GENETIC BASIS FOR ARTHROPOD LIMB DIVERSIFICATION

by

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DEDICATION

For my wife Amber and daughter Sara.

Thank you for being my beacon for those moments when I felt as though I had lost my way.

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ABBREVIATIONS

a1: antenna 1

a2: antenna 2

Abd-A: *Abdominal-A*

Ag: *Anopheles gambiae*

Am: *Apis mellifera*

Antp: *Antennapedia*

A/P: anteroposterior

Af: *Artemia franciscana*

btd: *buttonhead*

cad: *caudal*

co: coxa

Cs: *Cupiennius salei*

D/V: dorsoventral

dac: *dachshund*

DD1: Dachshund Domain 1

DD2: Dachshund Domain 2

Dl: *Delta*

Dll: *Distal-less*

Dm: *Drosophila melanogaster*

dpp: *decapentaplegic*

dsRNA: double stranded RNA

EGFR: Epidermal Growth Factor Receptor

en-: endite

endo: endopod

epi: epipod

esg: *escargot*

exd: *extradenticle*

exo: exopod

fe: femur

fng: *fringe*

Gg: *Gallus gallus*

Gm: *Glomeris marginata*

gn: gnathobase

hth: *homothorax*

I-B: insect-branchiopod

mn: mandible

mx1: maxillule

mx2: maxilla

n-EXD: nuclear-EXD

Of: *Oncopeltus fasciatus*

Ol: *Oryzias latipes*

Ot: *Onthophagus taurus*

P/D: proximodistal

Ps: *Porcellio scaber*

pt: pre-tarsus

RISC: RNA induced silencing complex

RNAi: RNA interference

rsd: *RNAi spreading defective*

Ser: *Serrate*

sid: *systemic RNAi defective*

siRNA: small interfering RNA

St: *Steatoda triangulosa*

t1-5: tarsal segment 1-5

Tc: *Tribolium castaneum*

ti: tibia

Tl: *Triops longicaudatus*

tr: trochanter

Ubx: *Ultrabithorax*

wg: *wingless*

Note: *Drosophila* gene and protein designations are used in *Triops*. Gene names begin with a lowercase letter when the gene is named for a recessive mutant phenotype and an uppercase when the gene is named for a dominant mutant phenotype. Gene symbol is an abbreviation of the gene name. Gene names and symbols are italicized.

Protein products are symbolized by the gene symbol, but this symbol is all in roman capital letters. When the full gene name is used for protein only the first letter of the name is capitalized.

ABSTRACT

Changes in the morphological character of appendages are essential to arthropod diversification and adaptation to a variety of living conditions. For instance, the fruit fly *Drosophila melanogaster* possesses cylindrical, uniramous (unbranched) walking legs that are well suited for terrestrial life, while *Triops longicaudatus* possesses paddle-like, multiramous (multibranched) limbs adapted to aquatic life. Comparative studies of limb patterning between different species of arthropods suggest that most animals utilize a conserved set of genes to construct a limb. How is this common set of genes used to produce morphological divergence? This question is addressed here by examining the spatiotemporal expression patterns of genes shown to establish distinct domains along the proximodistal (P/D) axis in arthropod species with legs morphologically distinct from *Drosophila* legs.

In this dissertation, I investigate the role of the limb patterning genes, *dachshund* (*dac*) and *homothorax* (*hth*), in patterning the appendages of the crustacean *Triops longicaudatus*. I examine the spatiotemporal relationships of the expression of these two essential limb-patterning genes individually and simultaneously with two previously reported leg patterning genes, *extradenticle* (*exd*) and *Distal-less* (*Dll*). I discovered that *Triops dac* and *hth,* as expected, are expressed during leg development. I verified a cell-to-cell association between HTH and nuclear-EXD (n-EXD), a spatial relationship that had only been conjectured to exist outside of *Drosophila.* This spatial relationship represents an ancient unchangeable constraint on limb patterning. HTH expression reported here in addition to previously reported EXD and DLL expressions suggests a

common, early subdivision of the leg into broad proximal and distal domains. However, the reiterated stripes of DAC expression found along the ventral axis do not support establishment of an intermediate leg domain but instead suggest that the ventral branches of the *Triops* limb are generated by a mechanism of segmentation not previously observed in other arthropod limbs. Additionally, I present a record of my attempts aimed at functional determination of genes believed to specify, pattern, or modify branchiopod appendages. Finally, I demonstrated that *dac* functions in the dung beetle leg to properly segment the tarsus as well as producing a structural modification, such as spiked protrusions.

CHAPTER 1

ARTHROPOD APPENDAGE ANATOMY, DEVELOPMENT, AND PATTERNING

Introduction

Arthropods are a speciose phylum composed of four extant sub-phyla: 1) chelicerates, featuring spiders and scorpions, 2) myriapods, characterized by centipedes and millipedes, 3) hexapods, composed of insects and their close relatives, and 4) crustaceans, represented by shrimps, crabs, and kin. Arthropods constitute over 85% of the described species in the animal kingdom and are distinguished by an exoskeleton, segmented body, and jointed appendages (Brusca and Brusca, 2003). Anybody looking at a beetle horn, grasshopper hind leg, or a fly antenna will notice that arthropod appendages have adopted diverse shapes and adapted to multiple functions over the course of evolution. Appendages are used for a variety of tasks, which include sensory perception, feeding, respiration, locomotion, mating, combat, and defense (Brusca and Brusca, 2003). The research reported in this thesis was designed to increase our understanding of the underlying developmental/genetic basis of the diversification of arthropod appendages. I sought to discover whether transformations in morphological form apparent in representative, extant arthropods could be explained by discrete and quantifiable changes in the developmental gene networks responsible for generating their morphology. Gross morphology has provided a foundation for our understanding of arthropod diversity; however, it is only through an exploration of the genetics underlying form that a better understanding of diversity may be achieved. Because of its amenability

for genetic manipulation and molecular investigation, the fruit fly *Drosophila melanogaster* became the model arthropod system for understanding the molecular basis of appendage development. By discovering many of the genes crucial for limb development in *Drosophila*, a gene network responsible for limb patterning was pieced together (Abu-Shaar and Mann, 1998; Cohen et al., 1989; Kojima, 2004; Lecuit and Cohen, 1997; Mardon et al., 1994; Wu and Cohen, 1999). Subsequently, the degree to which this gene network is used in other arthropod species was investigated by comparative studies in other arthropod species (Abzhanov and Kaufman, 2000; Angelini and Kaufman, 2004; Angelini and Kaufman, 2005a; Angelini and Kaufman, 2005b; Inoue et al., 2002; Prpic et al., 2003; Prpic and Tautz, 2003; Prpic et al., 2001; Schoppmeier and Damen, 2001), however the preponderance of the expression and function studies has been in legs morphologically similar to *Drosophila*. These studies, and my own work reported here for the multiramous leg of the tadpole shrimp *Triops longicaudatus*, show conservation of substantial portions of this limb patterning network, as well as some surprising differences. These differences may have a hand in shaping morphology, thereby providing a potential explanation for the genetic basis of morphological diversity. In this introductory chapter, I examine what is known about arthropod leg anatomy and development, highlighting how orthologs of genes participating in *Drosophila* leg development are used during leg development in other arthropods. In addition, I emphasize how RNA interference has been utilized in arthropods to investigate gene function. Furthermore, I discuss how available expression

and functional data may provide further insight into arthropod leg development and diversity.

Arthropod appendages (general features)

Arthropod appendages are an ideal system with which to explore diversity of form given that arthropods have evolved remarkably diverse appendages to accommodate countless niches and lifestyles. But before comparisons of arthropod appendages are made, a basic understanding of their morphology is needed.

When considering all arthropods (Table 1.1), appendages are composed of three basic elements: protopod, exopod, and telopod (Fig. 1.1; Boxshall, 2004; Brusca and Brusca, 2003; Huxley, 1877; Huxley, 1880). Subsequent modifications to this generic limb plan have produced the abundance of limb forms present in extant arthropods. Common alterations to this design include a reduced or lost exopod and/or telopod, additions or deletions of articulated segments, sub-division of segments into non-articulated units, changes in segment size and shape, and addition of endites or exites (Boxshall, 2004).

The protopod (termed coxopodite for insects) is the proximal most portion of the limb, an expansion of the body wall from which the distal limb elements arise. The protopod may be variably segmented in arthropods. In crustaceans, the protopod may be further sub-divided into as many as three segments, termed the pre-coxa, coxa, and basis. In insects, a sub-divided protopod is collectively referred to as a coxa. This rudiment is further defined as the proximal stem that bears the distal rami (branches), which are the telopod and exopod (reviewed in Boxshall, 2004).

The exopod (outer branch) is abundant in crustacean and trilobite fossils, however presence of the exopod is variable in extant crustacean species and virtually non-existent in other arthropod sub-phyla. Appendages missing an exopod but possessing the protopod and telopod are referred to as uniramous (unbranched), whereas appendages containing all three basic elements are called biramous (two-branches). The emergence of arthropods from aquatic environments to terrestrial life is associated with the loss of the exopod (Brusca and Brusca, 2003; reviewed in Giorgianni, 2004). Exopods may have encumbered mobility on land and as a consequence provided a competitive and survival disadvantage.

The telopod represents the limb proper (Snodgrass, 1935) and corresponds to the inner branch (alternatively called the endopod) in a biramous appendage. This element is composed of a variable number of both "true" and "false" segments. "True" segments are characterized by the innervation of distinct muscle attachments whereas "false" segments are not (Snodgrass, 1935).

A common feature in crustaceans but usually absent in hexapods, myriapods, and chelicerates, is the appearance of lobed protrusions from the leg. These lobes are carried on legs, predominantly the protopod, and are generically termed endites and exites. Endites are branches emerging from the medial surface while exites project from the lateral surface. The proximal-most endite is often referred to as a gnathobase and is morphologically distinct from other endites. The lateral lobe located on the pre-coxa or coxa is termed the epipod. During terrestrialization of arthropods, the epipod is believed to have been modified in different ways to form various structures, one being the insect

wing (Averof and Cohen, 1997; Damen et al., 2002). Addition of exites and endites to a limb with a protopod, exopod, and telopod produce what are termed multiramous (multibranched) limbs.

A defining characteristic of arthropods is the presence of a reiterated series of joints along the P/D axis of the leg. Joints sub-divide the leg into repeated units called segments. What is apparent from looking at arthropod legs is that they possess variability in segment number and size. The *Drosophila* leg is composed of ten segments. From proximal-to-distal they are the coxa, trochanter, femur, tibia, tarsal segments 1-5, and a claw-bearing pre-tarsus (Fig. 1.2; reviewed in Kojima, 2004). On the other hand, a typical crustacean leg has seven segments. From proximal to distal they are coxa, basis, ischium, merus, carpus, propodus, and dactylus (Fig. 1.2; Brusca and Brusca, 2003). However, within crustaceans the number of leg segments varies dramatically. In some cases, particularly in species with flexible limbs adapted for swimming in which the leg joints are highly modified, the precise number of segments is difficult to define. For example, the multiramous leg of *Triops longicaudatus* (Fig. 1.3) in some cases is described as having an unsegmented leg (Brusca and Brusca, 2003). However, as two joints are clearly recognizable in the *Triops* limbs, it can be argued that this limb has three distinct segments.

Because of this variability, it is sometimes difficult to know which segments in an insect leg are homologous to which segments in a crustacean leg (Fig. 1.3). For example, if the *Triops* leg is viewed as having three segments because of the presence of discernable joints, is the first segment homologous to the insect coxa, the second a fusion

of the trochanter through tibia, and the final segment a direct homolog of the tarsus? Any such designation would appear quite arbitrary based on comparison of the adult limb morphologies alone. Interestingly, Olesen et al. (2001) proposed that ventral branches (endites) of a phyllopodous leg, which is paddle-shaped and often contains a variable number of branches, are homologous to the segments of a uniramous leg. This proposal arose from a comparison of leg development and the expression of a gene known to be essential for leg patterning in two branchiopod crustaceans, one with a phyllopodous leg, *Cyclestheria hislopi*, and the other with a uniramous leg, *Leptodora kindtii*. Early ontogeny of trunk limbs are similar for both species where their limbs form as ventrally placed, elongated, sub-divided limb buds. In *Cyclestheria* the early buds correspond to the endites and endopod of the adult leg. However, the limb buds in *Leptodora* make up the segments in a segmented uniramous leg. It was initially hoped that a comparison of leg patterning genes across taxa would aide in the identification of homologous leg segments across species (see below).

Arthropod legs may be sub-divided into three general morphological categories: uniramous, biramous, and multiramous (Figure 1.1). Although hexapods and myriapods have appendages representing most or all of the three morphological classes, their legs, as well as those of chelicerates, are predominantly uniramous. On the other hand, crustaceans possess legs from all three morphological groups, thereby making them the ideal clade for examining leg diversity. In order to compare appendages between taxa, the basic underlying developmental and patterning process used for comparison are addressed below.

Leg development

Most arthropods derive their appendages from embryonic limb buds (Fig. 1.4A) that develop directly into adult appendages. Even within arthropods where legs develop from limb buds, differences exist in the extent that the limb primordium occupies the ventral trunk. Limb buds typically occupy only a portion of the ventral trunk in most arthropods, whereas the leg primordium in others like the branchiopod *Triops longicaudatus* occupies the entire ventral portion of the trunk (Fig. 1.4B). By contrast, *Drosophila* and other higher insects undergo metamorphic (holometabolous) development, and adult appendages are produced from imaginal precursor cells (Fig. 1.4C; Gilbert, 2000). These precursor cells form imaginal discs, internalized epithelial sheets of cells that invaginate and reside internally during *Drosophila*'s larval stages. During the larval stages of development, these specialized clusters of cells undergo simultaneous growth and patterning. The discs are transformed into their adult morphology during the pupal stage of the fruit fly life cycle. During metamorphosis the tissue of the imaginal discs evert. The central region of the discs corresponds to the distal region of the adult appendage, whereas the imaginal disc periphery corresponds to proximal limb structures and the body wall. Within holometabolous insects there exists variability in the degree, ranging from some to all, to which appendages are derived from this special population of cells (Svacha, 1992; Tanaka and Truman, 2005; Truman and Riddiford, 2002). Since the life cycle of the fruit fly differs significantly from most arthropods, comparing the development and developmental genetics of limb development across taxa raises a difficult, and at present, mostly unresolved question. Is *Drosophila*

larval leg development directly comparable to leg development in other arthropods or is the process by which imaginal discs are specified in the embryo the more relevant stage to compare to insects and other arthropods with directly developing appendages? The comparisons of leg patterning that have been completed to date suggest striking similarities in the expression of the leg patterning genes used by *Drosophila* during larval stages, thereby demonstrating that genes are not specific to metamorphic leg development and may be used to compare leg development across taxa.

Drosophila-the leg-patterning model

In order to understand how limbs in different species have evolved into morphologically diverse structures, it is important to understand and compare the genetic program underlying limb development in a large variety of species. Since *Drosophila* is the model to which other arthropod leg patterning is compared and more is known about how it patterns its leg, the genes underlying *Drosophila* leg patterning are examined here.

Leg specification: Drosophila leg development begins during embryogenesis as in other arthropods. In the fly, small clusters of epithelial cells are designated as the leg precursors and are referred to as the leg imaginal discs. The leg imaginal discs are distinguished by the expression of the zinc-finger transcription factor, *buttonhead* (*btd*), and the homeobox gene, *Distal-less* (*Dll*), both of which require Wingless (WG) signaling (Fig. 1.5-1; Estella et al., 2003; reviewed in Kojima, 2004; Kubota et al., 2003). Additionally, the activities of the Decapentaplegic (DPP) and Epidermal Growth Factor Receptor (EGFR) signaling pathways restrict the leg precursor cells dorsally and ventrally, respectively, to the ventrolateral region of the thoracic segments (Cohen, 1990;

Gonzalez-Crespo et al., 1998; Goto and Hayashi, 1997). The roles of these three signaling pathways during embryogenesis differ dramatically from their functions during larval stages, which are discussed below, where they guide growth and patterning of the developing leg.

Separation of leg and wing: After specification of the leg primordium, WG, DPP, and EGFR signaling within the leg disc establishes three distinct cell populations: the dorsal sector produces the wing and haltere, the medial region becomes the proximal leg, and the ventral sector creates the distal leg (Cohen et al., 1993; Kubota et al., 2000). *Dll* expression is restricted to the center of the leg primordium and is surrounded by a domain of cells co-expressing the genes *escargot* (*esg*) and *homothorax* (*hth*) (Fig. 1.5-2; Kubota et al., 2003). Expression of these genes roughly corresponds to the generic distal (telopod) and proximal (coxopod) domains of the developing leg. During this early stage of patterning, *esg* and *Dll* depend on input from WG, DPP, and EGFR signaling pathways (Kubota et al., 2000; Kubota et al., 2003). DPP and EGFR signaling pathways, which functioned to suppress leg development during the specification stage, are now essential components of leg development and P/D specification.

P/D elaboration: As mentioned previously, expression of the signaling molecules DPP and WG plays a role in setting up and early patterning of the imaginal disc. Additionally, they establish the dorsoventral (D/V) and P/D axes of the leg imaginal discs (Fig. 1.5-3; Brook and Cohen, 1996; Campbell et al., 1993; Lecuit and Cohen, 1997; Penton and Hoffmann, 1996; Struhl and Basler, 1993) during larval development. DPP is expressed in the dorsal-anterior portion of the *Drosophila* leg imaginal disc whereas WG

is expressed in the ventral-anterior portion. Overlapping gradients of secreted DPP and WG ligands differentially regulate the expression of the limb patterning genes *hth/extradenticle* (*exd*), *dachshund* (*dac*) and *Dll* (Fig. 1.5-3; Abu-Shaar and Mann, 1998; Lecuit and Cohen, 1997; Wu and Cohen, 1999). The primary P/D axis of the leg is sub-divided into a distal domain expressing *Dll* (Fig. 1.5-3; Cohen et al., 1989; Cohen and Jurgens, 1989), an intermediate *dac* expression domain (Fig. 1.5-3; Abzhanov and Kaufman, 2000; Inoue et al., 2002; Mardon et al., 1994), and a proximal domain expressing *hth* and *exd* (Fig. 1.5-3, 1.6; Abu-Shaar and Mann, 1998; Aspland and White, 1997; Erkner et al., 1999; Gonzalez-Crespo and Morata, 1996; Rieckhof et al., 1997; Wu and Cohen, 2000). In addition to morphogens, regulatory interactions between the P/D genes are essential for clear establishment of these discrete expression domains. These interactions involve mutually exclusive regulatory relationships, which exist between *hth* and *dac*, between the *hth* target gene *teashirt* and *dac*, and between *dac* and *Dll* (Abu-Shaar and Mann, 1998; Dong et al., 2001; Erkner et al., 1999; Wu and Cohen, 1999). Mutations of these broadly expressed genes produce deletions, or gaps, of leg segments, comparable to the phenotypes generated by the gap genes that function in embryonic segmentation (Campbell and Tomlinson, 1998; Cohen and Jurgens, 1989; Gorfinkiel et al., 1997; Mardon et al., 1994; Wu and Cohen, 1999). Hence these genes are collectively referred to as leg gap genes.

