoriation, with clutch quality declining markedly toward the end of the season; data not shown). This variance may also reflect our accruing experience with handling *P. opilio* embryos, although the experiments were conducted in parallel.

RNAi of *Po-Dll*

In situ hybridizations for *Dll* on embryos with strong *Dll* loss of function phenotypes (described below) showed no *Dll* expression in any of the wild type domains (Fig. 3A), confirming that our

RNAi treatment effectively abrogated *Dll* transcript accumulation. Of the embryos that survived injections of *Dll* dsRNA, 45% (n = 27) underwent a dramatic reduction in length of multiple prosomal appendages. In 89% of embryos with phenotypes (n=24), all prosomal appendages were shortened or nearly missing, retaining only the coxa, or the coxa and some of the trochanter (Fig. 4C, 4D). No labrum was observed in these embryos (Fig. 3B). We interpret these phenotypes as strong loss of function phenotypes of *Dll*. In the remaining 11% (n=3) of embryos with phenotypes, we observed a weak (or mosaic) phenotype, wherein only some appendages were shortened, typically only on one side (Fig. 5B, 5C).

Among severe *Dll* RNAi phenotype embryos, in contrast to endites in *DsRed*-dsRNA injected embryos (Fig. 4B), the pedipalpal and L1 endites did not undergo proximodistal elongation. As a result, the mouth was exposed, and the preoral chamber did not form (Fig. 4D, S1). In *DsRed*-dsRNA injected embryos, outgrowths of these endites formed a wild type preoral chamber (Fig. 4B). In addition, embryos with strong phenotypes featured a reduction of the ocularium, in contrast to *DsRed*-dsRNA injected embryos, which bore a prominent ocularium (Fig. 4A). In the most extreme case, the eyes were disposed flatly onto the prosomal dorsal scutum (Fig. 4C).

In wild type *P. opilio* embryos, *dac* is expressed at early stages in the central nervous system and posterior terminus, and at later stages in the trochanter and femur of the pedipalps and legs, and the proximal chelicera (Sharma et al. 2012b; Fig. 5A). In contrast, in *Dll*-RNAi embryos, *dac* was either absent (presumably due to the absence of appendage tissue; Fig. 5B) or, in those truncated appendages that did form, expressed in the distal-most portion, i.e., in the trochanter (Fig. 5B, arrowhead). In limbs that lacked the trochanters and thus consisted only of the coxa, *dac* expression was not observed in the appendages at all (Fig. 5B; non-specific staining in the termini of the coxae is due to cuticle deposition, but *dac* is not expressed in this region in earlier stages, as in the pedipalpal coxae in Fig. 5B). Non-appendage *dac* domains, including in the central

nervous system and the posterior growth zone, were not affected (Fig. 5B). In embryos with weak phenotypes, *dac* was consistently expressed in the medial appendage segments (Fig. 5C, 5D).

RNAi of *Po-dac*

In order to verify *dac* knockdown, we performed *in situ* hybridizations for *dac* on embryos injected with *dac* dsRNA (Fig. 6). Like uninjected embryos, *DsRed*-dsRNA injected embryos displayed the previously reported (Sharma et al. 2012b) *dac* expression domains in the nervous system, growth zone, and proximal chelicera, and the trochanter, femur, and proximal patella in legs and pedipalps. In contrast to *DsRed*-dsRNA injected embryos (Fig. 6A), *dac*-RNAi embryos typically lacked *dac* expression in the walking legs and pedipalps. However, some embryos retained *dac* expression in the pedipalps (Fig. 6C), and all embryos examined showed *dac* expression in the proximal segment of the chelicerae at levels comparable to those in control chelicerae, even when *dac* expression domains were disrupted or lost in other appendages (Fig. 6B, 6C).

Of the embryos that survived injections of *dac* dsRNA, 24 % (n = 16) underwent loss of one or more segments in the legs and pedipalps (Fig. 7). Determination of segmental identity was based on the shape of the podomeres, as follows. The trochanter is a short podomere with the length approximately equal to the width. The femur is an elongated (i.e., high length-to-width ratio) segment that is wider distally than proximally. The patella of the legs is the only podomere that has a curvature in its shape, forming the “double knee” characteristic of arachnids. The tibia is of approximately cylindrical shape, with its greatest width in the center of its length. The metatarsus and tarsus are the narrowest of all podomeres, and the tarsus is differentiated by the distal tarsal claw and being the longest podomere. We note that the segmental identities are inferences based

on shape and on *dac* expression in wild type embryos; presently, segment-specific markers are not known for embryonic appendage segments in harvestmen.

In two of these 16 embryos (12.5%), we observed that legs were missing a single segment corresponding to the patella, and the pedipalpal patella was either missing or reduced. We interpret this as a weak *dac* loss of function phenotype (Fig. 7E-F). In the remaining 14 embryos (87.5%) the legs and pedipalps lacked two segments, corresponding to the patella and femur; we interpret this as a strong *dac* loss of function phenotype (Fig. 7H-I). The total length of each appendage is reduced as a consequence of these losses, with dramatic shortening of pedipalps and all legs (Fig. 7, S2). Among the legs of embryos that showed strong phenotypes, the observed length difference (with respect to the length of the segments inferred to be missing) corresponded approximately to the combined length of the femur and patella (deviation from expected length difference: 2-15%). Among the pedipalpi of both strong and weak phenotype embryos, the reductions varied much more from expected length differences (up to 60%), which could be attributable to incomplete podomere deletions (e.g., reduced/fused patella in Fig. 7E; possible retention of portion of patella at the distal end of the tibia in Fig. 7H).

Fifteen of the 16 embryos (93.8%) with segmental losses in other appendages bore a three-segmented chelicera comparable to control chelicerae (Fig. 7, compare A and D). However, in a single embryo (6.2%) with a strong *dac* loss of function phenotype, the chelicerae symmetrically lacked the proximal segment and were shorter in length (Fig. 7D, compare A and G).

Discussion

Two motivations underlie the recent pursuit of *Phalangium opilio* as a system for evolutionary developmental study: taxon sampling and character sampling. First, studies of chelicerate development have largely emphasized two orders: spiders and mites. These lineages have enabled comparisons of developmental mechanisms with the other rami of the arthropod tree of life (pancrustaceans and myriapods) (e.g., Prpic et al. 2003; Prpic and Damen 2009; Janssen et al. 2010), but do not suffice to enable polarization of characters within Chelicerata. Study of a member of the chelicerate orders displaying plesiomorphic characters (e.g., book gills, three-segmented chelicerae), and attendant determination of ancestral states, is of particular interest in cases where (1) only one of the two current model lineages demonstrates an unusual developmental trait, exemplified by the role of *Dll* as a gap gene in a spider, but not in a mite (Khila and Grbic 2007; Pechmann et al. 2011); and (2) when both spiders and mites (as well as other derived orders) share a state that is not observed in other chelicerate orders, exemplified by the two-segmented chelicera that lacks a *dac* domain (Prpic and Damen 2004; Pechmann and Prpic 2009; Sharma et al. 2012b).

Second, *P. opilio* has several morphological characters that do not occur in well-established chelicerate species, but are of biological interest. These include the greatly elongated walking legs characteristic of many Opiliones; the numerous tarsomeres (tarsal articles) that subdivide the tarsi, enabling prehensility and grasping; sexually dimorphic cheliceral armature (which also occurs in many non-model spider species); and repugnatorial glands for repelling potential predators. Allometric growth of the appendages is of particular interest in the context of developmental genetics (see for example Mahfooz et al. 2004, 2007).

In the present study, we thus endeavored to develop functional genetic techniques in *P. opilio* for evolutionary inference and study of development in chelicerates. We utilized *Dll* and *dac* as case studies for testing the effectiveness of RNAi and demonstrate both conserved and derived roles of leg gap genes in this group.

Conserved roles of Dll and dac as leg gap genes

Truncation of the distal limb is the archetypal *Dll* phenotype in arthropods, and this has been corroborated in chelicerates (Cohen and Jürgens 1989; Beermann et al. 2001; Prpic et al. 2001; Schoppmeier and Damen 2001; Khila and Grbic 2007). However, identities of deleted segments in the chelicerate leg can only be inferred from spider data, based on embryonic gene expression patterns. In the spider, zygotic knockdown of *Dll* results in the loss of the leg distal to the trochanter, as inferred from the expression of *dac* in *Dll* knockdown embryos (Fig. 4 of Schoppmeier and Damen, 2001). Consistent with these data, we obtained harvestman *Dll* RNAi phenotypes with severe truncations in the distal limbs. As we were able to rear these embryos past the point of podomere formation, and even to assisted hatching, we were able to observe that only the coxa, or sometimes the coxa and trochanter, were retained in limbs with the strongest phenotypes. The loss of the femur and other distal podomeres is consistent with the expression of *Po-Dll* in both spiders and harvestmen. Furthermore, the loss of the trochanter, which does not express *Dll* in later stages, suggests that our RNAi experiments disrupted *Dll* activity at an earlier stage in development.

Consistent with this interpretation, *dac* expression in appendages of *Dll* RNAi embryos is restricted to the trochanter (where it is normally expressed) when this segment is retained. In severe phenotypes wherein trochanter outgrowth is lost, *dac* expression is not observed in the

appendages at all, suggesting that RNAi in these embryos disrupted *Dll* activity and prevented outgrowth prior to the regionalization of the appendages (Fig. 5B, left side of embryo).