Leg segmentation: Once limb development is initiated, proper axial and domain identities are maintained by the previously mentioned mutually antagonistic relationships between the leg gap genes (Abu-Shaar and Mann, 1998; Dong et al., 2001; Erkner et al.,

1999; Jiang and Struhl, 1996). The next step in leg development is to divide the leg further into segments. Despite the establishment of five discrete domains based on leg gap gene expression, these gene expression domains do not correspond one to one with distinct leg segments (Fig. 1.6; reviewed in Kojima, 2004). However, Rauskolb and Irvine (1999) suggested that the leg gap genes act alone or in combination to establish the reiterated rings of expression of the Notch ligands *Delta* (*Dl*) and *Serrate* (*Ser*) (Greenwald, 1998; Kimble and Simpson, 1997) as well as the Notch signaling modulator, *fringe* (*fng*) (Fig. 1.5-4; Irvine and Wieschaus, 1994; Panin and Irvine, 1998; Panin et al., 1997). These rings are expressed in a segmentally repeated pattern (Bachmann and Knust, 1998; de Celis et al., 1998) and their expression, as well as expression of activated Notch, corresponds to joints. In addition, null and temperature sensitive mutants of those genes resulted in a loss of distinct joints and subsequent fusions of leg segments (de Celis et al., 1998; Parody and Muskavitch, 1993; Shellenbarger and Mohler, 1978; Speicher et al., 1994). A temporal component to leg segmentation was also found to exist as demonstrated by the progressive increase in the number of *Dl*, *Ser*, and *fng* rings (Fig. 1.5; Rauskolb and Irvine, 1999) as the imaginal disc grows. This increase in the number of rings corresponds well with changes in the expression patterns of the leg gap genes during imaginal disc growth (Abu-Shaar and Mann, 1998; Campbell and Tomlinson, 1998; Gorfinkiel et al., 1997; Lecuit and Cohen, 1997; Wu and Cohen, 1999) where new exclusive and co-expression domains are added over time. Finally, Rauskolb (2001) showed that the leg gap genes are key regulators of Notch ligands and *fng* expression, further associating leg gap genes with the leg segmentation process. Therefore, it is

plausible that a similar relationship between leg gap genes and the Notch ligands exists in other arthropod legs.

Leg gap genes in other arthropods legs

As previously mentioned, *Drosophila* leg development is a derived condition and is not truly representative of the process for arthropods in general. Most arthropod appendages are directly derived from the outpocketing of the embryonic body wall. Nonetheless, commonalities in the patterning network underlying fruit fly leg development have been identified in other arthropod species. This section highlights leg gap gene expression in arthropods with morphologically similar (uniramous) and different (biramous and multiramous) legs.

Uniramous legs: Features of leg gap gene expression appear very well conserved throughout arthropods. *Dll* is expressed in the distal region of every major arthropod sub-phylum (Fig. 1.7; Abzhanov and Kaufman, 2000; Angelini and Kaufman, 2004; Beermann et al., 2001; Panganiban et al., 1997; Panganiban et al., 1994; Panganiban et al., 1995; Prpic and Tautz, 2003; Schoppmeier and Damen, 2001). In addition to the distal *Dll* domain, a proximal ring of expression has been observed in many insects, including *Drosophila*, as well as in the millipede (Fig. 1.7; Abu-Shaar and Mann, 1998; Abzhanov and Kaufman, 2000; Angelini and Kaufman, 2004; Beermann et al., 2001; Inoue et al., 2002; Jockusch et al., 2000; Mardon et al., 1994; Prpic et al., 2001; Rogers et al., 2002). These two *Dll* domains are commonly referred to as the "ring and sock" pattern of *Dll*. The proximal ring domain is associated with the femur-trochanter boundary and the distal sock domain corresponds to the distal tibia and tarsus. *dac* is

expressed in an intermediate leg location in every arthropod it has been studied in and is generally positioned between the *Dll* "ring and sock" where those domains are present (Fig. 1.7; Abu-Shaar and Mann, 1998; Abzhanov and Kaufman, 2000; Angelini and Kaufman, 2004; Beermann et al., 2001; Inoue et al., 2002; Jockusch et al., 2000; Mardon et al., 1994; Prpic et al., 2001; Rogers et al., 2002). In the fly, flour beetle, millipede, and spider co-expression of *hth*/*exd* is basically limited to the proximal leg region (Abu-Shaar and Mann, 1998; Cohen et al., 1989; Prpic et al., 2003; Prpic and Tautz, 2003; Wu and Cohen, 1999). These three domains pattern all uniramous legs in which the leg gap genes have been studied. However, differences in the details of the spatial relationships between the leg gap genes do exist and are described below.

As previously mentioned, *dac* is initially expressed exclusive of *Dll* in the *Drosophila* leg imaginal disc. As the limbs of *Drosophila* grow, overlap in *dac* and *Dll* domains increases substantially in the region corresponding to the distal tibia-basal tarsus (Fig. 1.6, 1.7; Lecuit and Cohen, 1997). On the other hand, the intermediate *dac* domain develops within a *Dll* domain in the beetle (Prpic et al., 2001), the myriapod (Prpic and Tautz, 2003), the spider (Prpic et al., 2003), and the pill bug (Abzhanov and Kaufman, 2000) where the pattern ultimately resolves into a *dac* domain flanked by a "ring and sock" pattern of *Dll* (Fig. 1.7). The initial spatial relationship between *dac* and *Dll* differs the fly from the other arthropods and may be one of the defining features distinguishing metamorphic leg development from legs derived from embryonic limb buds. Additionally, the myriapod and pill bug differ from other arthropods in that the entire intermediate *dac* domain lies within the *Dll* domain (Fig. 1.7). What emerges from the

dac and *Dll* expression data is that differences exist between the arthropod sub-phyla. One commonality in insects and spiders is the presence of a *dac* domain lacking *Dll* expression, which suggests an antagonistic relationship between *dac* and *Dll* like that in *Drosophila*. However, *dac* and *Dll* co-expression in the intermediate leg domain of the myriapod and crustacean shows that *dac*/*Dll* antagonism present in *Drosophila* is missing from these two species. The implications of this are discussed below.

One additional commonality emerges from the spatiotemporal patterns previously described for *dac* and *Dll* and that involves the activation of *dac* after *Dll* activation. In the *Drosophila* leg imaginal disc, *Dll* expression is activated long before the appearance of *dac*. *dac* is activated in the late second instar leg imaginal disc in a ring sandwiched between *hth* and *Dll* expression domains with minimal overlap with either gene (Abu-Shaar and Mann, 1998; Lecuit and Cohen, 1997). In *Tribolium*, *dac* expression appears in an intermediate domain buried within a *Dll* expression domain (Prpic et al., 2001). In all other arthropods where *Dll* and *dac* expression have been examined, *Dll* expression precedes *dac* expression in the telopod. Therefore, the activation of *Dll* before *dac* in the telopod appears to be a common and conserved theme in arthropods. This suggests that *dac* is activated later in leg development to pattern intermediate leg structures from the primary distal leg domain.

How does *hth*/*exd* expression relate to *dac* and *Dll* expression in other arthropod legs? Expression studies of *hth*, besides those in *Drosophila*, were limited to *hth* mRNA expression. In species where both *exd* and *hth* mRNA expression have been examined, functional HTH/EXD duplexes are conjectured to exist where the messages of both genes

are expressed. Where expression of both is known for a particular species, they are represented here as a functional unit. *hth/exd* is detected in the early leg primordium of *Drosophila* embryos and is subsequently expressed in a ring surrounding a central *Dll* expressing region (Abu-Shaar and Mann, 1998; Cohen et al., 1989; Wu and Cohen, 1999). As the limb grows, *hth/exd* expression is restricted to the proximal domain, which will ultimately become the coxa, trochanter, and extreme proximal region of the femur in the adult fly. This pattern is similar in other arthropod species. During the late third larval instar of *Drosophila*, *dac* and *Dll* expression overlap *hth*/*exd* expression in the distal trochanter and proximal femur primordium (Fig. 1.5, 1.7; Abu-Shaar and Mann, 1998; Rieckhof et al., 1997). In addition to its proximal domain of expression an intermediate band of *hth/exd* is detected in *Tribolium* and the spiders (Fig. 1.7; Abzhanov and Kaufman, 2000; Prpic et al., 2003) where it potentially overlaps with *dac* expression. When considering *hth* alone, *hth* expression appears to overlap entire *dac* domain in *Cupiennius* (Prpic et al., 2003) and *Glomeris* (Prpic and Tautz, 2003). As mentioned in *Drosophila*, *dac* and *hth* have mostly a mutually exclusive regulatory relationship, therefore *hth* expression in a region totally overlapping *dac* in the spider and millipede suggests the ability of *dac* to repress *hth* expression in these species appears to be lost. Again, the implications of this are discussed below.

Biramous and multiramous legs: To date, the only leg gap genes for which expression has been reported in biramous and multiramous leg morphologies are *exd* and *Dll*. EXD was seen only in the proximal (protopod) portion of the biramous leg (see biramous leg Fig 1.1; Giorgianni and Patel, 2004) whereas DLL was shown only in the

distal branches (exopod and telopod) (see biramous leg Fig. 1.1; Giorgianni and Patel, 2004; Panganiban et al., 1995). This is consistent with their proposed roles for establishing proximal and distal leg domains, respectively, in uniramous legs. In phyllopodous and multiramous legs, early expression of EXD and DLL appear to be used to define a single P/D axis as it does in *Drosophila* (Gonzalez-Crespo and Morata, 1996; Panganiban et al., 1995; Williams et al., 2002). Additionally, EXD and/or DLL were found expressed in every branch of the branchiopod leg, however, no common denominator was observed for patterning all of the leg branches. EXD/DLL patterns depended on position of the branch within the leg and stage of developmental maturity (Williams et al., 2002). Therefore, an unknown mechanism, which differs from primary growth and patterning of the P/D axis, was speculated to regulate branch growth in a multiramous leg (Williams et al., 2002).

The spatial organization of the leg gap genes described in *Drosophila* along the P/D axis appears relatively conserved in other arthropods, however differences in the details of the spatial relationships of the leg gap genes do exist. In uniramous legs, the described differences indicate that arthropod leg development is capable of tolerating numerous variations in the spatial relationships of the leg gap genes without dramatic alterations to morphology, such as the creation of leg branches. Although the differences may influence aspects of uniramous leg development such as growth, segment number, and segment morphology. However, in biramous and multiramous legs, morphological modifications like branches are associated with expression of the leg gap genes. Additionally, EXD and DLL expressions have suggested that, like uniramous legs, both

biramous and multiramous legs possess basic proximal and distal domains. Ultimately, adding expression data for more genes will improve comparisons between the three morphological categories.

Functional testing

Expression studies provide a basic understanding of the molecular pathways used for patterning. The development of gene knockdown approaches, such as RNA interference (RNAi), has allowed researchers to investigate gene function in systems where genetic manipulation has not been established in order to ascertain what gene functions are conserved or divergent. *hth* and *dac* have been functionally tested in *Oncopeltus* where fusion of proximal leg structures and loss/fusion of intermediate leg domains, respectively, were observed (Angelini and Kaufman, 2004). These phenotypes are consistent with mutations of these genes in *Drosophila* (Casares and Mann, 2001; Mardon et al., 1994). Additionally, the role of *Dll* in appendage development has been examined in numerous systems utilizing RNAi. RNAi of *Dll* in *Tribolium*, *Cupiennius*, and *Oncopeltus* produced legs lacking distal leg structures (Angelini and Kaufman, 2004; Bucher et al., 2002; Schoppmeier and Damen, 2001). The *Tribolium* phenotype was similar to those of isolated and maintained *Dll* mutant strains and the phenotypes generated in all species coincided well with anticipated phenotypes. RNAi was even shown to be a viable method for exploring gene function in the branchiopod crustacean *Artemia* (Copf et al., 2004). These examples demonstrate the value of RNAi as an investigative tool for function in arthropods.

Conclusion

Expression and functional data for patterning genes, such as *hth*/*exd*, *dac* and *Dll*, from a diverse sampling of insects and crustaceans, are key to understanding what aspects of the limb patterning network are conserved and what features are associated with altered morphologies. Although data suggest that limb formation is a conserved process, the degree to which the spatial domains and interactive relationships of the limb-patterning components exist in other arthropods varies. Additional differences, such as the association of patterning genes with leg segments, are also apparent within arthropods (Abzhanov and Kaufman, 2000). What emerges is a picture where uniramous morphology is capable of withstanding changes in the spatial relationships of the leg gap genes essential for patterning. These reported changes in the expression patterns of the leg gap genes along the P/D axis of the leg and their corresponding association with leg segments may represent phylogenetically significant differences in leg segment number as well as morphology. However, these observations are based on comparative studies on limb patterning genes in taxonomically diverse species with predominantly morphologically similar legs.

Even though studies in species with morphologically similar legs have proven valuable by illuminating what features of limb patterning are evolutionarily conserved or changing for generically similar shapes, the next step required to advance our understanding of how changes to the limb patterning network have led to diversity is to compare various aspects of this program amongst species from different taxa with morphologically diverse limbs. Multiramous legs are dramatically different from

uniramous legs, therefore predictions about P/D genes and their relationship to morphology are challenging. DLL and EXD were originally shown to divide *Artemia* and *Triops* leg initially into two basic domains, proximal and distal. How then would DAC and HTH expression relate to multibranched limb development and morphology? This question is addressed in chapter two of this thesis. A prediction for the role of HTH was relatively straightforward; HTH would mimic n-EXD and play a role in establishment of the proximal limb. *dac* expression and subsequent pattern were more of a mystery. Since the entire limb primordium appeared to express either DLL or EXD, *Triops* legs were conjectured to lack *dac* expression or expression would coincide with expression of one or both genes. However, based on leg gap gene expression in other arthropods, *dac* expression in the branchiopod legs might be expected to overlap with the proximal portion of the distal DLL domain along the P/D axis. DLL and n-EXD patterns have already shed some light on the relationships between multiramous and uniramous legs. Both appear to establish a proximal and distal leg domain early in leg development. By adding the expression patterns for DAC and HTH, perhaps better comparisons between these diverse limb morphologies will be possible.

Group	Class	Order	Species	Common name
Hexapods	Insecta	Diptera	*Drosophila melanogaster*	fruit fly
			Anopheles gambiae	mosquito
		Coleoptera	*Tribolium castaneum*	red flour beetle
			Onthophagus taurus	dung beetle
		hymenoptera	*Apis mellifera*	honey bee
		Lepidoptera	*Hyalophora cecropia*	giant silk moth
		Hemiptera	*Oncopeltus fasciatus*	milkweed bug
Crustaceans	Branchipoda	Notostraca	*Triops longicaudatus*	tadpole shrimp
		Anostraca	*Artemia franciscana*	brine shrimp
		Diplostraca	*Leptodora kindtii*	giant water flea
			Cyclestheria hislopi	clam shrimp
	Malacostraca	Isopod	*Porcellio scaber*	sow bug
		Mysida	*Americamysis bahia*	opossum shrimps
		extinct (fossil)	*Trilobite*	Trilobite
Myriapods	Diplopoda	Glomerida	*Glomeris marginata*	millipedes
Chelicerates	Arachnida	Araneae	*Steatoda triangulosa*	cobweb spider
			Cupiennius salei	hunting spider
		Ixodida	*Amblyomma americanum*	lone star tick
			Ixodes scapularis	blacklegged tick

Table 1.1. Table of arthropod species. The table shows the major arthropod groups and constituent species (with common names) that are discussed throughout this thesis.

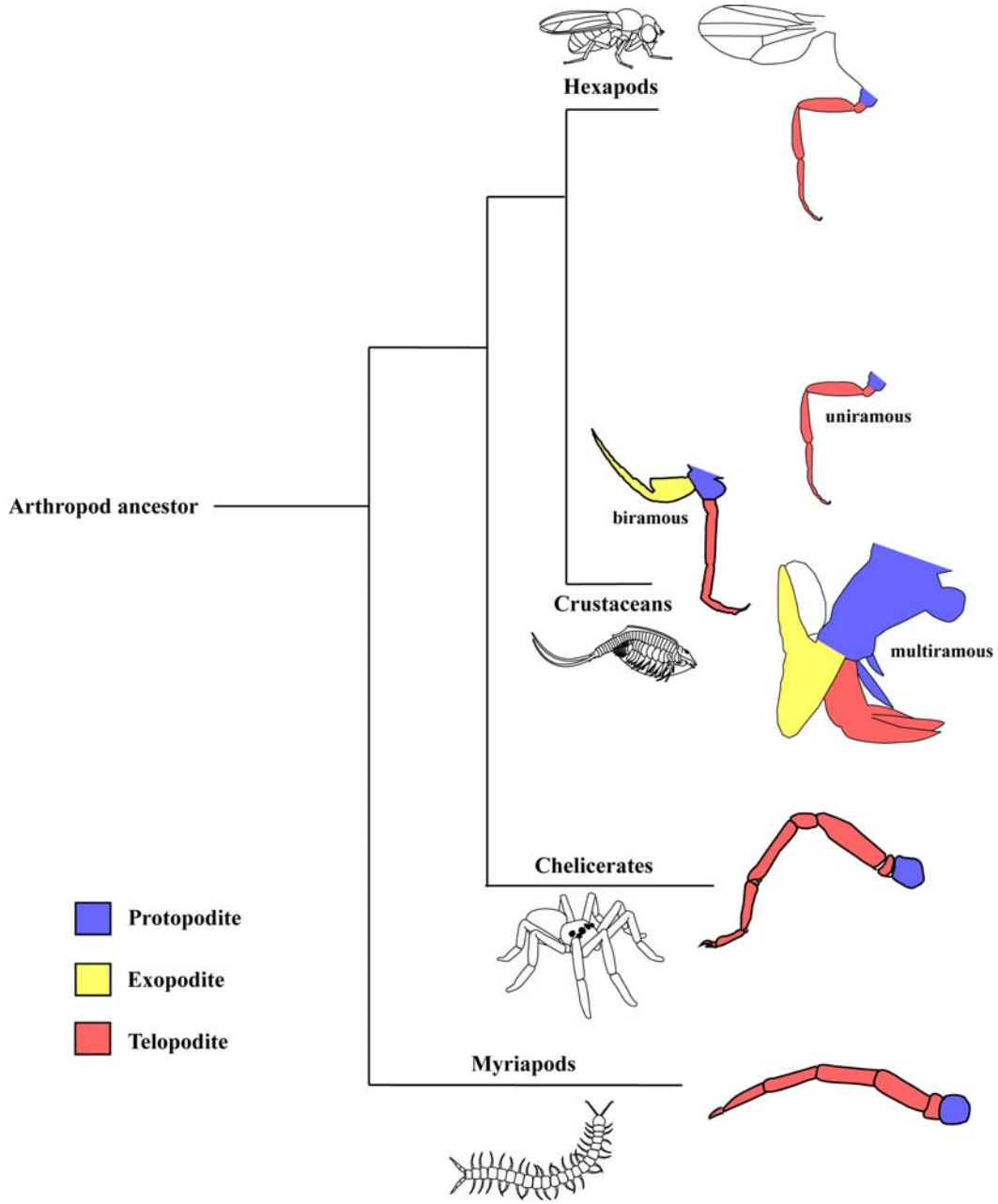


Fig. 1.1. Generic phylogram of arthropod limb morphologies. Major arthropod groups evolved their appendages from a common ancestor. Crustaceans are distinguished from other arthropods in having legs representing the three morphological categories: uniramous, biramous, and multiramous. The basic leg elements are highlighted, protopod (blue), exopod (yellow), and telopod (red).

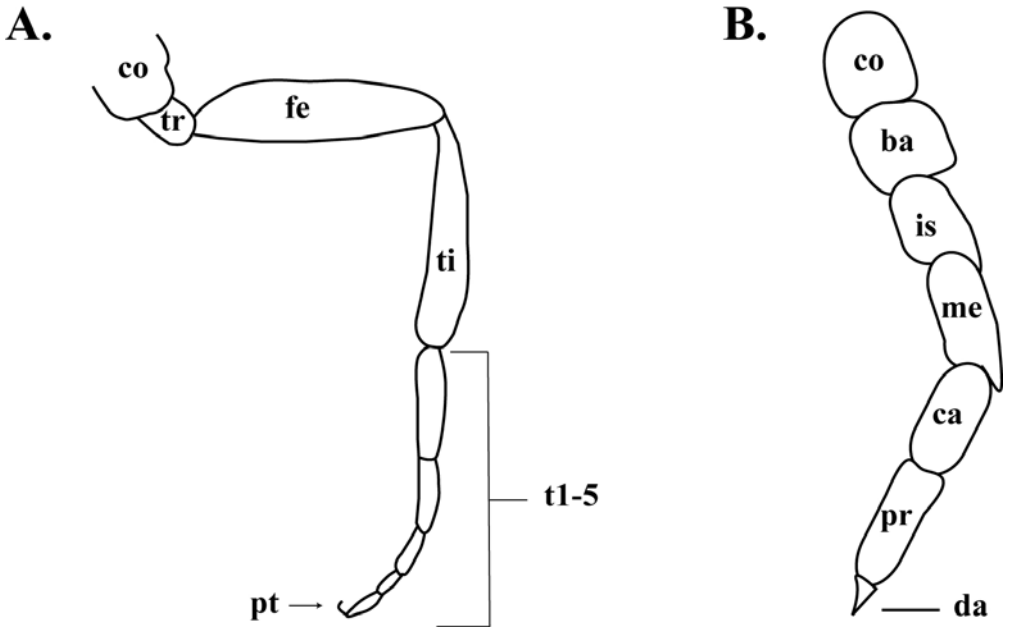


Fig. 1.2. Fruit fly and generalized crustacean leg anatomy. The *Drosophila* leg has ten segments. Listed from proximal to distal they are: coxa (co), trochanter (tr), femur (fe), tibia (ti), tarsus (t), and pretarsus (pt). The tarsus is further sub-divided into five segments. A generic crustacean leg has seven segments. Listed from proximal to distal they are: coxa (co), basis (ba), ischium (is), merus (me), carpus (ca), propodus (pr), and dactyl (da).

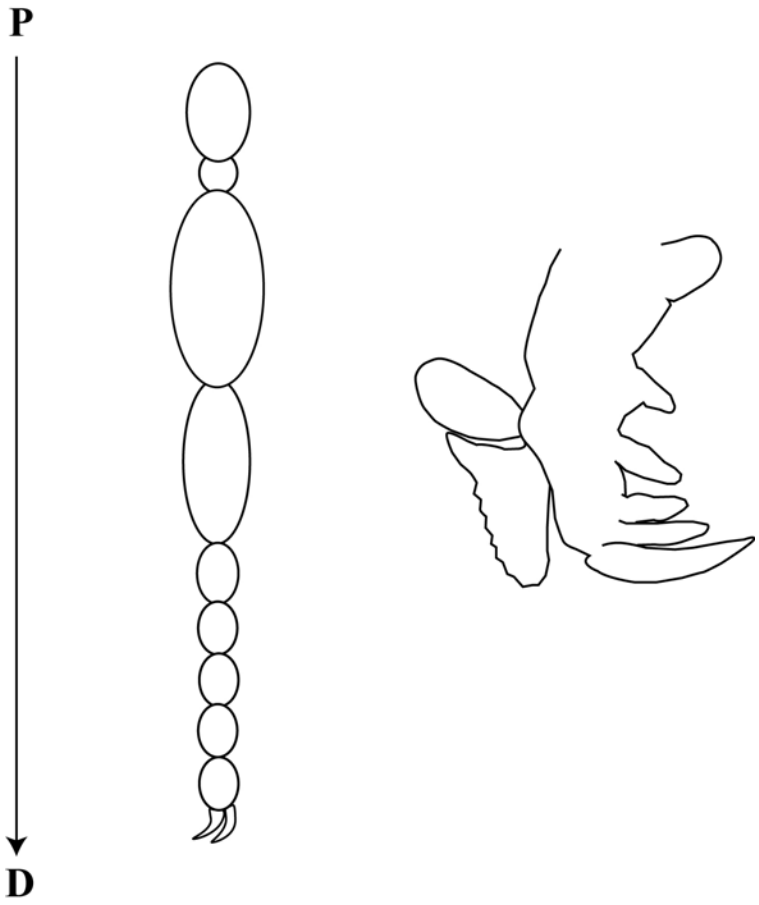


Fig. 1.3. Legs-*Drosophila* versus *Triops*. A *Drosophila* leg is represented on the left and a *Triops* leg is represented on the right. Based on morphology alone, these legs are difficult to compare.

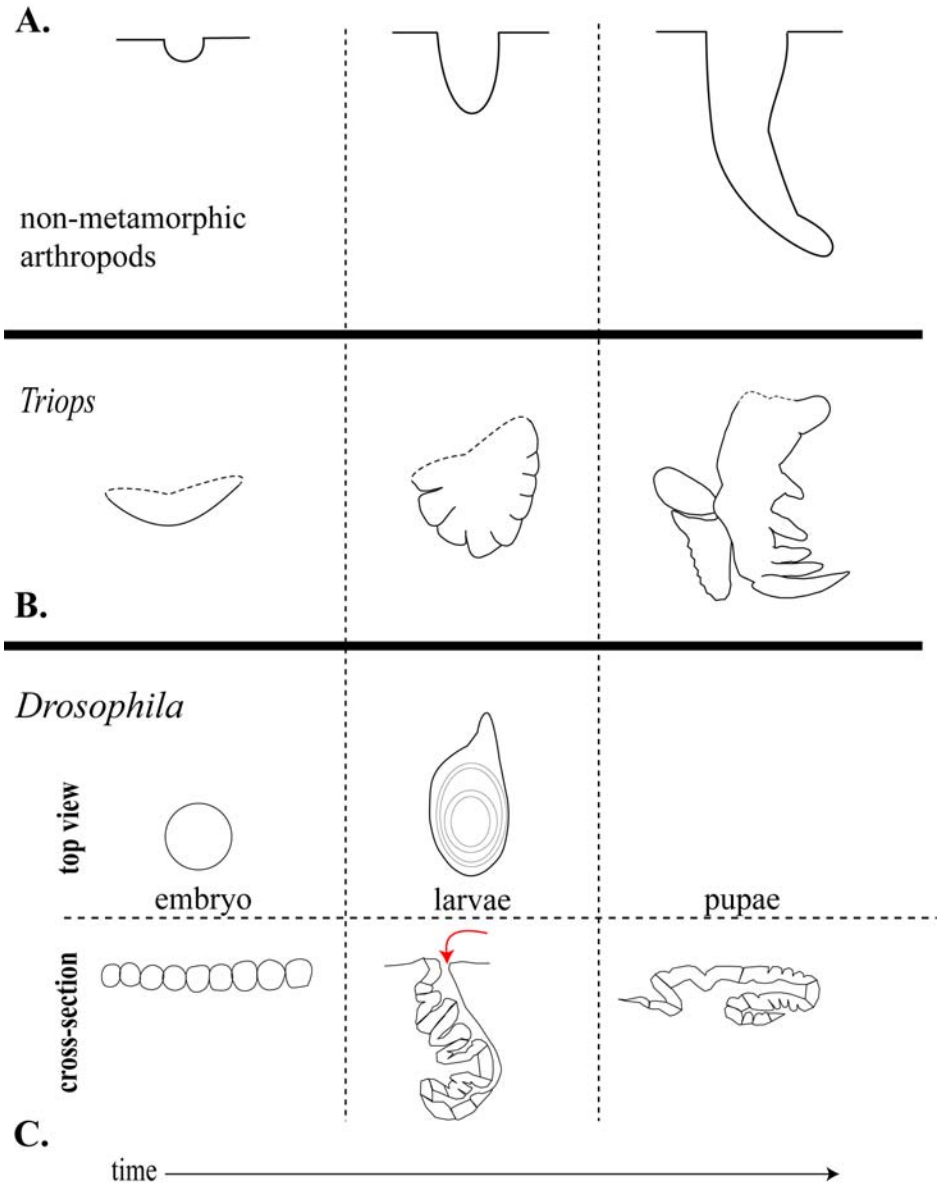


Fig. 1.4. Modes of leg development. (A) Non-metamorphic arthropods establish limb buds early in embryogenesis. The embryos legs buds undergo subsequent growth and patterning and eventually hatch with small versions of the adult appendage. (B) Limb buds of a *Triops* larva. Early limb buds are elongated and occupy the entire ventral body wall. (C) Metamorphic leg development. Leg primordium is specified during embryogenesis. Tissue invaginates (red arrow) and is followed by growth and patterning in the larval stage. Pupation results in eversion of patterned tissue into the adult structure.

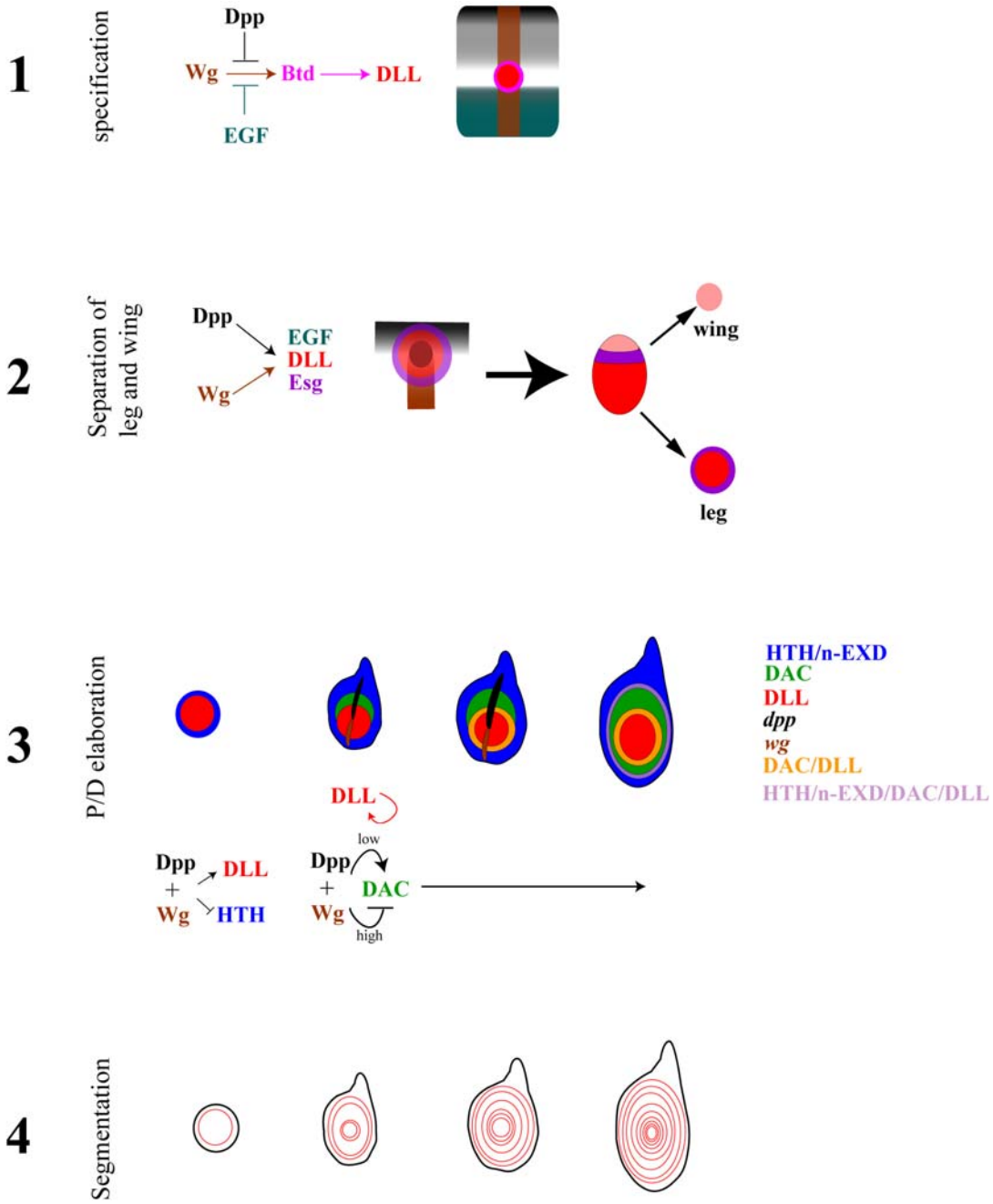

1
specification
Dpp
Wg
Btd
DLL
EGF
2
Separation of leg and wing
Dpp
Wg
EGF
DLL
Esg
wing
leg
3
P/D elaboration
HTH/n-EXD
DAC
DLL
dpp
wg
DAC/DLL
HTH/n-EXD/DAC/DLL
DLL
low
Dpp
+
Wg
DLL
HTH
Dpp
+
Wg
DAC
high
4
Segmentation

Fig. 1.5. *Drosophila* limb specification and patterning. In the illustration dorsal is up, ventral is down, and anterior is left. Leg patterning consists of four distinct stages: specification, separation of leg and wing primordia, P/D elaboration, and segmentation. (1) Leg primordium is established via Wg signaling and its size is restricted dorsally and ventrally by Dpp and EGF morphogen gradients, respectively. (2) After specification, Wg, Dpp, and EGF morphogen gradients further divide the primordium into three cellular populations. One population breaks away, migrates dorsally, and develops into the *Drosophila* wing imaginal disc whereas the other two cell populations define the proximal and distal domains of the leg imaginal disc. (3) This tier shows imaginal disc patterning during larval development. Dpp (black) and Wg (brown) diffuse from their expression domains thereby producing a Dpp+Wg morphogen gradient. The area of cells exposed to high levels of Dpp+Wg remains relatively fixed at the center of the disc and expresses DLL (red). DAC (green) is expressed in regions of the disc receiving low-moderate levels of both signals, whereas HTH and n-EXD (blue) are expressed in the disc periphery due to virtual absence of Dpp+Wg. During late third larval instar, disc growth pushes DLL-expressing cells in the perimeter of the central expression domain out of the high Dpp+Wg gradient, thereby resulting DAC expression. DLL expression is now independent of Dpp+Wg producing an overlap in DAC and DLL (orange). Co-expression of HTH/n-EXD, DAC, and DLL (violet) appears in the distal trochanter-proximal femur during late third instar. (4) Bottom panel tier shows the progressive expression patterns of the Notch ligands *Ser* and *Dl*, as well as the Notch signaling modifier *fng* (red rings) during leg imaginal disc development.