The expression of *dac* in wild type early stage embryos of both spiders and harvestmen was reported to be restricted to the trochanter and femur (Abzhanov and Kaufman 2000; Prpic and Damen 2004; Pechmann and Prpic 2009; Sharma et al. 2012b). However, in later stages of development, we observed that *P. opilio* embryos expressed *dac* in the proximal patella of all legs and the pedipalp as well (Fig. 5A). This expansion of the *dac* domain into the patella was previously overlooked in the harvestman, as only earlier developmental stages (comparable to the spider embryos in studies discussed above) were investigated (Sharma et al. 2012b). The overlap between the *Dll* and *dac* domains is therefore more extensive than previously thought. Consistent with these expression patterns, weak *dac* RNAi phenotypes exhibit a loss of the patella in the legs and pedipalp, whereas strong phenotypes exhibit a loss of both the patella and the femur in these appendages (Fig. 7).

Our determinations of which segments were lost in *dac* phenotypes are inferences based on the shape of the podomeres and the reduced total length of the appendage, the latter disfavoring an alternative explanation based exclusively on segmental fusions. Nevertheless, the assignments of segmental identity would benefit from corroboration by gene expression studies. One way to test the identities of the podomeres would be to utilize *exd*, which is expressed as a distal ring in the patella of wild type embryos, as a marker for this segment; however, we were unable to perform this test due to limitations in obtaining *P. opilio* embryos. We presently do not know of other appendage patterning genes that would serve as reliable markers of segmental identity in the harvestman. Moreover, because spiders possess two paralogs of *hth* and *exd*, each of which has a unique suite of expression domains (reviewed by Pechmann et al. 2010), the spider model may be of greater utility for this purpose.

This study nonetheless demonstrates two curious aspects of *dac* activity in the harvestman leg. First, a significant portion of the *Dll* domain overlaps the *dac* domain in the harvestman. Second, this overlap is dynamic, as the *dac* domain expands into the patella in later stages, a new observation not described earlier in chelicerates. A similar case has been reported in the beetle *Tribolium castaneum*, which bears two rings of *dac* in the legs (Prpic et al. 2001). In early stages, a single *Tc-dac* domain is proximal to the *Tc-Dll* domain and corresponds to the more proximal of the two *dac* rings. Later in development, the distal ring of *Tc-dac* expression arises within the *Tc-Dll* domain. Upon completion of appendage formation, the distal *Tc-dac* domain becomes significantly larger and overlaps with the *Tc-Dll* domain. Prpic et al. (2001) contended that the distal domain of *dac* comprised the primordia of the femur and tibiotarsus, and the proximal domain of *dac* likely comprised the coxal primordium.

Knockdown phenotypes of *T. castaneum* at metamorphosis (Suzuki et al. 2009; Angelini et al. 2012b) show that strong *dac* phenotypes have appendages with deletions of the distal femur, the entire tibia, and the proximal tarsomeres, which correspond to the distal embryonic *dac* domain described previously (Prpic et al. 2001). However, the proximal segments coxa and trochanter are not affected even in severe *dac* phenotypes, suggesting that the proximal *dac* domain in *T. castaneum* is not required for metamorphic appendage patterning. Moreover, strong *Tc-Dll* RNAi phenotypes also undergo severe truncations of the limb, up to the proximal part of the femur; the *Tc-Dll* RNAi deletion domain overlaps the entire distal *Tc-dac* domain, which does act as a leg gap gene (Angelini et al. 2012b). Functional data for *dac* activity in embryos of the milkweed bug *Oncopeltus fasciatus* are comparable to those of metamorphic *Tribolium*: knockdown of *Of-dac* results in the loss of the tibia and truncation of the femur in severe phenotypes, whereas knockdown of *Of-Dll* results in the loss of all structures distal to the femur (Angelini and Kaufman 2004). Thus, in both the beetle and the hemipteran, the domain deleted in response to

RNAi targeting *dac* lies almost entirely within the domain deleted in response to *Dll* knockdown, as in *P. opilio*.

We thus observe similarities in *dac* activity in beetles, true bugs, and harvestmen. However, we note that limitations in taxonomic sampling make inferences of *dac* domain evolution across arthropods tenuous: aside from *P. opilio*, functional data on *dac* function in arthropod appendage development are available only from the winged insects (*D. melanogaster*, *O. fasciatus*, *T. castaneum*, and onthophagine beetles; Moczek and Rose 2009). Nevertheless, our data support the conserved roles of *Dll* and *dac* as leg gap genes during limb development in *P. opilio*.

Dll functions in development of the preoral chamber in the harvestman

We previously reported expression domains of *Dll* in the pedipalpal and L1 endites in *P. opilio* and speculated that *Dll* was required to form the preoral chamber (Sharma et al. 2012b). The present study supports this hypothesis, as we observed that individuals with severe *Dll* RNAi phenotypes lacked the preoral chamber. These *Dll* domains are comparable to spider *Dll* domains in the pedipalpal endites (Schoppmeier and Damen 2001), which were previously hypothesized to be involved in forming the spider's "maxilla" (Prpic and Damen 2004; Pechmann et al. 2010). *Dll* expression in endites has been reported in various mandibulates (e.g., Panganiban et al. 1995; Williams 1998; Abzhanov and Kaufman 2000; Rogers et al. 2002; Giorgianni and Patel 2004; Jockusch et al. 2004). Among chelicerates, *Dll* expression has also been reported in the pedipalpal endites of the mite (thought to form the subcapitulum [the inferior part of the gnathosoma], the rutellum [a limb-like projection], and/or the corniculus [a typically horn-like process]; Evans 1992; Thomas and Telford 1999), and in the pedipalpal and all leg endites of horseshoe crabs (Mittmann and Scholtz 2001).

Schoppmeier and Damen (2001) illustrated images of severe phenotypes in spider *Dll* RNAi embryos, which seem to show the loss of the pedipalpal endites (Figs. 4B, 5B in Schoppmeier and Damen 2001), but the focus of the authors was on the distal parts of the limbs. Similarly, in RNAi experiments with the mite *Tetranychus urticae*, Khila and Grbic (2007) observed reduction of the pedipalpal lobes (endites) in a particular class of *Dll* phenotypes, in which the limbs were truncated and antibody staining confirmed effective *Dll* knockdown. Our data, in conjunction with those of Schoppmeier and Damen (2001) and Khila and Grbic (2007), further support the hypothesis that *Dll* is involved in the growth of chelicerate pedipalpal endites (previously discussed by Prpic and Damen 2004 and Pechmann et al. 2010).

These data also suggest that the spider “maxilla” and possibly the mite subcapitulum (and its projections, the rutellum and corniculus) are both homologous to the anterior part of the preoral chamber of harvestmen and scorpions. As with the gnathobases of the last pair of walking legs in the horseshoe crab (Manton 1977; Wyse and Dwyer 1973), all of these structures are involved in feeding. It is therefore tempting to reconstruct an evolutionary history of chelicerates that is characterized by progressive reduction in the number of outgrown endites, with mouthparts becoming increasingly specialized (e.g., suctorial and piercing mouthparts of derived arachnids). However, paleontological data disfavor a progressive reduction in endite number across the chelicerate tree (Weygoldt, 1998). Moreover, the placement of several orders, both extinct and extant, in chelicerate phylogeny is not definitive (Shultz, 2007; Dunlop 2010; Dunlop et al. 2012; Giribet and Edgecombe 2012). Further study is therefore required both for understanding how endites are formed in the mouthparts of the various arachnid orders and for inferring character evolution of the mouthparts based on a resolved arachnid phylogeny.

Dll is required for the growth of the ocularium in the harvestman

We previously postulated that pronounced *Dll* domains in parts of the eye fields (the semilunar lobes) of the harvestman could be involved in patterning the ocularium (Sharma et al. 2012b). However, this hypothesis was speculative, given that *Dll* domains occur in head lobes of other chelicerates that do not form eye mounds, where these domains are often associated with sensory structures (Thomas and Telford 2000; Abzhanov and Kaufman 2000; Mittmann and Scholtz 2001; Schoppmeier and Damen 2001). Here we observed that in *P. opilio* *Dll* RNAi embryos that developed to the normal hatching time, the ocularium was completely absent, with the eyes developing flatly on the prosomal carapace. We presently do not have the toolkit to investigate neurogenetic defects resulting from knockdown of *Dll*, as little is known about harvestman neurogenesis. However, future studies should investigate the role of *Dll* in chelicerate neurogenesis, given that *Dll* has been previously implicated in patterning chelicerate sensory structures and *Dll* domains have been reported in the head lobes of every chelicerate order studied (Mittmann and Scholtz 2001; Schoppmeier and Damen 2001; Khila and Grbic 2007).

The involvement of *Dll* in forming the ocularium demonstrates another case of cooption of leg gap genes to form non-appendage outgrowths, exemplified by the sexually dimorphic horns of onthophagine beetles (Moczek and Nagy 2005; Moczek et al. 2006; Moczek and Rose 2009). Among harvestmen, the ocularium is sexually dimorphic in some species and of highly variable morphology in multiple unrelated harvestman families (Giribet et al. 2010; Sharma and Giribet 2011). The genetic basis for the sexual dimorphism is unknown, and we are presently unable to investigate it further here, because the structure is not sexually dimorphic in *P. opilio*.