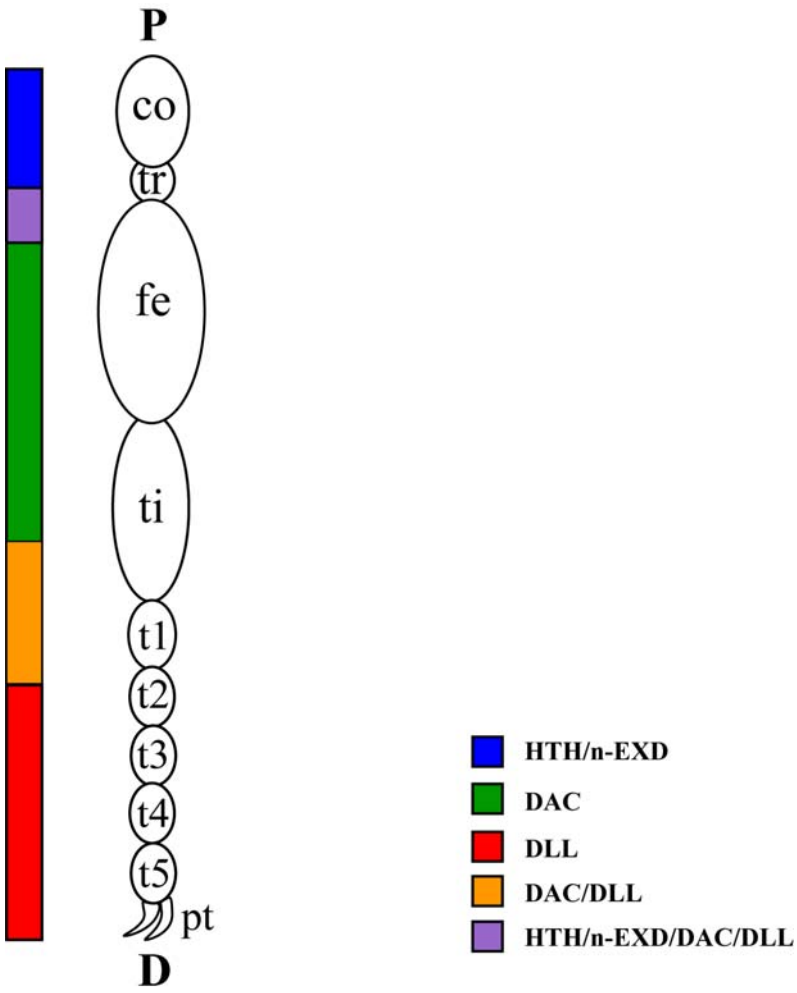


Fig. 1.6. Fruit fly leg anatomy and leg gap gene expression domains. The *Drosophila* leg has ten segments. Listed from proximal to distal they are: coxa (co), trochanter (tr), femur (fe), tibia (ti), tarsus (t), and pretarsus (pt). The tarsus is further sub-divided into five segments. HTH/n-EXD (blue), DAC (green), and DLL (red) are expressed in the proximal, intermediate, and distal leg domains, respectively. Expression of the leg gap genes overlaps in two distinct domains. All four genes are co-expressed in the distal trochanter and proximal femur (violet) whereas only DAC and DLL are co-expressed (orange) in the distal tibia and proximal tarsal segments.

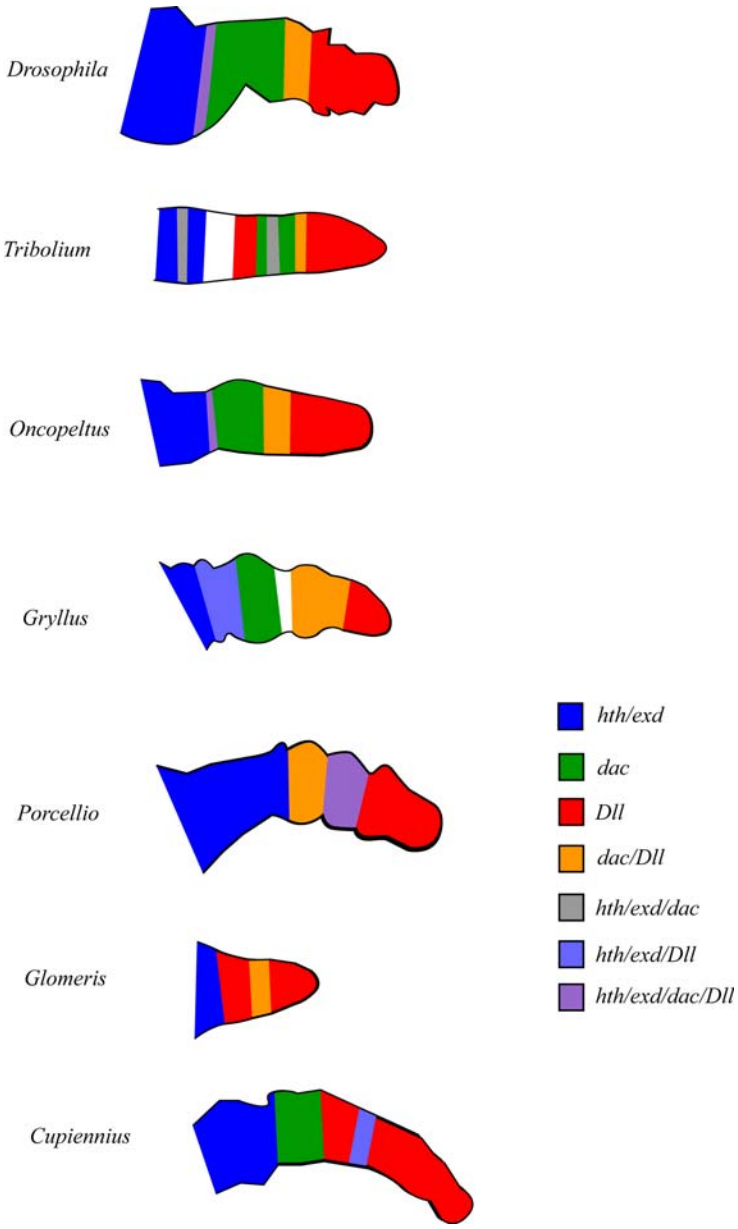


Fig. 1.7. Leg gap genes expression in various arthropod legs. *hth*/*exd* (blue), *dac* (green), *Dll* (red), *dac*/*Dll* (orange), *hth*/*exd*/*dac* (gray), *hth*/*exd*/*Dll* (faded blue), *hth*/*exd*/*dac*/*Dll* (violet). Only regions where *hth*/*exd* are co-expressed are represented in *Drosophila*, *Tribolium*, *Gryllus*, *Glomeris*, and *Cupiennius*. Only *exd* expression is shown for *Porcellio*, whereas only *hth* is shown for *Oncopeltus*.

CHAPTER 2

DACHSHUND, HOMOTHORAX, AND PATTERNING OF *TRIOPS* APPENDAGES

Summary

The branchiopod *Triops longicaudatus* has leaflike thoracic appendages with multiple medial and lateral branches. The limbs are flexible and do not have the typical joints observed in most arthropod appendages. Here I explore the degree to which the *Triops* limb, with its highly modified morphology, is patterned by the same network of genes used to pattern the uniramous, multi-jointed insect appendage. Key to establishing the proximodistal pattern of the insect leg are the genes *exd*, *hth*, *dac,* and *Dll*. These genes encode transcription factors that when functionally tested; define the identity of proximal, intermediate, and distal segments within developing legs. It has been previously reported that nuclear-EXD (n-EXD) and DLL expression patterns subdivide the early *Triops* limb into discrete proximal and distal domains, suggesting that despite the marked differences in morphology, the *Triops* leg is patterned by a mechanism that first defines large regional domains along the P/D axis. However, I find no evidence to support the early establishment of an intermediate domain of DAC expression. By contrast, DAC is detected prior to the onset of DLL expression, and is expressed along the medial flank of the developing leg. Two early, broad regions of expression resolve over time into a series of reiterated stripes that correlate with the distal side of each medial branch. In the most proximal and distal medial branches, the expression extends from the medial edge of the limb through the central and lateral regions. Thus I propose

that each of these stripes corresponds to a boundary between leg segments that are either incompletely, or not separated by joints. The expression pattern of DAC suggests it plays a critical role in segmenting the *Triops* leg. A similar pattern of expression is observed in the second, but not the first antenna. Finally, I show that HTH expression in *Triops* overlaps cell-to-cell with n-EXD expression, thereby providing the first substantiation in arthropods, outside of *Drosophila*, of the ancient and conserved relationship of HTH/EXD.

Introduction

Arthropods are a speciose phylum composed of four extant sub-phyla: chelicerates, myriapods, hexapods, and crustaceans. What makes this group extraordinary is the diversity of jointed appendages that have evolved to accommodate countless niches and lifestyles. While appendages exist in a myriad of shapes, they may be divided into three general categories: uniramous (unbranched), biramous (two branches), and multiramous (many branches). Although hexapods, chelicerates, and myriapods have limbs representing most or all of these morphological classes, their legs are predominantly uniramous. On the other hand, representatives of all three types of legs are found within the crustaceans, thereby making them an ideal group for investigating diversity of appendage form.

A paradigm for leg development has been established through genetic studies in *Drosophila* (reviewed in Kojima, 2004). During *Drosophila* larval stages, leg disc growth combined with the overlapping morphogen gradients established by the diffusible ligands Wingless (WG) and Decapentaplegic (DPP) splits the leg primordium into three

domains. In the leg disc where Wg/Dpp activity is low or absent, HTH is expressed and cooperates with EXD to establish the proximal leg domain (Rieckhof et al., 1997; Wu and Cohen, 1999). DAC expression is a response to moderate doses of WG/DPP activity (Lecuit and Cohen, 1997) and patterns the intermediate leg domain (Mardon et al., 1994). DLL expression is activated in response to a high dose of Wg/Dpp activity (Cohen et al., 1993; Diaz-Benjumea et al., 1994) and specifies distal leg structures (Cohen et al., 1989). *hth/exd*, *dac*, and *Dll* are thus initially expressed in mutually repressive domains along the P/D axis. As the leg imaginal disc continues to grow, regions of co-expression between these genes develop. A region expressing both DAC and DLL emerges between the DAC and DLL domains (Abu-Shaar and Mann, 1998) and subsequently, DAC expands proximally and overlaps with HTH. This is followed by the emergence of a new ring of DLL in this DAC/HTH domain (Abu-Shaar and Mann, 1998; Diaz-Benjumea et al., 1994; Gorfinkiel et al., 1997; Wu and Cohen, 1999). Thus, *hth/exd*, *dac*, and *Dll*, in both uniquely expressed and co-expressed regions, function at the early steps of a genetic pathway that defines positional fates (Abu-Shaar and Mann, 1998) and determines leg segment number, size, and morphology (reviewed in Kojima, 2004; Rauskolb, 2001). The leg gap genes are proposed to lie upstream of the genes *Serrate*, *Delta*, and *fringe*. These genes encode ligands and a modulator of Notch signaling and are essential for joint formation in the *Drosophila* leg (Rauskolb, 2001). HTH and DAC were demonstrated to activate *Ser* expression whereas DLL represses it (Rauskolb, 2001). Perhaps the leg gap genes regulate the establishment of segments in other arthropods via the same mechanism.

The same suite of genes used to pattern legs is employed to design and build other appendages, such as antennae and mouthparts, in all arthropods. In *Drosophila*, the general positions of *hth*/*exd*, *dac*, and *Dll* along the P/D axis in the antenna are similar to what they are in the leg. *hth* is commonly referred to as an antennal selector gene due to the transformation of antenna into leg in *Drosophila* null for *hth* (Dong et al., 2001), however, the function of *dac* in antennal patterning is relatively unknown. In another insect, *Oncopeltus*, antennae fail to form altogether in *hth* knockdown and *dac* knockdown has no effect on antenna development (Angelini and Kaufman, 2004). During arthropod mouthpart development, *dac* and *hth* are expressed in the developing mouthparts of every arthropod in which their expression has been examined, with the exception of *dac* in *Drosophila* (Abzhanov et al., 2001; Angelini and Kaufman, 2004; Joulia et al., 2005; Prpic et al., 2003; Prpic and Tautz, 2003; Prpic et al., 2001). EXD and DLL were previously shown to be expressed in *Triops* head appendages; therefore HTH and DAC are expected to be expressed as well.

Arthropod limb diversity has been investigated predominantly by examining to what degree the well-established *Drosophila* limb-patterning paradigm is conserved across arthropod taxa. A common theme emerging is the apparent conservation of a role for the leg gap genes *hth*/*exd*, *dac*, and *Dll* in subdividing the developing leg into distinguishable domains (Angelini and Kaufman, 2004; Inoue et al., 2002; Kojima, 2004; Prpic et al., 2003; Prpic and Tautz, 2003; Prpic et al., 2001). *hth*/*exd* expression is restricted to the proximal leg of all arthropods, *dac* is expressed an in intermediate domain, and *Dll* is found distally. Notably however, the relative sizes of their expression

domains vary and there is no correspondence between the boundaries of their expression domains and particular segments. Additional, proximal bands of *dac* expression have been observed in *Tribolium*, *Porcellio*, and *Cupiennius* (Abzhanov and Kaufman, 2000; Prpic et al., 2003). Details of the timing and overlap of expression also vary. For example, *dac* is expressed in an intermediate domain but is typically initially activated within the *Dll* expressing region. Despite the overall conservation of the relative positions of the leg gap genes along the P/D axis of the leg, the dependence of leg gap gene activation on the combined activity of DPP and WG signaling appears to be limited, even within the holometabolous insects (Angelini and Kaufman, 2005a; Ober and Jockusch, 2006). To what degree the process of leg segmentation downstream of the leg gap genes resulting in the fully segmented leg is conserved in evolution is not known. Precisely where the boundaries of leg gap genes would be expressed in the flexible, leaf-like *Triops* trunk appendages is difficult to predict. A *Triops* trunk limb differs from other arthropod limbs in two ways: 1) it has multiple branches; 2) it also has a reduced number of joints. Only two atypical joints can be identified: one at the base of the endopod and the other near the base of the dorsal epipod (Fig. 2.1B-blue arrowhead and open arrowhead, respectively). Despite the presence of joints, *Triops* legs are considered unsegmented (Terri Williams, personal communication; Brusca and Brusca, 2003). However Olesen et al. (2001) proposed that the medial branches of leaflike legs correspond to the segments. Conversely, the typical jointed *Drosophila* leg is composed of ten segments; from proximal-to-distal they are the coxa, trochanter, femur, tibia, tarsal segments 1-5, and claw bearing pretarsus (reviewed in Kojima, 2004). Consequently, it

is difficult to assign homologies of the different regions of the leg to the segments of other crustacean/arthropod legs, which are clearly delineated by joints. EXD and DLL expression patterns suggested establishment of generic proximal-distal limb domains as demonstrated by initial mutually exclusive expression, DLL in the lateral sector of the limb bud and EXD everywhere else (Nulsen and Nagy, 1999; Williams et al., 2002). This demonstrates an unusual degree of similarity at the molecular level in spite of a lack of overt morphological homology between the multiramous *Triops* leg and the uniramous legs of other arthropods. However, Williams et al. (2002) predicted that expression of *dac* in the developing branchiopod leg would either not exist or it would overlap with DLL and/or EXD since every region of the *Triops* leg expressed one of the two or both together in the stages examined, whereas HTH is predicted to be coincident with nuclear EXD (n-EXD) based on past *hth* and *exd* studies (Abu-Shaar and Mann, 1998; Rieckhof et al., 1997).

I find, as predicted, that DAC is not expressed in a large central domain in the early leg. I find that DAC expression is initiated in a proximal and distal domain along the medial flank of the limb, and evolves into stripes of expression that correspond to the distal region of each medial endite. The reiterated DAC expression pattern provides further evidence for a segmenting process in multiramous legs. Finally, strong proximal DAC expression in the *Triops* leg correlates with the gnathobasic character of the trunk appendages. While the multiramous *Triops* leg is a morphological novelty, it still possesses features of gene expression found in uniramous legs in addition to patterns unique to multiramous legs.

Materials and Methods

Animal rearing and fixation

Triops longicaudatus (Wards) cysts and detritus were sprinkled into an aquarium of dechlorinated tap water and hatched within 24h. Larvae were preserved for 1h in detritus conditioned aged tap water containing 5.5% formaldehyde, pH ~6.2 for DAC antibody stains and preserved as in Williams et al. (2002) for other antibody stains and *in situs*. Preserved animals were transitioned into methanol, and stored at -20°C. *Artemia franciscana* (Wards) cysts were hatched in artificial seawater.

A lab established colony of *Americamysis bahia* was maintained in an aquarium of artificial seawater. Embryos were removed from the brood pouches of gravid females, preserved for 1h in filtered artificial seawater containing 4% formaldehyde, transitioned into methanol, and stored at -20°C.

Cloning and sequencing

Total RNA was extracted from *Triops* and *Artemia* larvae, and *Americamysis* embryos by homogenizing a stacked body volume of 60-100 µl (in a 1.7 ml eppendorf tube) in 1.0 ml TRIzol (Invitrogen) with a Polytron PT3000 (Brinkmann). Two non-overlapping regions of the *Triops dac* gene were cloned using cDNA created from first strand synthesis using standard RACE poly-T primers (Sigma-Genosys) and Clontech's MatchMaker2 kit. The first region was cloned using nested degenerate 3'-RACE with the following primer sets: first round-DacR1 (AARGTIGCIGTIGAYAAYGC) and Adaptor Primer 1 (AAGGATCCGTCGACATCGAT), second round-DacR2

(GARAARRCIGARYTIAARATG) and Adaptor Primer 2 (CGACATCGATAAACTAGGGA). The second region of *Tldac* (1178 bp) as well as the single region of *Artemia dac* (*Afdac*-accession number DQ452571) and *Americamysis dac* (*Abdac*-accession number DQ452573) was cloned utilizing nested degenerate primers designed by Prpic et al. (2001). Products were cloned into pCR-II-TOPO (Invitrogen) and sequenced three times by automated sequencing (GATC DNA Sequencing Facility) in both directions. The sequence for the gap between the two non-overlapping *Tldac* regions was acquired by designing gene specific primers based on sequence data from the two clones flanking the gap. The gap segment was subsequently cloned into pCR-II-TOPO (Invitrogen) and sequenced as above. *Tldac* cDNA fragments spanning from the 3' end of the Dachshund Domain 1 (DD1) coding region into the 3'UTR were pieced together to yield one contiguous sequence (*Tldac*-accession number DQ452572). *Triops Dll* (*TlDll*-accession number DQ923136) was cloned with the following degenerate primer set: *Tl Dll* Forward1 (GGGCAAGAAGATGCGGAARCCNMGNAC) and *Tl Dll* Rev1 (CGCCGGTTCTGGAACCADATYTT) and *Triops caudal* (*Tlcad*-accession number DQ923135) was cloned with the following degenerate primer set: Cad 1F (ACNMGVACNAARGACAARTAYCG) and Cad 1R (AACCADATYTTNACYTGNCYATC). Primers for both genes were designed against conserved amino acid residues using available DLL and CAD protein sequences, respectively. Products were subsequently treated as above. Sequence analyses and

orthology assignments were performed using the NCBI BLAST [blastx] (Altschul et al., 1990) program and GCG/Seqlab (Wisconsin Package).