Dll in the labrum

As mentioned previously, the nature of the labrum is controversial (Popadić et al. 1998; Budd 2002; Kimm and Prpic 2006; reviewed by Scholtz and Edgecombe 2006; Posnien et al. 2009).

Consistent with data reported in other chelicerates (Thomas and Telford 1999; Abzhanov and Kaufman 2000; Schoppmeier and Damen 2001; Pechmann and Prpic 2009; Khila and Grbic 2007), the labrum of *P. opilio* strongly expresses *Dll* and *Dll* RNAi embryos lack the labrum. However, *Dll* (as well as other leg gap genes) is also involved in the development of non-appendage derived structures, (e.g., bristles and sensory structures; Sunkel and Whittle 1987; Cohen and Jürgens 1989; Williams et al. 2002). Inversely, some appendage-derived structures do not express *Dll* (e.g., insect mandibles, spider book lungs), making this gene an unreliable marker for limb homology. Consequently, our data can neither support nor disfavor the homology of the labrum and arthropod limbs.

dac and the three-segmented chelicera

Wild type *P. opilio* display a three-segmented chelicera that expresses *dac* in the proximal segment, consistent with the hypothesis that the deutocerebral appendage possessed a tripartite domain structure in the last common ancestor of arthropods (Sharma et al. 2012b). Among the 16 *dac* RNAi embryos displaying pedipalp and walking leg phenotypes, a single embryo was observed with a pair of identical two-segmented chelicerae, which were morphologically similar to the chelicerae observed in such chelicerate orders as Solifugae and Pseudoscorpiones (Fig. 5G) (Sharma et al. 2012b). In this phenotype, the decreased length of the appendage and the presence of a chela are consistent with the loss of the proximal segment, wherein *dac* is strongly expressed in wild type embryos (Sharma et al. 2012b). These data, albeit limited, are consistent with our previously proposed hypothesis on the mechanism of the evolutionary transition from the three- to the two-segmented cheliceral type (Sharma et al. 2012b).

The mechanism for the evolutionary transitions between cheliceral types is of great interest due to the recent discovery of a fossil synziphosurine (stem xiphosuran) with elongated, antenniform

cheliceræ (*Dibasterium durgae*; Briggs et al. 2012). At least four distinct segments were reported in the chelicera of this fossil chelicerate species, and the flexion of the appendage suggests numerous cheliceræ articles, possibly comparable to antennules in crustacean antennae. In extant chelicerates, members of a paraphyletic group at the base of Chelicerata (e.g., sea spiders, horseshoe crabs, scorpions, and harvestmen) bear three-segmented chelicerae (called chelifores in sea spiders; four-segmented chelifores also occur in some extant species [members of the genera *Achelia*, *Ammothella*, and *Eurycyde*], and the fossil sea spider *Palaeoisopus problematicus* putatively bears five-segmented chelifores; Arango 2002), whereas the members of derived arachnid orders (e.g., Tetrapulmonata) bear two-segmented chelicerae (reviewed by Sharma et al. 2012b).

This hypothesis of a reductive trend in the chelicera has been previously proposed and formally treated in phylogenetic analyses (Dunlop 1996; Wheeler and Hayashi 1998; Giribet et al. 2002; Shultz 2007). The trend has been extended to the “great appendages” of Cambrian fossil arthropods, which some authors have treated as homologous to chelicerae owing to the inclusion of chelate podomeres (Chen et al. 2004; Haug et al. 2012). However, homology of the Cambrian “great appendage” and the deutocerebral appendage of extant arthropods is controversial, as the former is alternatively interpreted to be protocerebral in origin, whereas the chelicerae of extant Chelicerata are demonstrably deutocerebral (Telford and Thomas 1998; Budd 2002; Jäger et al. 2006; Brenneis et al. 2008). In addition, chelate appendages have evolved convergently several times in many extant arthropods (e.g., pedipalps of scorpions, pseudoscorpions and ricinuleids; great chelae of malacostracan crustaceans and many crustacean pereiopods; the first walking legs of dryinid wasps), disfavoring homology statements based on the chelate condition. Finally, the evidence of a reductive trend has not been conclusively demonstrated even within Early Cambrian arthropods by Chen et al. (2004) or Haug et al. (2011), insofar as these appendages have not been mapped onto a numerical (i.e., cladistic) phylogenetic tree, but rather a non-

analytical hypothetical scenario of cheliceral evolution based largely on cheliceral segment number. In any case, inasmuch as developmental genetics (let alone functional studies) are not feasible in extinct Early Cambrian arthropods, neither the homology statement between appendage types nor the mechanism of reduction in these appendages can be tested.

By contrast, the incidence of more than three segments in the chelifores of fossil and extant pycnogonids, the antenniform chelicera of *Dibasterium*, and the disposition of cheliceral morphology across the phylogeny of arachnids are all analytically consistent with a reductive trend in the chelicera (phylogenetic analyses of Dunlop 1996; Wheeler and Hayashi 1998; Arango 2002; Giribet et al. 2002; Shultz 2007; Briggs et al. 2012). If the portion of the chelicera basal to the chela—the distal-most pair of segments, which are articulated to form a pincer—is homologous among the chelicerates and has undergone progressive reduction, then this putative trend may explain the remarkable morphological disparity between the mandibulate antenna and the chelicera, which are both appendages of the deutocerebral segment (Telford and Thomas 1998; Brenneis et al. 2008). Consequently, the proximal cheliceral segment of such lineages as harvestmen, horseshoe crabs, and sea spiders may represent the remnant of the elongate, flexible portion of the chelicera present in stem Merostomata, such as *Dibasterium*. However, as the expression of leg gap genes in a three-segmented chelicera has only been observed in the harvestman to date, it remains to be tested whether the proximal segment(s) is consistently associated with *dac* expression in other chelicerate lineages, i.e., whether *dac* is a reliable marker for proximal segment identity in the chelicera.

Although our sample size was limited (n = 16 embryos with *dac* phenotypes) due to the difficulties of obtaining large numbers of embryos during the *P. opilio* breeding season, it is nevertheless surprising that only one embryo was obtained with a cheliceral phenotype, whereas the other limbs reliably underwent deletions of one or more segments. This apparent dichotomy

may be attributable to incomplete knockdowns of *dac*. *In situ* hybridizations of *dac* RNAi embryos with a *dac* probe showed that whereas *dac* expression in the legs was reliably disrupted or entirely inhibited by injection of *dac* dsRNA, expression in the chelicerae was always observed (and sometimes in the pedipalps as well; 6B, C). Correspondingly, embryos exhibiting *dac* phenotypes in the legs and pedipalps also formed wild type chelicerae in which the proximal segment retained *dac* expression, even when *dac* was disrupted in the other appendages (Fig. 6B). Our sample sizes for the *dac* experiments do not suffice to examine why *dac* is not easily knocked down in the chelicerae. It may be, for example, that severe *dac* phenotypes missing a portion of the chelicera also undergo neurogenetic defects that limit survivorship. Although we report it here and score it as a “strong” cheliceral phenotype, we are unable to rule out injection artifacts and thus cannot interpret this datum unambiguously. We note, however, that the symmetry of the phenotype (a pair of two-segmented chelicerae) disfavors the possibility of an injection artifact. Furthermore, no such teratology has been reported in any other Opiliones (Juberthie, 1964), lending support to our interpretation that loss of *dac* function was responsible for the loss of a cheliceral segment in our experiments. As *P. opilio* is a wild-caught and seasonal species, the corroboration of this result requires future experiments. The function of *dac* should also be investigated in other lineages with three- and four-segmented chelicerae (e.g., horseshoe crabs, pycnogonids). If our model were correct, *dac* will be expressed in the proximal cheliceral segments and abrogation of *dac* expression in such lineages would also result in the formation of a two-segmented chelicera. Investigation of the four-segmented condition of some pycnogonids’ chelicerae may also elucidate how the proximal region elongates and adds segments in these lineages. This may constitute an exciting avenue for discovery of the developmental basis of the antenniform chelicera, providing a link between the disparate deutocerebral appendages of mandibulates and chelicerates.

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Figure legends

Fig. 1 (A) Phylogeny of Chelicerata indicating topological placement of harvestmen and spiders, the sole chelicerate orders wherein expression domains are known for all four leg gap genes. Topology derived from Giribet et al. (2001), Shultz (2007), and Giribet and Edgecombe (2012). (B) Diagram of adult male *Phalangium opilio*, lateral view of prosoma. (C) Diagram of adult male *Phalangium opilio*, ventral view of prosoma. Coxal apophyses of the pedipalp (Cxa Pp) and first walking leg (Cxa L1) form the preoral chamber (POC). Labels in boldface text in (B) and (C) indicate structures of relevance to the present study. Ch = chelicera; Cx 1-4 = coxa 1-4; Oc = ocularium; Pp = pedipalp; lb = labrum.

Fig. 2 Percentage distribution of phenotypes upon injection of *Dll*-dsRNA (left) and *dac*-dsRNA (right); see Table S1 for numerical breakdown. Phenotypes are color coded as indicated in the legend.