Phylogenetic analysis

Tl-DAC, *Af*-DAC, and *Ab*-DAC putative protein sequences were aligned with DAC orthologs from species in diverse taxa using CLUSTAL W (Thompson et al., 1994) utilizing the BLOSUM 62 residue comparison matrix (Henikoff and Henikoff, 1992) assigning a gap-opening penalty of 10 and a gap extension penalty of 0.2. A phylogram was generated using parsimony criteria, TBR with 2000 random additions, and non-parametric bootstrap analysis with 1000 replicates (200 random additions/replicate) as implemented in PAUP 4.0 b10 (Swofford, 2003).

Pattern/profile analysis

Putative DAC protein sequences from *Triops* and *Drosophila* were searched with Eukaryotic Linear Motif (Puntervoll et al., 2003) for investigating candidate functional sites.

in situ hybridization

in situs were performed as described by Nulsen and Nagy (1999) with the following modifications. *Tldac* (1178 bp), *TlDll* (118 bp), and *Tlcad* (109 bp) DIG-labeled sense and anti-sense probes were synthesized with T7 and SP6 RNA polymerases (Roche), using PCR amplified *Tldac*, *TlDll*, and *Tlcad* clones as template. *Tldac* probes were subsequently hydrolyzed for 20 minutes at 65°C using one volume 2X carbonate buffer (80 mM $NaHCO_3$, 120 mM Na_2CO_3, pH 10.2). Prehybridization was for 3-4 hours

at 60°C. Denatured riboprobe solution (80°C) was added to the animals, and animals in solution were slow cooled to 60°C and subsequently hybridized at 60°C overnight. Post hybridization wash times were reduced to 3X quickly with hybridization buffer (60°C); 4X, 15 min. at 60°C; 4X, 10 min., 60°C with 2X SSC + 0.1% CHAPS (60°C); 3X with PBS+0.1% Tween (PBTw) at room temperature. Specimens were then blocked in PBTw+ 2% BSA for 1 hour at room temperature. Larvae were incubated 2 hr at RT in PBTw + 2% BSA with alkaline phosphatase conjugated anti-DIG antibody (Roche). *Triops* were washed and the signal detected as previously described.

Antibodies and Immunohistochemistry

Polyclonal antibodies: Rabbits were immunized 5 times over 3 months (Cocalico Biologicals) with protein translated from the *Tldac* clone (1178 bp), sub-cloned into pET23a (Novagen) with a C-terminal His tag and purified on NTA-Ni agarose (Novagen or Qiagen). The Dac antibodies were affinity-purified on a column of *Triops* DAC protein coupled to CNBr resin and eluted with a low pH Glycine buffer. The affinity-purified antibody was diluted to 1:15,000. A rabbit polyclonal antibody to the *Drosophila* Homothorax protein (Kurant et al., 1998) was diluted 1:1000. Mouse monoclonal antibodies to Extradenticle (B11M) and Ultrabithorax/Abdominal-A (FP6.87) (gifts from R. White) were used at 1:5 and 1:10 dilutions, respectively. HRP and Cy2 conjugated α-rabbit and Cy3 conjugated α-mouse secondaries (Jackson) were used at 1:200.

Immunostaining: Performed as described in Williams et al. (2002).

Western blot: *Triops* larvae (Naupliar stages 1-4) and *Drosophila* embryos (0-16h, 23°C) were ground up in sample buffer and extracts subsequently boiled 5 min. Samples were centrifuges 1 min., maximum speed in a tabletop micro-centrifuge to clarify the supernatant. 10 μl of the supernatant was loaded into a 10% SDS-PAGE and electrophoresed in a mini-protean II (Bio-Rad). Proteins were transferred to a nitrocellulose membrane and probed with rabbit α-HTH serum [Diluted 1:40,000 in 5% non-fat dry milk in PBS+0.1% Triton (PBT)] or affinity purified rabbit α-*Tl*-DAC [Diluted 1:20,000 in 5% non-fat dry milk in PBS+0.1% Triton (PBT)], o/n at RT. Blots were washed 3X quickly with PBT then 6X, 5 min., PBT. Membranes were incubated with Donkey α-rabbit-HRP (Jackson: Diluted 1:10,000 in 5% non-fat dry milk in PBT), 2h, RT and then washed 3X quickly with PBT then 6X, 5 min., PBT. Proteins were detected using ECL detection system (Amersham) exposure to KODAK film (Chemiluminescence Biomax).

Results

Triops larval and limb development

Triops embryos are unusual in that they exist in developmental stasis and are encapsulated in an environmentally resilient cyst. Upon hatching, the *Triops* larva (~600 mm length) has a segmented head and trunk with ~6-10 visible segments (Fig. 2.1A). The *Triops* head possesses five appendages, which include (anterior to posterior): antennae 1 and 2, mandible, maxillule, and maxilla (Fig. 2.1A). Three discrete regions define the trunk; a segmented portion bearing limbs, a segmented domain having little to

no limb primordia, and a terminal non-segmented region (Fig. 2.1A). Larval development proceeds from anterior to posterior and provides a graded series of limb maturity in the trunk (Fig. 2.1A,B). Early *Triops* larval limb buds differ from arthropods with uniramous or biramous legs in that the primordium occupies the entire ventral surface of the trunk. Posterior trunk appendages are at early developmental stages while anterior legs are more mature (Fig. 2.1B). *Triops* trunk appendages are morphologically similar with the first and eleventh limbs modified for sensory and reproductive needs (Fryer, 1988; Williams and Müller, 1996). The developing multibranched *Triops* limb is composed of eight branches emerging from the leg stem (Fig. 2.1B), which develop into the future [listed ventral (medial)-dorsal (lateral)]: gnathobase (gn), endite-4 (en-4), endite-3 (en-3), endite-2 (en-2), endite-1 (en-1), endopod (endo), exopod (exo), and epipod (epi) (Fig. 2.1B). The primary P/D axis of the limb is highlighted (Fig. 2.1B-purple arrow).

Isolation of the Tldac, Afdac, and Abdac genes

To explore the role of leg gap genes in *Triops* limb development we cloned a *dac* ortholog. The contiguous *Triops dac* sequence is 1494 bp. *dac* sequences were also cloned from *Artemia franciscana* and *Americamysis bahia* to facilitate design of a polyclonal antibody. The cloned *Artemia dac* is 981 bp and *Americamysis dac* is 1086 bp.

All full-length DAC proteins contain two conserved domains termed Dachshund Domain 1 and 2 (DD1-DD2) (Davis et al., 1999). The C-terminal portion of DD1 from various representative species was used to illustrate the high degree of conservation in

this domain (Fig. 2.2A). Outside these domains, DAC sequences are divergent, although sequence similarities may be detected in closely related species. We found another 14 amino acid domain in the predicted *Triops* and *Artemia* sequences, WENCRAAYEDIVKH (referred to here as the insect-branchiopod motif) (*Dm*DAC-amino acids 449-462**)** with one amino acid difference (alanine or valine substituted for an isoleucine, indicated with an underline) (Fig. 2.2B). This domain was previously reported as being present in the predicted amino acid sequences of *dachshund* orthologs cloned from insect species but absent from other sequences (Moczek et al., 2006). Interestingly, this motif is found in the two branchiopods we studied and correlates well with the proposed phylogenetic relationship between branchiopods and insects (Fig. 2.2B,C; Regier et al., 2005).

Western blot detection of DAC and HTH

To verify the specificity of both antibodies western blots on *Triops* and *Drosophila* extracts were performed. *Drosophila* Dachshund protein has a predicted molecular weight of 113.7 kDa. Three strong bands detected in *Triops* and two in *Drosophila*, with bands of lesser intensity detectable in both species (Fig. 2.3A).

Western blots, utilizing antiserum generated against the entire *Drosophila* HTH protein (Kurant et al., 1998), were used to assess specificity and cross-reactivity. The *Drosophila* Homothorax protein has a predicted molecular weight of 49.6 kDa. Three major protein bands (molecular weights ~65, 50, and 28 kDa respectively) were detected in extract from *Drosophila* embryos and one prominent band in extracts from *Triops* larvae (Fig. 2.3B-yellow arrowhead). Although the ~50 kDa protein band most closely

matches the predicted molecular weight of HTH, however, (Kurant et al., 2001) reported when HTH was overexpressed in transgenic flies, the higher molecular weight band (~65 kDa-red arrowhead) was upregulated, suggesting this is the fully processed protein. A protein of similar molecular weight is detected in *Triops*.

DAC and EXD expression during trunk limb development

DAC expression is first detected in the whole embryo in the posterior segmented region of the trunk. It is expressed as a single bilateral cluster (Fig. 2.4A-white arrow) located in the medial region of the emerging limb bud (Fig. 2.4B). This corresponds to the future gnathobase and endites 4 and 3 (Fig. 2.2B). These cells lie both anterior and posterior to the apical edge of the limb bud. As the limb grows and differentiates from the body axis, the size of this cluster increases, and a second cluster appears in a more distal medial region of the limb bud (Fig. 2.4B; Fig. 2.4E-black arrows) in the second posterior-most trunk segment and coincides with the appearance of *Dll* expression in the lateral leg bud (Fig. 2.4E-red arrows). This lateral expression will become endites 1 and 2, as well as a small domain at the base of the endopod (Fig. 2.4J-white arrowhead).

With further limb development, the initial proximal cluster of DAC expressing cells splits into two distinct regions of expression (Fig. 2.4F-yellow and red arrowheads). A broad proximal stripe of expression begins at the base of the gnathobase and extends to the proximal side of the epipod. This domain encircles the entire anterior-posterior axis (yellow arrowhead, Fig. 2.4J). The second region comprises the remaining cells that originate at the proximal apical edge of the limb bud (red arrowhead, Fig. 2.4J). With growth of the limb, non-DAC expressing cells appear within this domain, and the DAC-

expressing cells appear to populate the distal side of each endite and extend into the stem of the limb (Fig. 2.4N-white arrows) forming an iterative pattern.

The entire proximal domain of young *Triops* limbs express n-EXD (Fig. 2.4C,G,K). During early leg ontogeny, DAC and n-EXD largely overlap in the proximal medial region (yellow arrows, Fig. 2.4D,H,L). Conversely, DAC is exclusive of n-EXD in the distal medial primordium (Fig. 2.4D,H,L). As the leg matures a new DAC domain appears between the two domains described above. Here DAC is void of n-EXD and corresponds to endite-2 (Fig. 2.4H,L-magenta arrows). n-EXD is seen in the distal limb during later leg development (Fig. 2.4O). Expression is prominent in the base of the endites (Fig. 2.4O-pastel cyan arrows) and weaker laterally in the endites, perhaps due to strong DAC expression (Fig. 2.4O,P-green arrows).

In the mature limb, DAC continues to be expressed in a proximal stripe (Fig. 2.3N) and a lateral stripe in each endite (Fig. 2.4N). Intriguingly, DAC is found where many sclerites (hard plates of the exoskeleton) meet (Fig. 2.3M-red, blue, and yellow lines). The region between the endopod and epipod has now grown substantially and continues to express DAC, with moderate levels of n-EXD expression (Fig. 2.4N,O-white asterisk). The distal limb joint (Fig. 2.2B-blue dashed line and blue arrowhead) is bounded on the proximal edge by a row of DAC expressing cells (Fig. 2.4N), whereas the proximal joint (Fig. 2.2B-blue dashed line and blue open arrowhead) is asymmetrically bound by DAC on its proximal-ventral and distal-dorsal edges (Fig. 2.4I-red line).

HTH and EXD expression during trunk limb outgrowth

HTH expression is detected throughout the trunk (Fig. 2.5A) with robust HTH expression observed in a reiterated stripe, 1-3 cells wide, in the posterior portion of each segment of the segment-bearing region (Fig. 2.5A-red arrow). Early in limb development HTH is expressed across the proximal limb from regions corresponding to the future gnathobase, endite 4 and the medial portion of endite 3 (Fig. 2.5B) to the presumptive epipod and the lateral portion of the exopod (Fig. 2.5B). As the limb matures, HTH is expressed in the basal half of the gnathobase and each endite (Fig. 2.5E) with stronger expression located in the medial portion of the endites and the lateral region of the gnathobase. Expression in the epipod and exopod becomes limited to their bases (Fig. 2.5E). In older limbs, HTH is detected everywhere in the limb stem including the domain proximal to the endopod where DAC is strongly expressed (Fig. 2.5H). HTH is absent from the epipod, distal exopod, endopod (with the exception of several cells associated with muscle attachment in the proximal endopod), and the distal domains of the medial branches. HTH is expressed at high levels in the dorsal body wall (Fig. 2.5E). n-EXD expression alone (Fig. 2.5C,F,I) resembles the previously mentioned HTH pattern and is described in the previous section.

DAC and HTH expression in Triops larval head appendages

Antennae: Triops has two antennae: a small, unsegmented first antenna (anterior-most) and a second antenna with two medial branches, one positioned proximally and the other intermediately (Fig. 2.2A). In *Drosophila*, DAC is expressed in the intermediate domain along the P/D axis of the antenna completely overlapping with both the proximal HTH domain and the distal DLL domain (Dong et al., 2001). The *Triops*, first antennae

lack DAC expression (Fig. 2.6A-yellow outline), whereas HTH is expressed ubiquitously (Fig. 2.6B-yellow outline). DAC is detected in the proximal branch in the second antennae and is present in the primary axis as well as the distal branch at the location where the distal branch emerges from the appendage (Fig. 2.6A-white outline). HTH is observed up to the region where the distal branch extends from the limb. Distal to that point, HTH is ubiquitous but more prominent along the ventral surface where setae protrude from the appendage (Fig. 2.6C-white arrow).

Mouthparts: dac is expressed in the mouthparts of every arthropod with the exception of *Drosophila*, whereas *hth* is detected in the mouthparts of all arthropods surveyed (Angelini and Kaufman, 2004; Prpic et al., 2003; Prpic and Tautz, 2003). In *Triops*, DAC is expressed in all mouthparts (Fig. 2.6A). The mandible accommodates two domains: the proximal segment and the distal teeth (Fig. 2.6A-delineated by a blue line). DAC is detected in a proximal and distal region of maxillule and a basal domain in the maxilla (Fig. 2.6B-violet and black outlines). HTH is found throughout the maxilla (Fig. 2.6B-inset-black outline). The mandible and maxillule express HTH everywhere but the distal tip (Fig. 2.6B-red, blue, and violet arrows).

Discussion

We investigated HTH and DAC expression during *Triops* appendage development. Our data on the expression of HTH and DAC, along with previously published data for EXD and DLL (Williams et al., 2002), show a commonality in the expressed genes associated with specification of the P/D axis between multiramous and uniramous legs. Despite the remarkable differences in the shape of the early branchiopod

limb primordia from the primordia seen in other arthropod groups, the early primordia contain discrete HTH/n-EXD and *Dll* expression domains. Additionally, although the *Triops* leg lacks the typical jointed segments seen in most arthropod legs, the reiterated expression of DAC and *wg* (Nulsen and Nagy, 1999) suggest a segmentation mechanism that subdivides the medial region of the leg into a series of branches. We propose that the leaf-like, branched morphology of the *Triops* leg is generated by a modification of the mechanism that segments the uniramous insect leg.

DAC sequence: function and phylogeny

We found that the amino acid sequence WENCRAAYEDIVKH is conserved in both insects and branchiopod *dac* sequences, but is not present in other arthropod or vertebrate sequences. This conservation suggests that this peptide sequence has an important role in DAC protein function. Tavsanli et al. (2004) investigated the function of discrete regions of the *Drosophila* DAC protein, using a set of deletion constructs (Tavsanli et al., 2004). They assayed the ability of these constructs to rescue null phenotypes, translocate to the nucleus, and induce ectopic eye development. Their study showed that the region containing the insect-branchiopod (I-B) specific motif was essential to augment the function of DD1 containing constructs when DD2 was removed. The I-B motif and additional downstream amino acid sequences of *Drosophila* and *Triops* DAC as well as the DD2 region of all animals for which the sequence is known form coiled-coil domains. These domains mediate appropriate protein-protein interactions (Burkhard et al., 2001). By scanning the I-B motif with an algorithm utilized on Eukaryotic Linear Motif (ELM), we found that the I-B motif contains potential PDZ

and SH2 domain binding (or ligand) motifs, which function in mediating interactions for the assembly of large multi-protein complexes (facilitated by PDZ domain containing proteins) and propagation of signaling cascades by promoting protein-protein interactions (via SH2 domain containing proteins). Thus, it is likely that within the insect-branchiopod lineage DAC forms protein complexes not found in other metazoans. These unique protein complexes likely confer a function to DAC, common to insects and branchiopods that are absent from other arthropods. In *Drosophila*, DAC has been demonstrated to directly interact with Eyes Absent, with which it forms a complex required for eye development. In the *Drosophila* leg, DAC acts to repress *hth* and *Dll* expression and has been shown to regulate expression of the Notch ligands *Serrate* and *Delta* as well as Notch signaling modulator *fringe* (Rauskolb, 2001). What DAC physically interacts with to mediate these functions is still unknown. In mouse and chick, DAC has been reported to interact with components of the Transforming Growth Factor-β (TGF-β) signaling pathway in the leg where it functions as a repressor of TGF-β mediated transcriptional control (Kida et al., 2004). *dpp* is a member of the TGF-β gene superfamily. An association of *dpp* and the formation of leg joints have been suggested based on *dpp* expression patterns in grasshopper, cricket, and spider (Jockusch et al., 2000; Niwa et al., 2000; Prpic et al., 2003) legs. Therefore, it is possible that DAC interacts with orthologous components in arthropod legs and affects segmentation. Studies using co-immunoprecipitation assays might yield binding partners essential for universal DAC functions in arthropod legs or perhaps a protein interaction unique to insects and branchiopods.