Fig. 3 Expression of the *Phalangium opilio Distal-less* gene in controls and *Dll* RNAi embryos. (A) Stage 17 embryo injected with DsRed-dsRNA. *Po-Dll* is expressed in the distal parts of the chelicerae, pedipalps, and legs. Additional *Dll* domains include the labrum, the pedipalpal and L1 endites (arrowheads), and the central nervous system of the prosoma (arrowheads). (B) Stage 17 embryo injected with *Dll*-dsRNA (strong phenotype). Expression of *Po-Dll* is not observed. Note loss of the chelicerae and labrum, revealing the mouth. Pedipalpal and L1 endites form, but do not form outgrowths in later stages (arrowheads). Pedipalps and legs lack all podomeres except for the coxae. (A'-B') Counterstaining of embryos shown in (A-B) with Hoechst 33342. Scale bars for all figures are 200 μ m. L1-4: leg 1-4; other abbreviations as in Fig. 1.

Fig. 4 *Distal-less* RNAi disrupts formation of the ocularium and preoral chamber of *P. opilio*. (A) Hatchling injected with *DsRed*-dsRNA, in lateral view. Bracket indicates the ocularium, a dorsal outgrowth bearing the eyes. (B) Same hatchling as in (A), in ventral view, showing components of a wild type preoral chamber. Dotted lines indicate outlines of the left pedipalpal and L1 coxae. Black arrows indicate outgrown L1 endites. (C) Hatchling injected with *Dll*-dsRNA, in lateral view. Note the absence of the ocularium, with the eyes flush with the scutum. (D) Hatching injected with *Dll*-dsRNA, in ventral view. Dotted lines indicate outlines of the left pedipalpal and L1 coxae. Note absence of outgrown endites and exposed mouth. Scale bar for all figures are 200 μm . Abbreviations as in Figs. 1, 3.

Fig. 5 Expression of the *Phalangium opilio dachshund* gene in strong and weak *Dll* RNAi phenotypes. (A) Stage 17 embryo injected with *DsRed*-dsRNA showing wild type expression domains of *dac*. Brackets indicate expression domain of *Po-dac* in the proximal part of the patella in pedipalps and walking legs. (B) Stage 19+ embryo injected with *Dll*-dsRNA (strong phenotype), in ventral view. *Po-dac* is expressed in the head, in the nervous system, and in the posterior terminus. *Po-dac* is also expressed in the single remaining trochanter of the right L4 (arrowhead). Asterisks indicate non-specific staining due to cuticle deposition at the termini of the coxae, as observed in previous studies (Sharma et al. 2012a, b). (C) Stage 13 embryo injected with *Dll*-dsRNA (weak phenotype), in ventral view. In this weak phenotype, all appendages except for the right L4 are truncated to some degree, with more consistent truncations on the left side. Note the retention of the labrum and one of the chelicerae. *Po-dac* is expressed in the medial regions of appendages. (D) Same embryo as in (C), in lateral view. In truncated appendages, *Po-dac* is expressed in the distal-most part of the remaining appendage. Scale bars for all figures are 200 μm . Abbreviations as in Figs. 1, 3.

Fig. 6 Expression of the *Phalangium opilio dachshund* gene. (A) Stage 12 embryo injected with *DsRed*-dsRNA in lateral view, showing wild type expression domains of *dac* in appendages. Asterisks indicate non-specific staining due to cuticle deposition in the tips of the chelicerae. (B) Dissected stage 12 embryo injected with *dac*-dsRNA. *dac* expression is observed in the chelicera and pedipalp (arrowheads) and in part of the opisthosomal ventral ectoderm, but not in the legs. (C) Stage 18 embryo injected with *dac*-dsRNA. *dac* is expressed in the proximal segment of the chelicera (bracket) and in the eye fields, in spite of disrupted expression in the pedipalps. Scale bars for all figures are 200 μm . Ef = eye field; other abbreviations as in Figs. 1, 3.

Fig. 7 Appendage mounts of stage 20 control (A-C) and *dac*-dsRNA (D-I) injected embryos. Weak *dac* phenotypes are characterized by the reduction or loss of the patella in the pedipalp (E) and leg (F). Strong *dac* phenotypes are characterized by the loss of the patella and femur in the pedipalp (H) and leg (I). A single embryo bore a two-segmented chelicera (G), in contrast to the three-segmented chelicera observed in remaining RNAi embryos (D) and controls (A). Arrowheads denote locations of segment boundaries. Scale bars for all figures are 50 μm . Chela = union of secondary and mobile segments; Fe = femur; Mt = metatarsus; Pa = patella; Pr = proximal segment; Ta = tarsus; Ti = tibia; Tr = trochanter.

Fig. 8 Summary of RNAi phenotypes in the appendages of *P. opilio*. Top: Functional domains of *Dll* and *dac* in the walking leg. Gradient of *dac* expression in the patella indicates late stage onset of expression. Lighter shading of *Dll* in trochanter indicates retention of this segment in some embryos scored as having a strong phenotype. Note that *dac* is expressed in the trochanter, but this segment is not lost in *dac* RNAi phenotypes. Middle: Functional domains of *Dll* and *dac* in the pedipalp. These are identical to their respective domains in the walking leg. Bottom: Functional domains of *Dll* and *dac* in the chelicera. Lighter shading of *Dll* in proximal part of

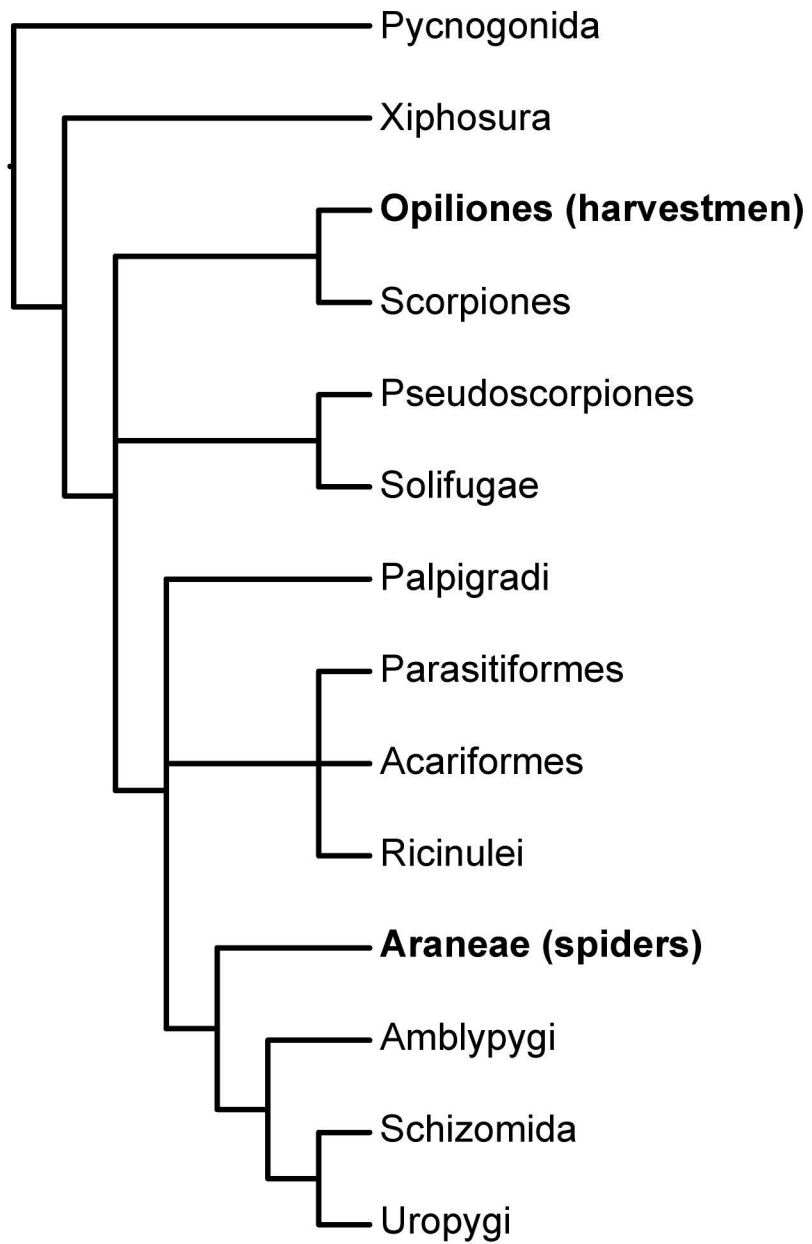
proximal segment indicates in retention in some embryos scored as having a strong phenotype. A possible role for *dac* function in the chelicera is discussed in the text. Px = proximal segment; Sa = secondary article; Da = distal article.

Fig. S1 Detail of ventral prosomal complex of *Dll*-dsRNA injected embryos. (A) Strong phenotype with retention of coxae only (dotted lines). Note absence of coxal apophyses and exposure of the oral cavity (M). (B) Strong phenotype with retention of coxae (dotted lines) and trochanters (brackets for two legs on the right side of the embryo). Note retention of basal-most part of chelicerae, absence of coxal apophyses, and exposure of the oral cavity. Scale bars for all figures are 200 μm

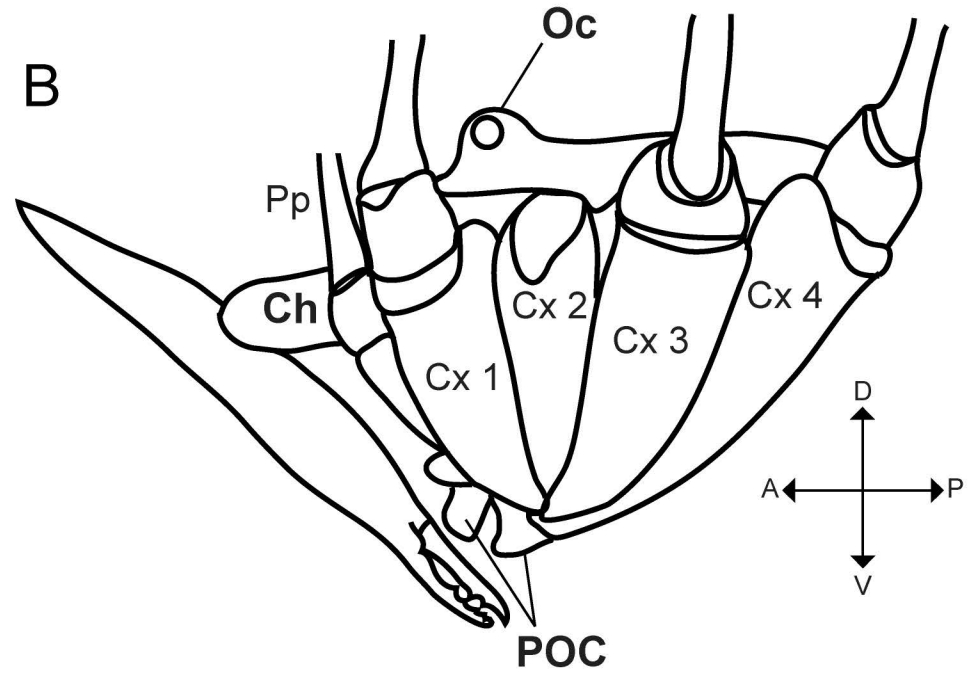
Fig. S2 First walking leg (L1) mounts of stage 20 control (A) and *dac* dsRNA (B, C) injected embryos. Arrowheads denote locations of segmental boundaries.

Table S1 Raw data of distribution of phenotypes upon injection of *Dll*-dsRNA and *dac*-dsRNA.