The finding that the WENCRAAYEDIVKH sequence is conserved only between insects and branchiopods is also interesting when compared to their phylogenetic relationship. Relationships between major arthropod groups, especially the establishment of hexapods and crustaceans as sister groups, have been supported for more than 15 years (Regier et al., 2005), however, which crustacean taxa constitute the sister taxa to the insects remains unresolved (Giribet et al., 2001; Mallatt et al., 2004; Regier and Shultz, 2001). Regier et al. (2005) proposed that hexapods and branchiopods are sister taxa and the *dac* amino acid tree presented here (Fig. 2.2C) supports that notion.

The Dachshund protein

We observed the presence of prominent bands much smaller than the predicted molecular weight of DAC on a Western with both *Triops* and *Drosophila* protein extracts. This suggests that differential function of the DAC protein could be facilitated either by the generation of multiple transcripts or post-translational processing of both the *Drosophila* and *Triops* DAC proteins.

Serial homology of second antenna and trunk limbs

Prpic et al. (2001) found that *dac* expression in mouthparts and legs of *TcDll* mutant, which lack distal leg elements, was similar. They proposed that *dac* expression could provide additional cues about homologies between head and thoracic appendages. The *Triops* second antenna is similar in morphology to the trunk limbs in that it possesses a primary P/D axis with medial branches. Additionally, DAC expression in the second antenna and trunk limbs is similar in pattern. Although no *TlDll* mutant strain of *Triops*

exists in the lab, DAC expression may be used here as evidence to further support the serial homology of head and trunk appendages, the second antenna in particular.

DAC correlates with the gnathobasic nature of appendages

The most striking difference between the expression of *dac* in the *Triops* and *Drosophila* limbs is the prominent proximal expression domain observed in *Triops* (Fig. 2.4F,J,N; Lecuit and Cohen, 1997). In *Triops*, the proximal most ventral branch of the leg is used to grapple food and ferry it to the mouth, in essence functioning as a peripheral mouthpart. This gnathobasic function of trunk limbs is considered a primitive characteristic in arthropods (Boxshall, 2004). In *Tribolium* and other arthropods with uniramous legs, the proximal leg is not used in the manipulation of food, and as such; typically lacks a proximal *dac* expression domain. When present proximally, *dac* expression is always weaker than its intermediate expression domain and no functional significance has been correlated with it. However, *dac* is typically expressed in the proximal regions of insect mouthparts (Angelini and Kaufman, 2004; Prpic et al., 2003; Prpic et al., 2001). *dac* expression in the mandible and proximal leg are preserved in a *Tribolium Dll* mutant strain, while the intermediate *dac* domain is eliminated. The preservation of the proximal leg domain in the *Dll* mutant strain as well as similarities in *dac* expression between the mandible and resulting truncated legs support a gnathobasic character of the coxa of the thoracic legs as well given its relationship to the mandible. Proximal *dac* expression may represent a vestige of the ancestral gnathobasic condition of the leg. Perhaps a shift toward uniramous leg morphology eliminated the necessity for

proximal trunk leg domains in acquiring and manipulating food or maybe the ventral branches were either a detriment to or not essential for terrestrial life.

Leg gap gene variation is associated with branch morphological diversity

Branch patterning in a multiramous limb differs from the patterns observed when a uniramous limb is induced to branch artificially. When branches are artificially induced in the uniramous limb of *Drosophila*, e.g. by ectopic expression of *wg* in a region expressing high levels of *dpp* or vice versa, the morphology of the resulting branch is similar in character to the original leg (Campbell and Tomlinson, 1995; Campbell et al., 1993; Gorfinkiel et al., 1997; Jiang and Struhl, 1996; Struhl and Basler, 1993). Ectopic branches also appear to invoke the same gene-patterning network used in the development of the original leg at a new time or place in development (Estella et al., 2003). In multiramous limbs, the branches have different morphologies and possess distinct gene expression patterns. Williams et al. (2002) noted this lack of developmental identity between the branches of a multiramous limb. A characteristic combination of the P/D genes with distinct spatial relationships pattern each branch of the *Triops* leg, and branches with similar morphology, such as the endites, have similar leg gap gene expression patterns. Since the gene patterns in the branches of *Triops* legs are diverse and do not resemble patterns seen in leg-like outgrowths, they are more than likely produced by an alternative patterning paradigm. This same conclusion was reached concerning the underlying developmental mechanisms of the branched mouthparts of many insects (Giorgianni and Patel, 2004; Jockusch et al., 2004).

For the most part, comparing patterns of genes used in leg development across arthropod taxa, the overwhelming conclusion has been that these genes cannot be used to define homologous parts of the appendages between divergent groups of arthropods. However, some exceptions to this generality have been proposed. In particular, the insect wing was proposed to have its origins either from modifications of a branch on a multiramous ancestral appendage or as a novel outgrowth of the body wall (Averof and Cohen, 1997). Averof and Cohen (1997) showed that two genes known to have wing specific functions in insects were also expressed in the epipod branches of multiramous legs, thereby supporting the hypothesis that wings evolved from dorsal branches of a multibranched limb. In our study, one interesting gene expression observation relates to the epipod. HTH/EXD are ubiquitously expressed in the epipod during early stages of epipod ontogeny but become restricted to the base of the maturing epipod. This pattern resembles what was described for HTH expression in relation to patterning the *Drosophila* wing (Azpiazu and Morata, 2000; Casares and Mann, 2001) and thus providing further evidence for that proposal.

Evolutionary relationship between a multiramous leg and the uniramous leg

Although multiramous and uniramous legs differ significantly from each other, we have sought to establish commonalities in patterning between the two in addition to noting patterning differences. Our data suggest that such a straightforward comparison of leg gap gene expression patterns between mature *Triops* and uniramous legs is not tenable due to the degree in which expression of all the leg gap genes overlap in the branch structures. At the earliest points in development, we have found that early

primary patterning of the P/D axis of the *Triops* leg involves a subdivision into two distinct "gap" domains, similar in many ways to that observed in *Drosophila*. However, in addition to this we have found evidence that suggests a unique pattering process that subdivides the medial region of the leg into iterated domains.

In addition to this lateral domain of DAC expression, we observe repeated stripes of DAC expression in the medial region of the leg. An early, large single stripe of DAC expression evolves into a series of smaller stripes, similar in many ways to the maturation of the *wg* expression pattern in the *Triops* leg (Nulsen and Nagy, 1999). The expression of DAC and *wg* in reiterated stripes along the region of the leg that will give rise to the four medial branches suggests medial branch structures are patterned by an iterative or segmental process. The medial branch structures may represent rudimentary segment boundaries, which fail to complete morphogenesis required for the formation of a fully jointed segment boundary. Interestingly, in their analysis of leg development in another branchiopod, Olesen et al. (2001) suggested that medial endites correspond to leg segments. This proposal was based on observations of early limb ontogeny of the crustacean *Leptodora kindtii*. Early leg development in *Leptodora* is similar to the multiramous limb in that the primordium takes up the entire ventral region of the thoracic segment and is followed by the formation of multiple branched structures. *Leptodora* leg development differs from leg development in other branchiopod species in that as the limb matures, the limb is liberated from the body wall thereby producing a uniramous leg (Olesen et al., 2001). Based on ontogeny of the leg and expression of the gene *Dll*, Olesen et al. (2001) proposed that the medial branch structures are directly homologous

to the segments of a uniramous limb. One inconsistency in their model was the expression of DLL in virtually every leg segment, compared to its restriction to the distal segments of the *Drosophila* leg. They proposed that lateral branches with persistent DLL expression corresponded to distal limb elements, whereas proximal branches with transient DLL expression were compared to the proximal segments of uniramous limbs. Early DAC and HTH/EXD expression in *Triops* endites support this association whereas later expression refutes it. Perhaps *Triops* represents a heterochronic version of *Leptodora*, where the final stage of leg development in which the limb separates from the body wall does not occur. Conversely, *Leptodora* limbs may not be comparable to multiramous limbs and may simply represent a uniramous leg with a novel ontogeny. As such, they would lack the defining spatiotemporal relationships observed between the P/D genes in true multibranched limbs. How *exd*, *hth*, and *dac* fit into *Leptodora*'s novel limb developmental scheme is unknown, therefore, examination of their patterns during *Leptodora* limb ontogeny may provide clues further relating uniramous and multiramous limbs.

HTH and EXD: ancient molecular relationship and HOX co-factors

Mutational analyses in *Drosophila* reveal that both *hth* and *exd* produce similar phenotypes (Abu-Shaar and Mann, 1998; Azpiazu and Morata, 2002; Kurant et al., 1998; Rieckhof et al., 1997), thereby demonstrating that they need each other to function. Whether reducing or eliminating the function of one will phenocopy a knockdown or knockout of the other in other arthropods is unknown. Existing expression data suggests that they do require each other to function but expression in other species outside of

Drosophila has been limited to mRNA expression for *hth* (Inoue et al., 2002; Prpic et al., 2003) and mRNA (Prpic et al., 2003) or protein for *exd* (Abzhanov and Kaufman, 2000; Giorgianni and Patel, 2004; Inoue et al., 2002; Jockusch et al., 2000). Here we show that HTH and EXD protein expressions match the expected pattern, thereby providing further evidence for their mutual requirement for function. Unfortunately, functional studies in other arthropods have been predominantly limited to *hth*, therefore it is unknown whether *hth* and *exd* would produce similar or differing mutational phenotypes or have functions in the developing leg independent of one another. Based on expression data across taxa and mutational analysis in *Drosophila*, knockdowns of each, separately and together, would therefore be expected to show the same phenotypes.

Interestingly, HTH/EXD nuclear co-localization coincides well with the anterior boundary of *caudal* expression (Fig. 2.7A,B). *caudal* is a homeobox gene shown to be involved in posterior development of animals (Copf et al., 2003; Copf et al., 2004; Dearden and Akam, 2001; Macdonald and Struhl, 1986; Moreno and Morata, 1999; Schulz et al., 1998). HTH/EXD expression also correlates with the posterior limit of expression of the trunk/abdominal determining *Hox* genes, *Ultrabithorax* (*Ubx*) and *Abdominal-A* (*Abd-A*) (Fig. 2.7C). *Hox* genes play crucial roles in establishing regional identities along the A/P axis and HTH/EXD are essential HOX co-factors (Rieckhof et al., 1997; Ryoo and Mann, 1999; Ryoo et al., 1999), even though HOX proteins have been demonstrated to affect gene expression in the absence of HTH/EXD function (Galant et al., 2002). Perhaps most tissues or developmental domains lacking HTH/EXD in *Triops* constitute regions not under autonomous HOX influence. Therefore, the

posterior extent of HTH/EXD expression may represent the limit of autonomous *Ubx*/*Abd-A* influence along the A/P axis as well as the limit of body regions competent to develop appendages.

Summary

We have shown that DAC and HTH are associated with adaptations to leg morphology, such as various branch structures seen in the *Triops* leg. On the other hand, there is one basic process that involves input from the leg gap genes that appears common to arthropod legs regardless of morphology and that is the establishment of three generic domains along the P/D axis. Additionally, we discovered the presence of an iterative mechanism that produces medial branches in the *Triops* leg that is similar to the iterative process in uniramous legs that establishes individual leg segments. Despite these noted similarities and differences based on leg gap gene expression, there are still many questions left to resolve, such as; 1) what lies upstream of the leg gap genes and how is the patterning network changing to produce morphological diversity, 2) do other crustaceans with multiramous legs have similar spatiotemporal relationships among the leg gap genes, 3) how do *dac* and *hth* expression relate to biramous leg morphologies, and 4) what patterning constraints are on limb development?

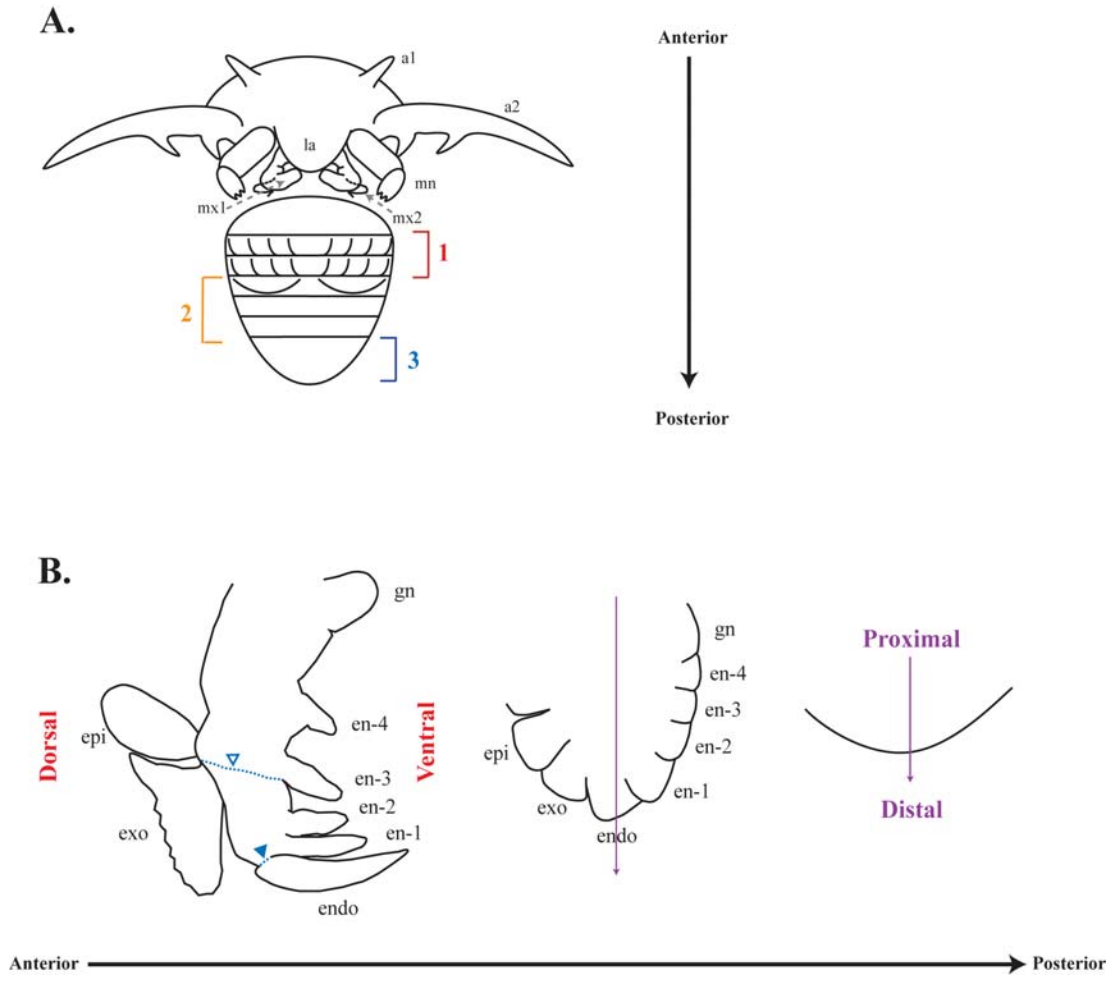


Fig. 2.1. Gross anatomy of *Triops* larva and leg. (A) *Triops* first stage nauplius (anterior up-posterior down). Five head appendages are indicated: antenna 1 (a1) and 2 (a2), mandible (mn), maxillule (mx1), and maxilla (mx2). Additionally, a structure referred to as the labrum (la) is represented as well. The trunk may be sub-divided into 3 general domains, regions 1, 2, and 3. (B) Trunk appendages at various stages of development [left-to-right: late stage anterior appendage-early limb bud]. Dorsal-ventral and proximal-distal axes are noted. Joints are represented with blue dashed lines and branches are labeled. Proximal joint (blue open arrowhead) and distal joint (blue arrowhead).

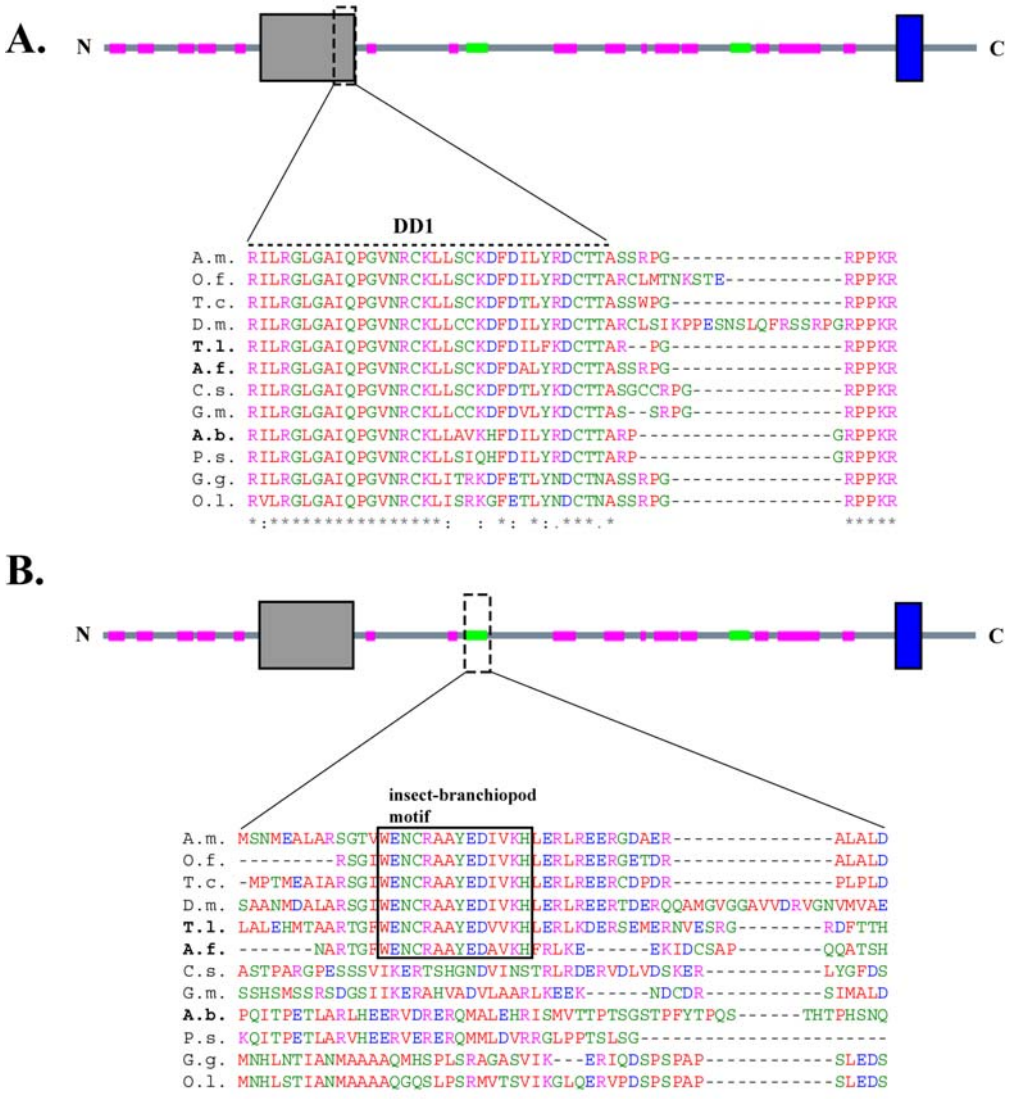


Fig. 2.2. Alignment of the predicted amino acid sequence of the *Triops*, *Artemia*, and *Americamysis dac* gene clones with orthologs from diverse taxa. (Legend follows.)