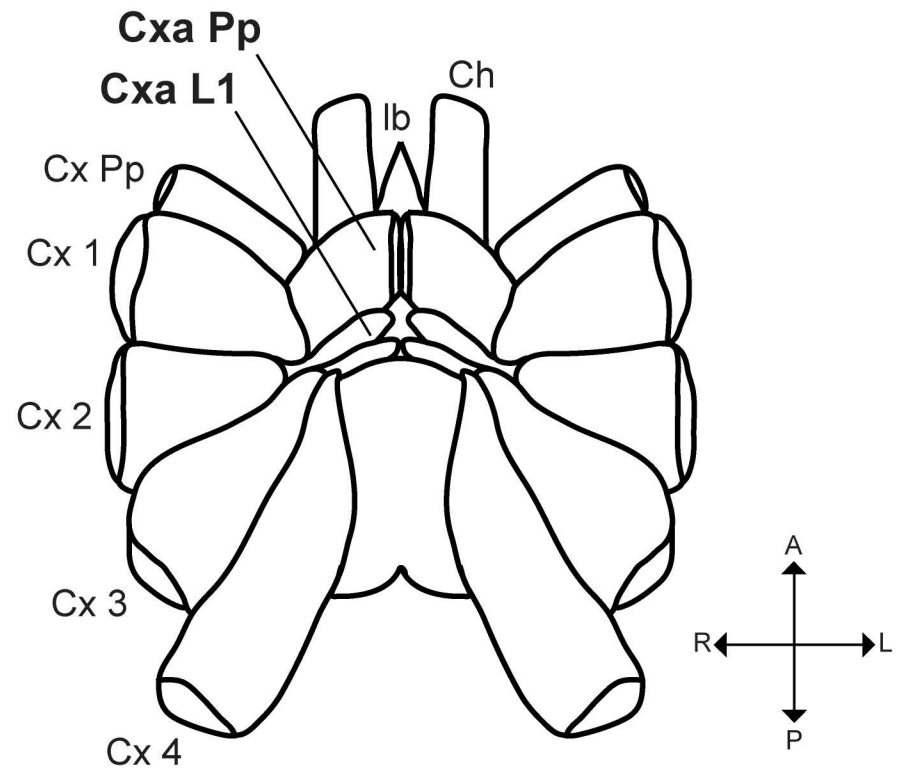
A



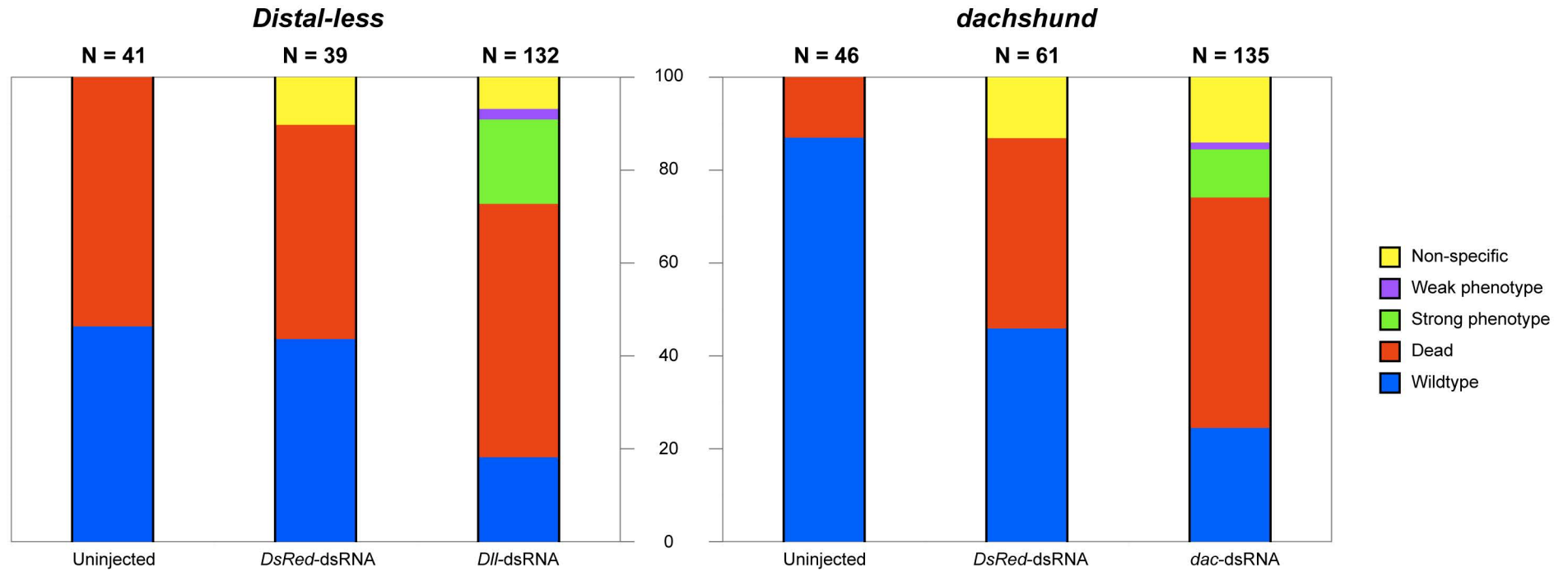
B



C

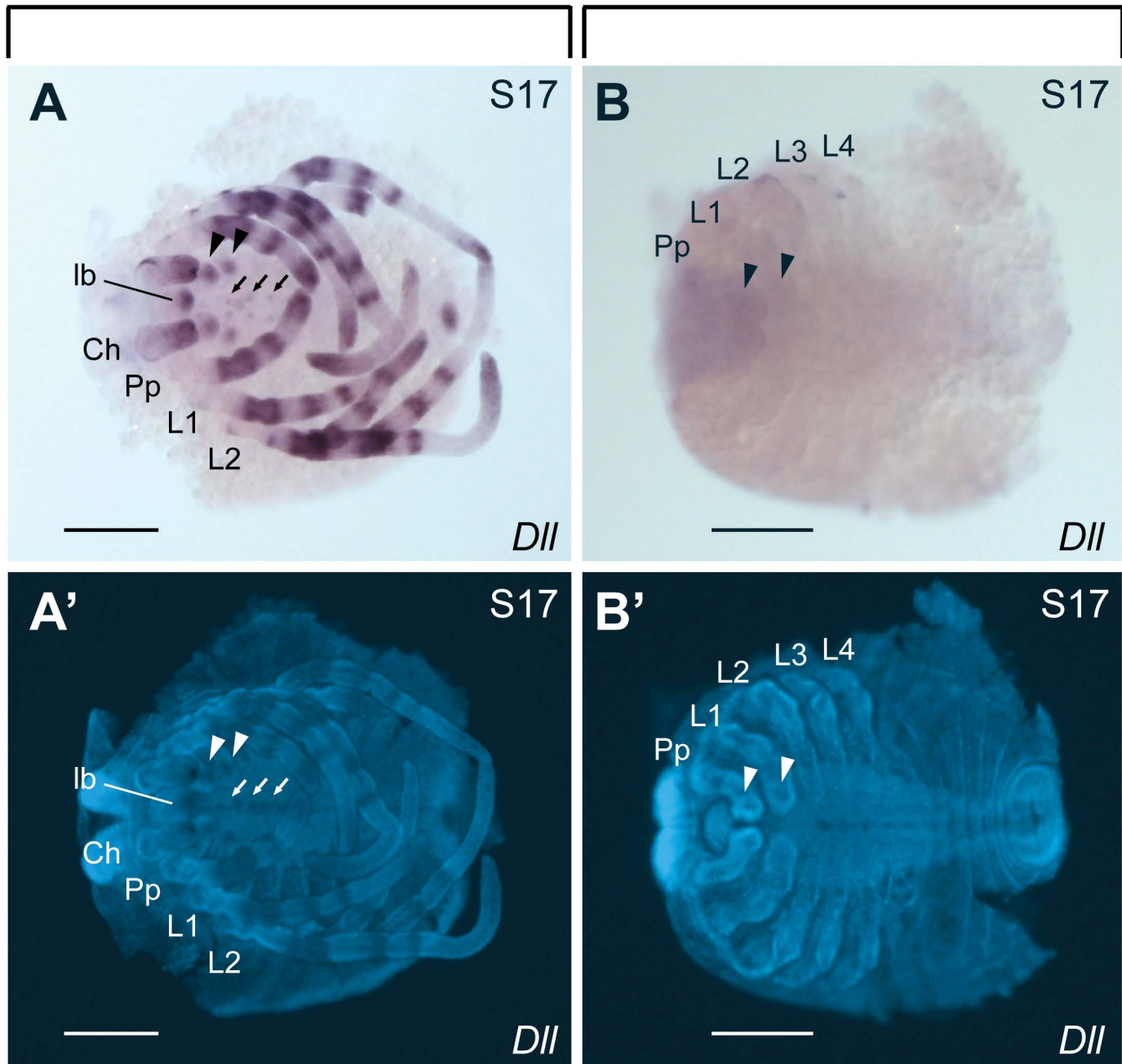


Sharma et al., Figure 2

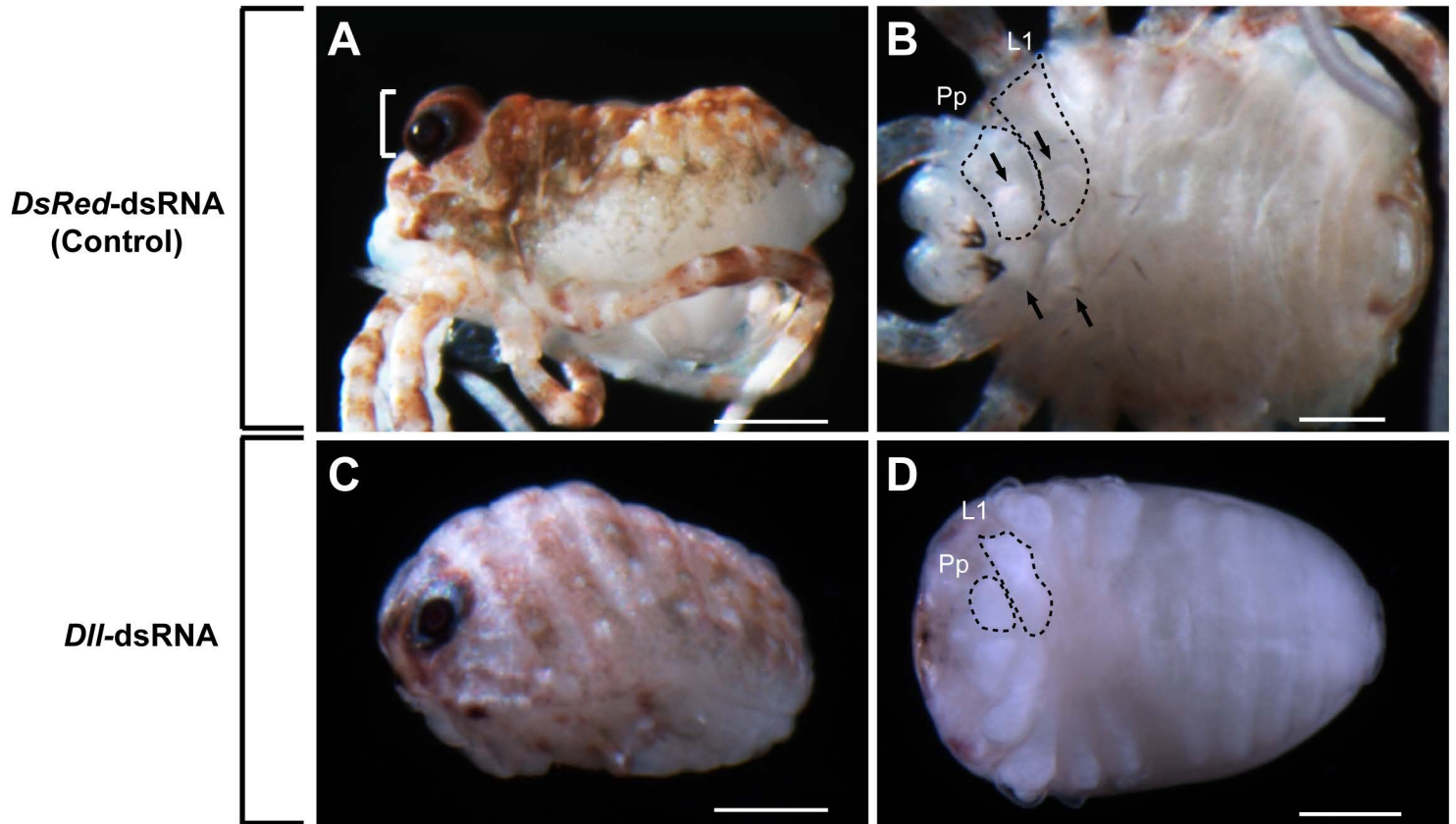


DsRed-dsRNA (Control)