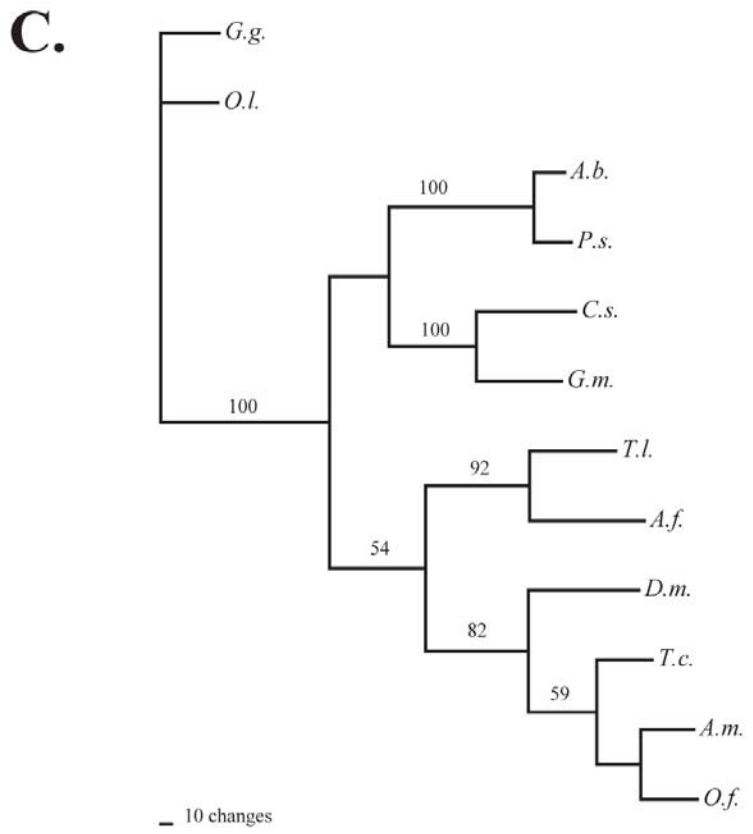


Fig. 2.2. Alignment of the predicted amino acid sequence of the *Triops*, *Artemia*, and *Americamysis dac* gene clones with orthologs from diverse taxa. (Legend follows.)

Fig. 2.2. Alignment of the predicted amino acid sequence of the *Triops*, *Artemia*, and *Americamysis dac* gene clones with orthologs from diverse taxa in (A) C-terminal portion DD1 and (B) region containing insect-branchiopod conserved sequence and (C) Phylogram of Dachshund protein sequences. A cartoon representation of the *Drosophila* DAC protein is depicted with each aligned region to provide a visual of where those domains lie within the protein. Abbreviations for animals and accession numbers: D.m. (*Drosophila melanogaster*-AAC46506.1), T.c. (*Tribolium castaneum*-CAC84070.1), A.m. (*Apis mellifera*-XP_394482.2), O.f. (*Oncopeltus fasciatus*-AAS93632.1), P.s. (*Porcellio scaber*-AAK58707.1), C.s. (*Cupiennius salei*-CAD57736.1), G.m. (*Glomeris marginata*-CAD82906.1), G.g. (*Gallus gallus*- AAF22354.1), and O.l. (*Oryzias latipes*-CAC48006.3). An asterisk represents region of identity, vertical double dot shows a conserved substitution, and semi-conserved substitution is demonstrated by a single dot. (C) Single most parsimonious phylogram of arthropod and two vertebrate *Dachshund* protein sequences. Only sequences lying within the DD1 and DD2 domains (Davis et al., 1999) were used for the phylogenetic analysis. Numbers along branches represent bootstrap values. DD2 is not represented in the alignment.

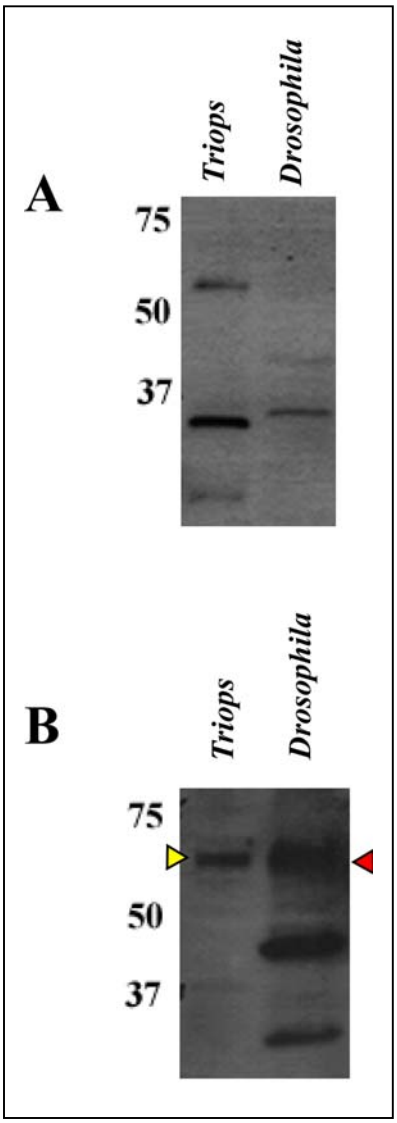


Fig. 2.3. Western blot of *Triops* and *Drosophila* extract probed with DAC α-serum and HTH α-serum. (A) DAC polyclonal antibody detected three prominent bands (two smaller than 37 kDa and one ~60 kDa) in *Triops* larval extract (stage 1-4) whereas two distinct bands (~32 and 42 kDa) were detected in *Drosophila* embryo extract (0-16h). (B) HTH α-serum detected only one distinct band (~65 kDa-yellow arrowhead) in *Triops* larval extract (stage 1-4) whereas three distinct bands were detected in *Drosophila* embryo extract (0-16h). Red arrowhead in *Drosophila* lane indicates upregulated band in transgenic flies.

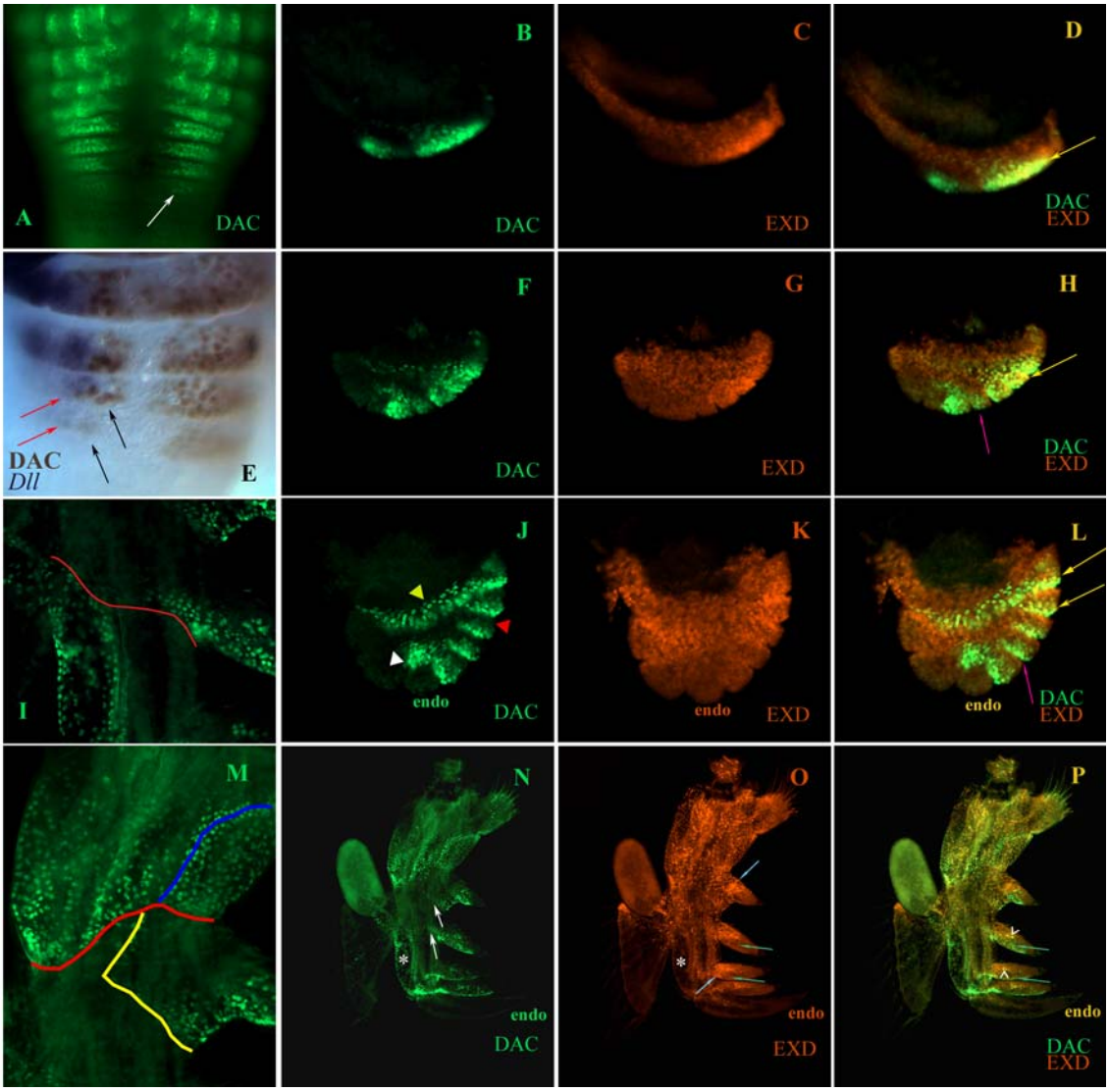


Fig. 2.4. Ontogeny of DAC and EXD expression in the *Triops* leg. (Legend follows.)

Fig. 2.4. Ontogeny of DAC and EXD expression in the *Triops* leg. [limb orientations: left (dorsal), right (ventral), and up (body wall)]. DAC (green) and EXD (red). (A) DAC expression in the posterior trunk of a *Triops* larva [ventral view, larva oriented anterior up]. (B,F,J,N) DAC, (C,G,K,O) EXD, and (D,H,L,P) DAC/EXD expression during early limb ontogeny examined in dissected trunk legs at various stages of development. (A) DAC is initially detected in posterior trunk segments (white arrow). (E) DAC expression in the distal limb (black arrows) coincides with the appearance of *Dll* mRNA expression (red arrows) [posterior trunk-lateral view, larva oriented anterior up]. DAC is detected predominantly in three domains: (J) proximal (yellow arrowhead); ventral (red arrowhead); and distal-medial (white arrow). (C,G,K) EXD resides across the entire proximal region of the leg. (D,H,L) DAC/EXD (yellow) co-expression is found primarily in the proximal-ventral (yellow arrows) leg location in immature limbs. (H,L) A DAC domain, void of n-EXD, intercalates between the proximal-ventral and distal domains (magenta arrows). (N) In 96h legs, DAC extends into the leg stem (white arrows) and is expressed in the dorsal limb domain between the endopod and exopod (white asterisk). (O) n-EXD is weakly expressed between the endopod and exopod (white asterisk), strong in the basal portion of the endites (pastel cyan arrows), and faint in the lateral region (O,P-light green arrows). (P) Robust co-expression is seen in a small number of cells within each endite (white carets) of older legs. (I,M) DAC is associated with proximal joint (I-red line) and sclerite boundaries (M-red, yellow, and blue lines).

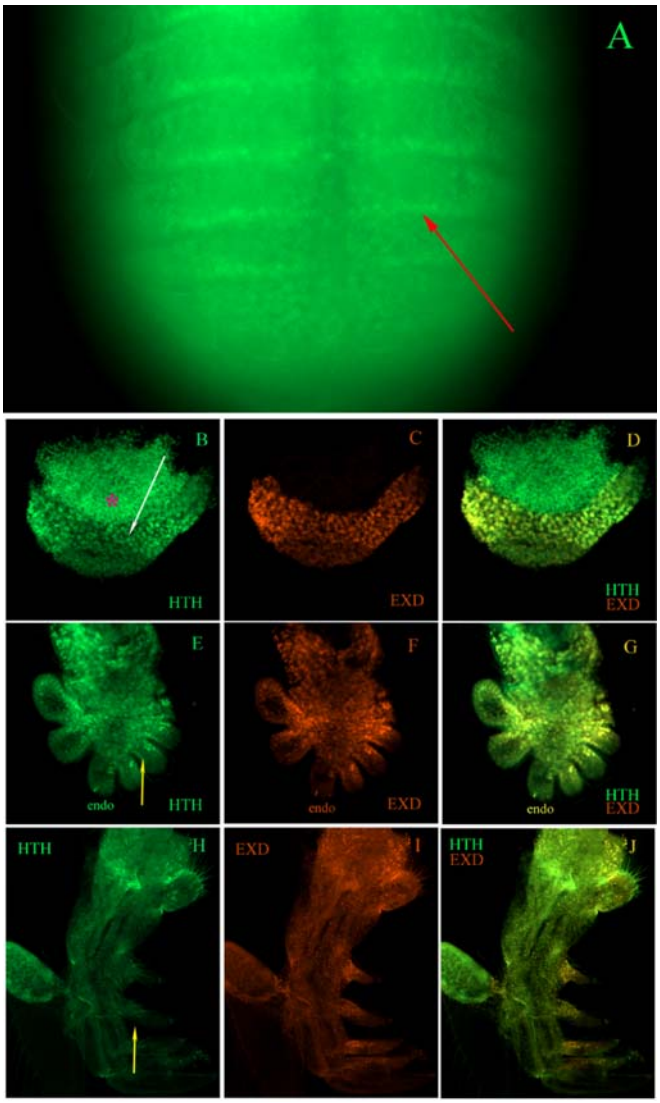


Fig. 2.5. Ontogeny of HTH and EXD expression in the *Triops* leg. *Triops* legs at various stages of development [limb orientation: left (dorsal), right (ventral), and down (distal)]. (A) HTH (green) is expressed everywhere in the trunk [larva oriented anterior up]. (B) HTH (green) is expressed in a swathe across the entire proximal leg (white arrow). (E,H) Weak HTH in the lateral endites (yellow arrows). (C,F,I) EXD (red) in the same limb series as HTH. (D,G,J) HTH/EXD (yellow) are co-expressed everywhere.

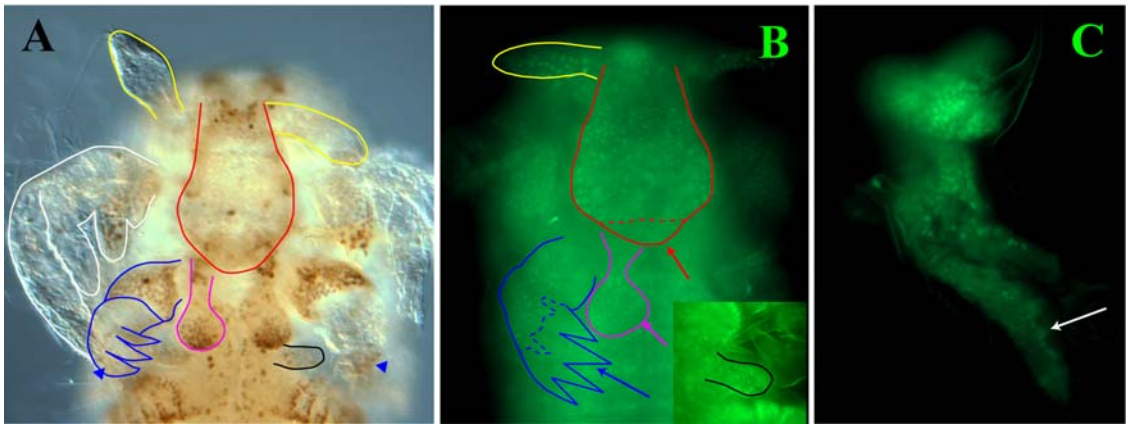


Fig. 2.6. DAC and HTH in *Triops* head appendages and labrum. (A) DAC protein expression (HRP detection), ventral view of the head. The following head structures are highlighted: a1 (yellow), a2 (white), la (red), mn (blue), mx1 (violet), and mx2 (black). DAC is absent from a1 but present in all other head appendages. Expression in the mandible is limited to the proximal segment except for mild expression in the mandibular teeth (blue arrow heads). (B) HTH is expressed virtually ubiquitously throughout the *Triops* larval head. Head appendages are highlighted as with DAC. Dashed lines and arrows demarcate the distal tips of the la, mn, and mx-1 (red, blue, and violet, respectively). (C) HTH is detected throughout a2. Distal expression is generally weaker but appears upregulated in regions corresponding to setal growth (white arrow).