Dll-dsRNA

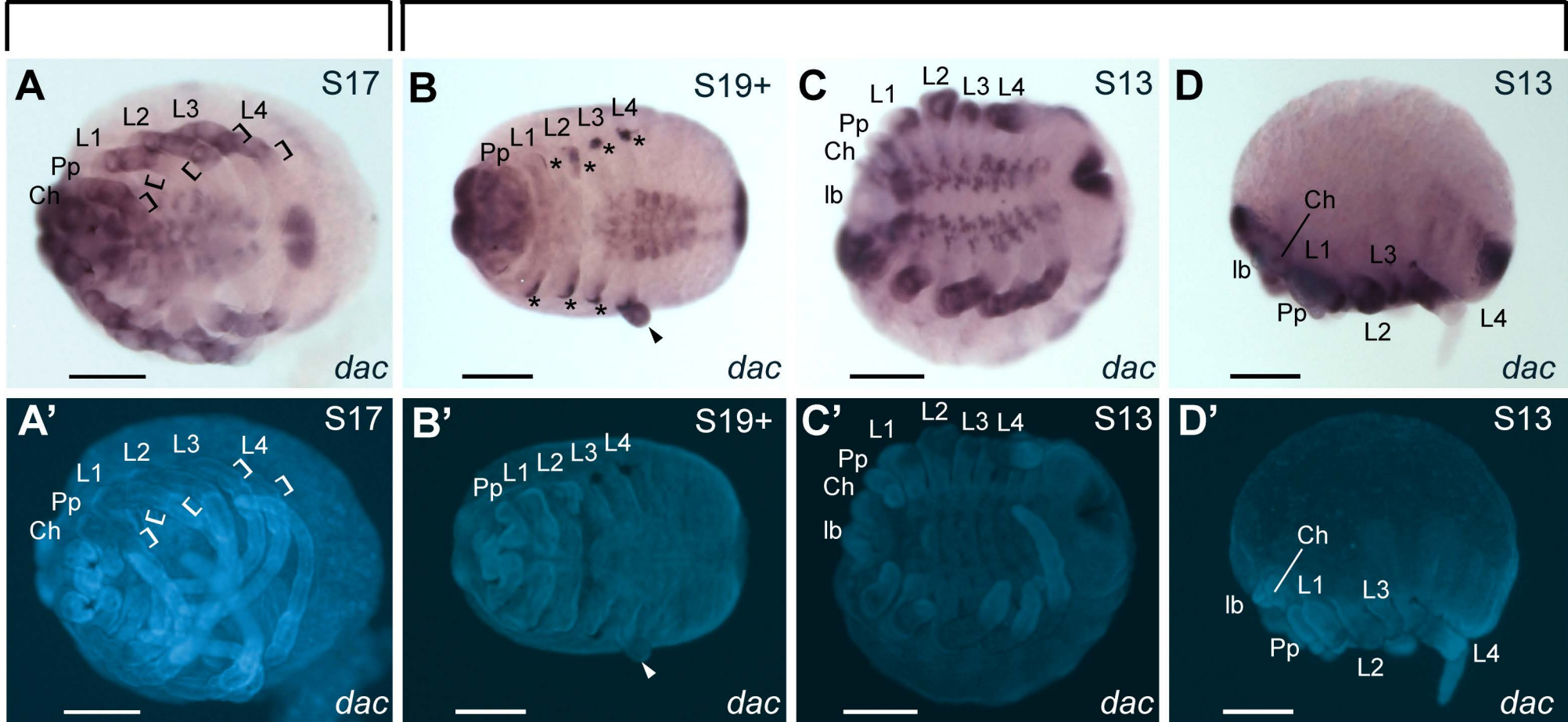


Sharma et al., Figure 4

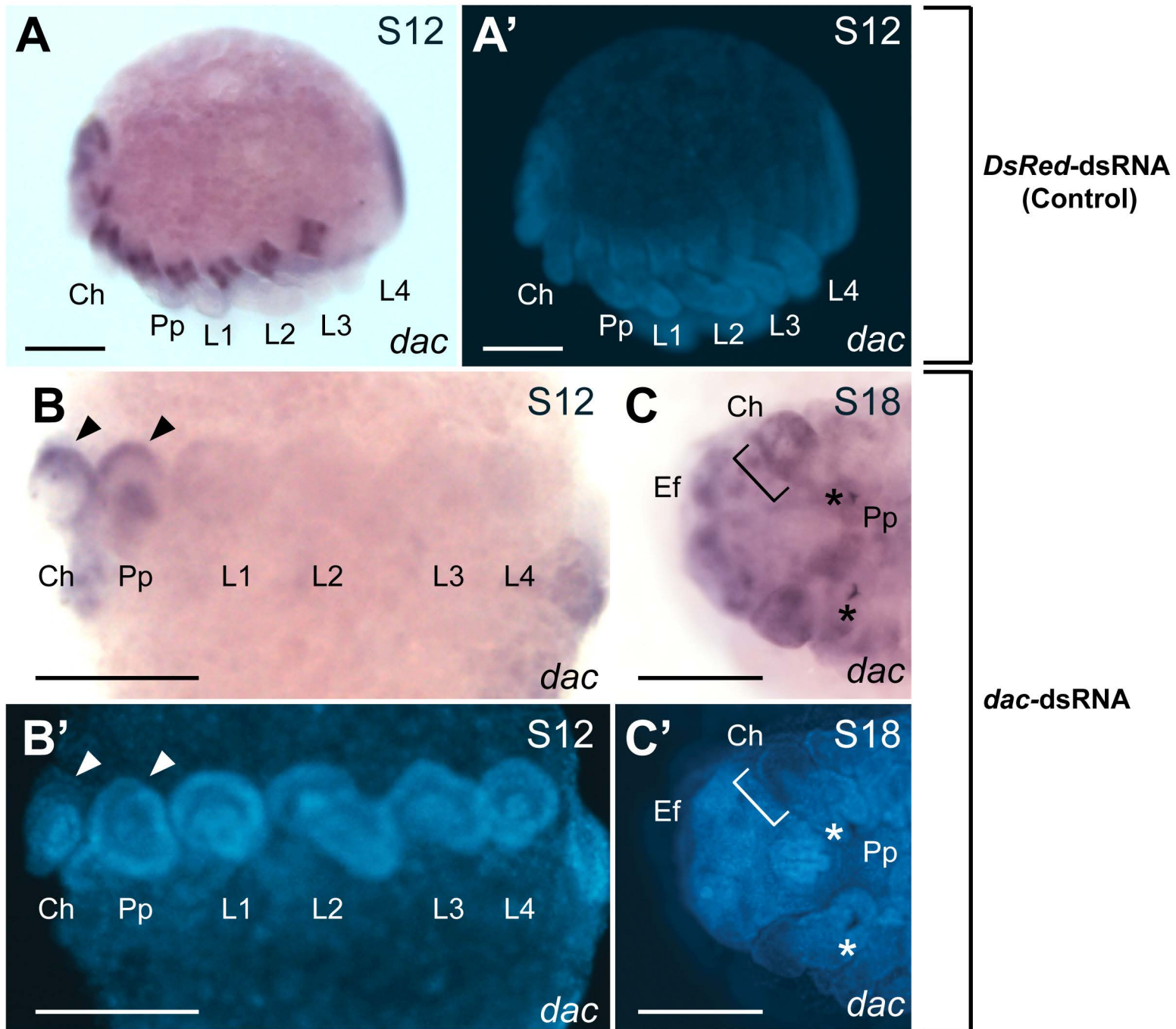


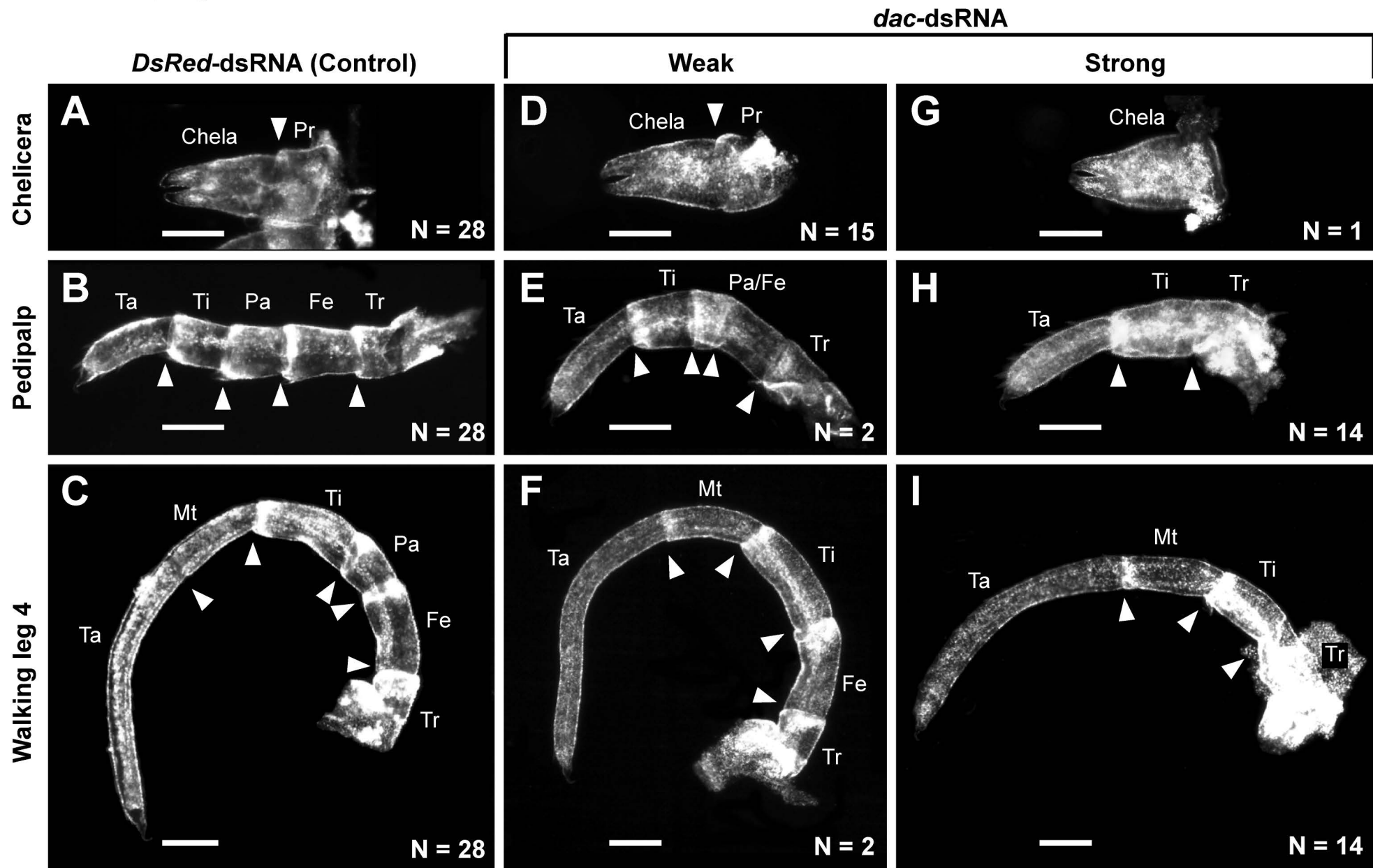
DsRed-dsRNA (Control)

Dll-dsRNA

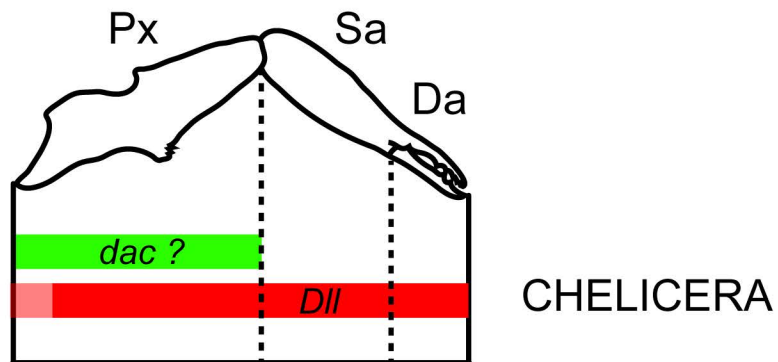
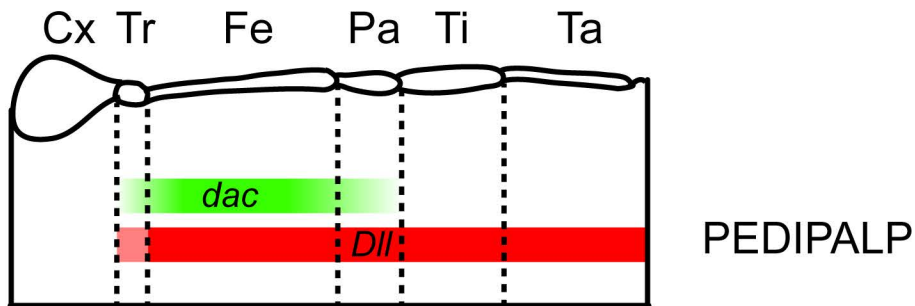
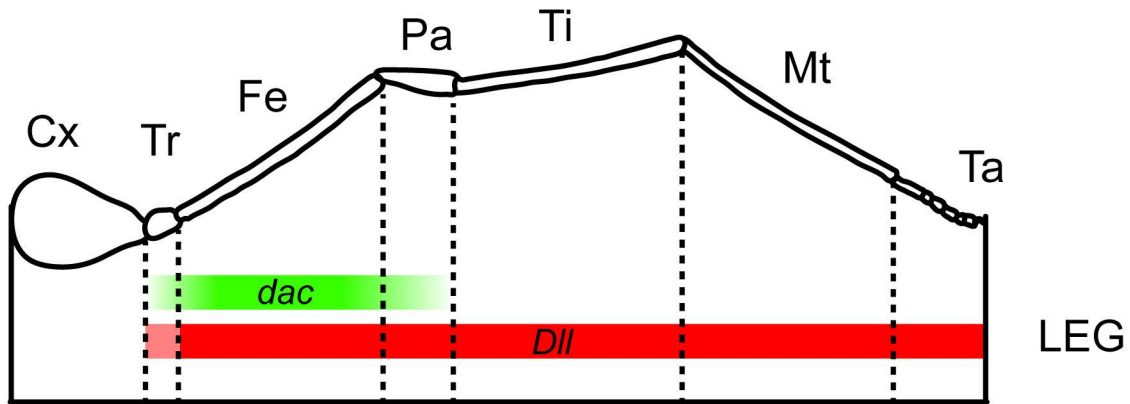


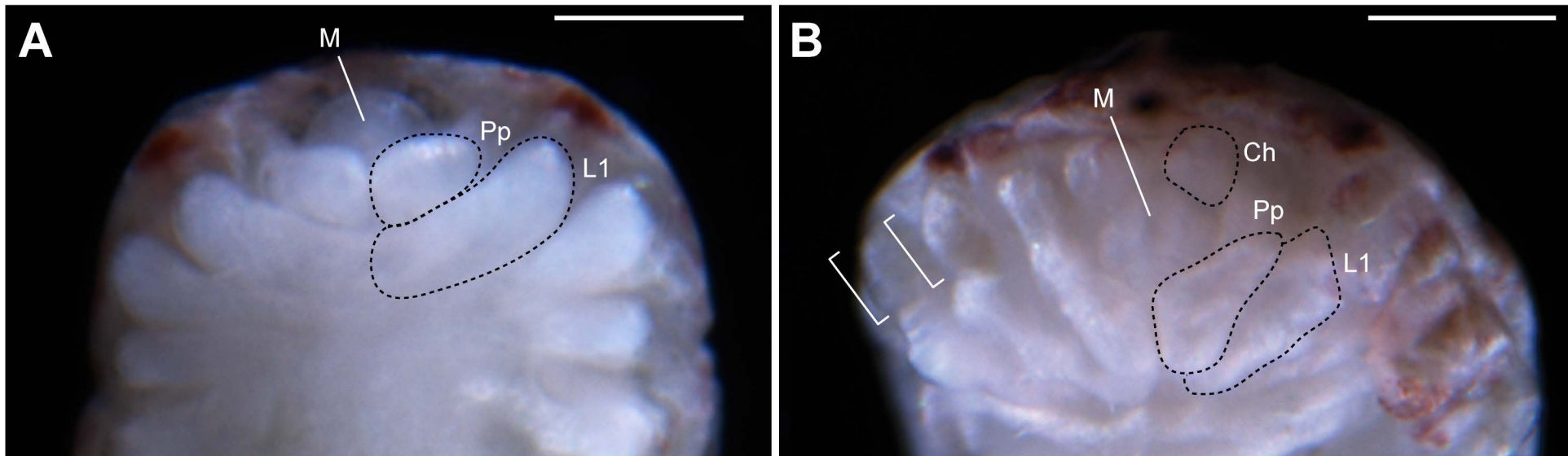
Sharma et al., Figure 6





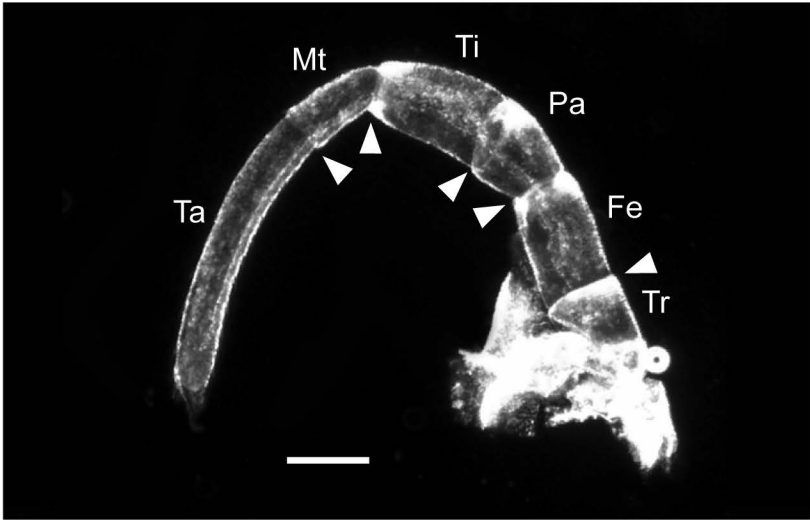
Sharma et al., Figure 8



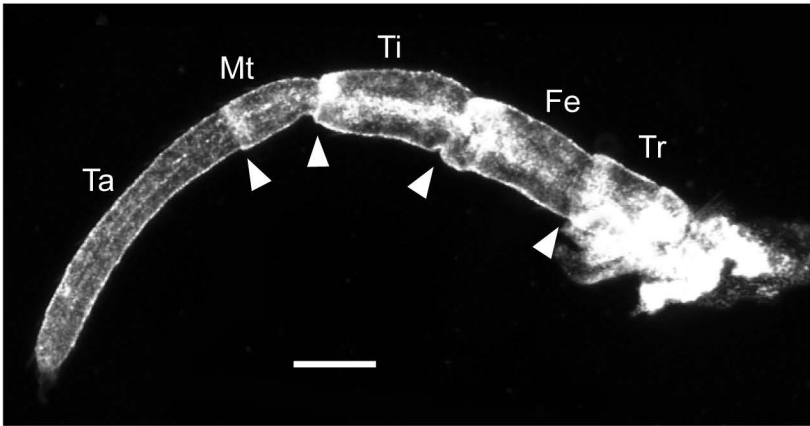


Sharma et al., Figure S2

DsRed-dsRNA
Control



dac-dsRNA
Weak phenotype



dac-dsRNA
Strong phenotype

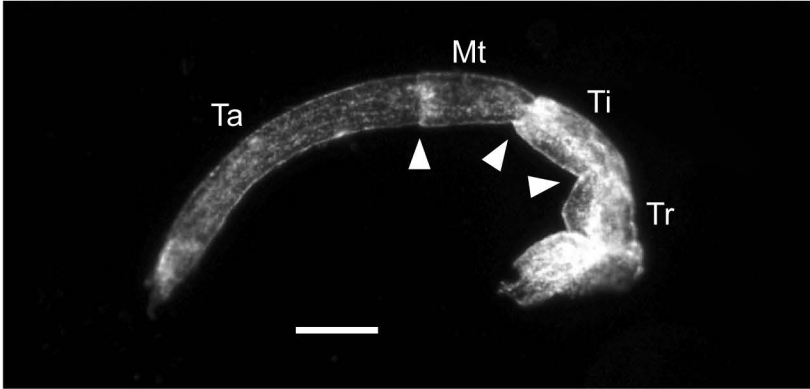


Table S1

	Normal	Dead	Strong phen.	Weak phen.	Non-spec.		Total
<i>Distal-less</i>	19	22	0	0	0	Uninjected	41
	17	18	0	0	4	<i>DsRed</i> -dsRNA	39
	24	72	24	3	9	<i>Dll</i> -dsRNA	132
—————							
<i>dachshund</i>	Normal	Dead	Strong phen.	Weak phen.	Non-spec.		Total
	40	6	0	0	0	Uninjected	46
	28	25	0	0	8	<i>DsRed</i> -dsRNA	61
	33	67	14	2	19	<i>dac</i> -dsRNA	135