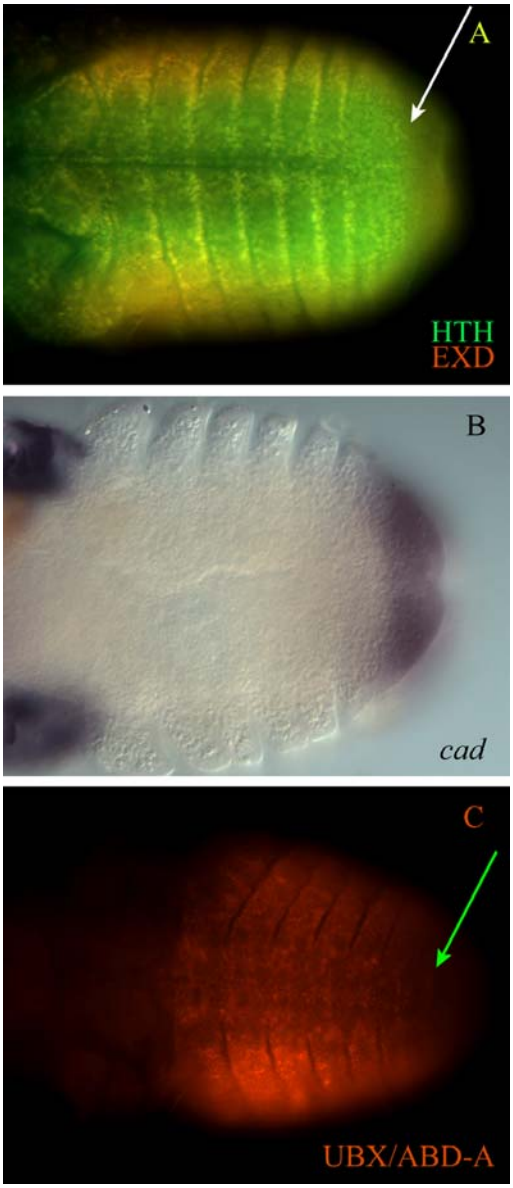


Fig. 2.7. Comparative expression of HTH/EXD, *Tlcad*, and UBX/ABD-A in a stage-1 *Triops* nauplius trunk. (A) HTH (Green)/EXD (Red) proteins are co-expressed (Yellow) throughout the segment bearing region of the trunk and extends into the non-segmented region (white arrow). (B) *Tlcad* mRNA detected with a riboprobe generated using a 109 bp EST. (C) UBX/ABD-A (Red) proteins are detected in the trunk and their posterior most expression is similar to HTH/EXD (green arrow).

CHAPTER 3

FUNCTIONAL EXPLORATION OF RNAI IN TWO NON-MODEL ARTHROPOD SPECIES-*TRIOPS LONGICAUDATUS* AND *ARTEMIA FRANCISCANA*

Summary

RNA interference (RNAi) has been shown to be an effective method for exploring gene function in many animal systems. RNAi takes advantage of a primitive defense mechanism possessed by most multi-celled organisms, presumably evolved to protect the host organism against invasion by dsRNA viruses by targeting viral mRNAs for destruction (Ding et al., 2004). By injecting dsRNA synthesized from a cloned gene, investigators are able to misdirect this viral defense machinery to target endogenous messages, thereby reducing gene dosage and potentially producing phenotypic consequences (Aljamali et al., 2002; Fire et al., 1998; Hannon, 2002). This chapter chronicles my efforts to invoke this powerful biological process to study the functional role of various patterning genes on multiramous limb development in both *Triops* and *Artemia*. Unfortunately, experiments in *Triops* failed to generate reproducible phenotypes despite employing various methods for introducing dsRNA into the animal. Nonetheless, phenotypes were reproducibly generated by microinjecting dsRNA using the *Artemia caudal* (*Afcad*) gene replicating the results published by the Averof lab (Copf et al., 2004). I was, however, not able to induce mutant phenotypes in *Artemia* with several other limb-patterning genes that have previously been shown to have functional roles in limb determination or patterning in other arthropods. Probable explanations for the inability to produce observable phenotypes are discussed.

Introduction

Expression patterns only provide a first impression of what role a gene plays in development or other processes. Generally, two additional approaches are taken to unmask the biological relevance of a gene—over-expression (ectopic expression) and under-expression (knockdowns or nulls). Over-expression provides insight into two aspects of gene function: 1) what happens if you increase abundance of a gene in a spatially appropriate fashion, and 2) what the consequence of gene expression outside the wild-type expression domain is. On the other hand, a simple way to view the function of an individual gene is to explore the consequences of removing its expression from the organism. Both ectopic expression and knockdowns also provide insight into what phenotypic novelties are possible in evolution if regulation of a particular gene were altered. For instance, naturally occurring regulatory changes in the *Pitx1* gene are correlated with fully formed, reduced, or missing pelvic structures in the threespine stickleback fish (Shapiro et al., 2004). This demonstrates how a change in the expression of one gene has an overt impact on morphology. Over-expression studies in non-model arthropods are challenging but are becoming increasingly feasible with the use of specific promoters to drive gene expression or the use of transposable elements to integrate genes of interest into the genome (Lewis et al., 1999; Oppenheimer et al., 1999; Pavlopoulos and Averof, 2005). By contrast, RNAi has been employed to examine the consequences of reducing gene expression in a diverse number of arthropod taxa with a variable degree of success (Table 3.1).

RNAi finds its roots in plant biology when researchers investigating petunia coloration discovered genetic manipulation intended to enhance flower color inadvertently suppressed it (Napoli et al., 1990; van der Krol et al., 1990). These observations were something of an enigma until Fire and others (1998) set out to investigate the structural and delivery requirements of interfering RNA in the nematode worm *Caenorhabditis elegans*. Prior to their study, anti-sense hybridization of the introduced RNA to the message was believed to be the underlying interfering mechanism. Remarkably, they found that dsRNA produced substantially more potent interference than either the sense or anti-sense strands alone. These discoveries prompted deeper probing of the underlying mechanism of dsRNA-induced gene silencing. Subsequently, numerous arthropods have been shown to support RNAi, which include the fruit fly (Kennerdell and Carthew, 1998), milkweed bug (Hughes and Kaufman, 2000), mosquito (Levashina et al., 2001), giant silkmoth (Bettencourt et al., 2002), red flour beetle (Bucher et al., 2002), tick (Aljamali et al., 2002), honeybee (Beye et al., 2002), spider (Stollewerk et al., 2003), and the brine shrimp (Copf et al., 2004). Since introduction of dsRNAs into species from a variety of taxa have produced gene specific defects the biological pathways required for RNAi to function were thought to exist in most eukaryotic organisms.

Components of the RNAi pathway have been identified via screening plants, fungi, worms, cell culture, and cell free systems deficient for various aspects of RNAi (Tijsterman et al., 2002). By combining phenotypes and biochemical characteristics of a numerous mutants, a picture of the RNAi process becomes visible (Fig. 3.1). In *C.*

elegans, *sid* (systemic RNAi defective)/*rsd* (RNAi spreading defective) mutants illuminated biological components essential for systemic RNAi (Tijsterman et al., 2004; Winston et al., 2002). *sid* and an *rsd* encode a transmembrane protein required for passive cellular uptake of dsRNA (Feinberg and Hunter, 2003; Winston et al., 2002), whereas other *rsd* mutants suggest endocytosis aides in dsRNA internalization (Tijsterman et al., 2004). *Drosophila* lacks an endogenous *sid-1* homologue and yet *Drosophila* S2 cells are capable of taking up dsRNA and using it for effective gene silencing. By using cultured *Drosophila* cells, Saleh and others (2006) demonstrated a receptor-mediated endocytotic process took up the dsRNA. Once dsRNA enters the cell, an RNase III nuclease family member (Bernstein et al., 2001), Dicer, cleaves the dsRNA into smaller chunks ~22 nt (small interfering RNA-siRNA) (Hamilton and Baulcombe, 1999) in length. siRNA next associates with the RNAi enforcer, RISC (RNA induced silencing complex), which is a protein-RNA nuclease complex that recognizes and endonucleolytically destroys target mRNAs (Hammond et al., 2000; Nykanen et al., 2001) thereby diminishing gene expression.

Originally, RNAi studies in worms involved injecting dsRNA into the worm's gonad or body cavity and observing phenotypes in progeny. Since those pioneering days of RNAi, simple and effective techniques of large-scale introduction of dsRNA into worms have been developed. Tabara and Mello (1998) discovered that merely soaking worms in a solution containing dsRNA was capable of inducing knockdown phenotypes, whereas Timmons and Fire (1998) ingeniously crafted a method for inducing dsRNA expression in the worm's bacterial diet. While these routes of dsRNA introduction are

potently effective in *C. elegans*, injection into the body (or egg) is the most common avenue utilized in arthropod species. Unlike most terrestrial dwelling arthropods in which function has been investigated, *Triops* provided an opportunity to explore gene function via RNAi by employing all three available dsRNA delivery techniques due to its aquatic habitat and ability to eat bacteria (Fig. 3.2).

dsRNA may be introduced at various stages of an animal's life. Depending on the organism and the developmental stages it undergoes, these include egg/embryo, larva (predominantly for holometabolous insects and certain arthropods like *Triops* and *Artemia*), and adult (Fig. 3.3). Egg/embryonic RNAi is typically used when studying process related to larval or adult development, anatomy, or physiology, whereas dsRNA introduction at the larval stage is often employed when investigating features related or leading up to the adult. Beginning at the adult level of development has advantages over dsRNA introduction at earlier developmental stages. RNAi not only has the capability of impacting the adult condition but it has the potential of passing to the germ line in females, thereby producing phenotypes in embryonic offspring, and depending on the type and severity of the knockdown, possibly larval and adult phenotypes of the next generation. Because *Triops* develops from a cyst arrested at the gastrula stage and this cyst is an obligate phase of the life cycle, *Triops* is only open to dsRNA introduction at the larval and adult stages.

Before RNAi is attempted, predictions may be made about the potential phenotypes of *Triops* or *Artemia* leg patterning genes likely to be produced by gene knockdowns, based on expression patterns and their functional roles in other arthropods.

Below I outline what I anticipated to find using RNAi with leg patterning genes in the branchiopod crustaceans.

dac expression and mutant phenotypes were previously described in chapter 2 (Angelini and Kaufman, 2004; Mardon et al., 1994). Based on my own results and all prior published work, reduced *dac* expression would be expected to eliminate or fuse leg joints in *Triops* and abolish ventral branch structures. An additional role for *dac* during development, not previously mentioned, is in development of the nervous system (Mardon et al., 1994; Prpic et al., 2001), therefore defects in eye development, the ganglia of the central nervous system or in the peripheral sensory structures, e.g. the setae in the leg branches, would be anticipated in both *Triops* and *Artemia*.

Dll is necessary for limb development beyond the ground-state of the body wall and loss of *Dll* function results in loss or fusion of distal leg elements (Angelini and Kaufman, 2004; Beermann et al., 2001; Cohen et al., 1989; Gonzalez-Crespo and Morata, 1996; Schoppmeier and Damen, 2001). *Dll* expression is not only linked to distal limb structures but is associated with the peripheral nervous system as well (Williams et al., 2002). Because of these characteristics, reduced *Dll* expression is predicted to manifest in *Triops* as a loss of the endopod/exopod and conceivably eradication or reduction of setae.

dpp and *wnt-1/wg* have mutually antagonistic yet complementary roles in *Drosophila* leg development. Lowering or suppressing *dpp* in *Drosophila* yields a range of leg phenotypes from loss of distal leg elements or ventralization of the dorsal leg (Jiang and Struhl, 1996; St Johnston et al., 1990), whereas knockdown of *dpp* in

Tribolium results in virtually wild-type legs (Ober and Jockusch, 2006). On the other hand, down regulating *wnt-1/wg* in *Drosophila* has produced dorsalization of the ventral axis or a complete lack of legs (Jiang and Struhl, 1996; Johnston and Schubiger, 1996). Additionally, lowering *wg* expression eliminated leg development in *Tribolium* yet no leg phenotype was apparent in *Oncopeltus* (Angelini and Kaufman, 2005a; Ober and Jockusch, 2006). Based on these observations, leg phenotypes seen in *Drosophila* or no defects at all are foreseeable.

caudal (*cad*) is a homeobox gene that plays a role in establishing the A/P body axis (Macdonald and Struhl, 1986; Moreno and Morata, 1999). As development proceeds, *cad* is restricted to a growth zone from which future segments are formed (Copf et al., 2004; Schulz et al., 1998), therefore loss of posterior growth and differentiation were anticipated phenotypes in *Triops*, as seen in *Artemia* (Fig. 3.4).

Mutations in the *Antennapedia* (*Antp*) and *Ultrabithorax* (*Ubx*) genes generate "homeotic" phenotypes and frequently manifest as body regions adopting a more anterior identity. *Antp* mutants partially transform legs into antennae in *Drosophila* whereas in *Oncopeltus* a complete transformation of leg into antenna is seen in severe cases (Angelini et al., 2005; Casares and Mann, 1998; Emerald and Cohen, 2004). *Ubx* produces a transformation of third thoracic segment into second thoracic identity in *Drosophila*, whereas ectopic limbs with leg features appear on the first abdominal segment in *Tribolium* and *Oncopeltus* (Angelini et al., 2005; Lewis et al., 2000; Lewis, 1963). Although both are expressed in largely overlapping domains, in insects they define distinct segment types in the thorax and abdomen, whereas in *Artemia* (and *Triops,*

Nagy unpublished) they are associated with a predominantly uniform thoracic region (Averof and Akam, 1995). Copf et al (2006) demonstrated that leg development in the *Artemia* abdomen was accompanied by ectopic activation of posterior *Hox* genes *Ubx*/*Abdominal-A* (*Abd-A*), suggesting they may be necessary for leg development in the trunk. Yet it is not known whether it is *Ubx*, *Abd-A*, or both that are potentially required. Therefore, "homeotic" transformations or loss of trunk appendages would be likely in larvae injected with *Ubx*/*Abd-A* dsRNA.

Materials and Methods

Animal husbandry

Artemia cysts were purchased from Ward's Natural Sciences whereas *Triops* cysts were purchased from a variety of vendors, which include Ward's Natural Sciences, Fawcett Hobby Shop (Utah), and *Triops* Inc. *Triops* received from Ward's and Fawcett's were packaged in bags of detritus and cysts from *Triops* Inc were packaged in egg vials free of detritus. *Artemia* cysts were sprinkled into small aquariums with freshly prepared artificial seawater and allowed to hatch at room temperature. *Triops* were reared under a variety of conditions based on the dsRNA introduction method being explored (conditions elaborated within method).

dsRNA synthesis

cDNA was PCR amplified with M13 forward & reverse primers. Product was column purified and used as a template for the transcription reactions. Sense and α-sense strands were transcribed in two separate reactions. *Triops dac*, *dpp*, *Dll*, *Ubx* short and

long, and *Artemia dac* EST templates were transcribed with SP6 and T7 RNA polymerases (Roche) whereas *Triops wnt-1* and *Artemia wg* and *cad* cDNA templates were synthesized with T3 and T7 RNA polymerases. Reactions were treated with DNase I and the RNA strands were subsequently purified using either RNeasy (Qiagen) or a TRIzol®:chloroform extraction followed by an ethanol-LiCl precipitation. Purified/resuspended were quantified using the nanodrop (ND1000-spectrophotometer) and the RNA strands were combined in equal quantities and annealed in a thermocycler (Perkin-Elmer) by incubating at 85°C 10 minutes and then shutting off the machine and allowing the dsRNA to slow cool for 20 minutes. dsRNA was quantified using the nanodrop.

Soaking RNAi

Soaking buffer mediated: Pure *Triops* cysts (*Triops* Inc) are treated with a 50% bleach solution (in aged tap water) with continuous mixing for 2 min. to digest the outer woody shell (alveolar layer) of the cyst. Embryos are subsequently washed several times with M9 solution (43.6 mM Na_2HPO_4, 22.0 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl). Embryos washed once with soaking buffer (10.9 mM Na_2HPO_4, 5.5 mM KH_2PO_4, 2.1 mM NaCl, 4.7 mM NH_4Cl, 3 mM Spermidine, 0.05% gelatin). 25 embryos were allocated per well in a 96 well round bottom micro-titer plate. Buffer used to transfer animals into the well was removed and replaced with 50 µl of a dsRNA solution (dsRNA dissolved in soaking buffer: volume of dsRNA to achieve 200-1000 ng/µl, a volume of concentrated soaking buffer stock to achieve above stated concentrations, and water to reach 50 µl). Three control incubations were performed: 1) yeast tRNA (500

ng/μl in soaking buffer) was used as a control RNA, 2) soaking buffer only (aged tap water and soaking buffer—volumes comparable to dsRNA and soaking buffer), and 3) aged tap water only. Larvae permitted to develop until a portion had developed through the 2^{nd} – 3^{rd} larval molt. All animals were preserved for 20 minutes in a 4% formaldehyde solution (PEMFA). Preserved specimens were either transitioned into methanol (for storage) or washed with PBTween several times with an extended wash containing the nuclear stain, Hoechst, stepped into an 80% glycerol solution, mounted on a slide and visualized on the Zeiss.

Liposome mediated: Lipofectamine (Invitrogen): used at 1 μl/μg dsRNA to achieve a final volume of 50 μl ($[dsRNA]_f$ = 200 ng/μl) and placed on bleach treated embryos. Animals allowed to hatch and preserved once a portion of the hatchlings had reached the third larval stage. Clonfectin (Clontech): reconstituted as described by manufacturer, used at 1 μl/μg dsRNA ($[dsRNA]_f$ = 100 ng/μl), and placed on bleach treated embryos. Animals monitored and preserved as in Lipofectamine treatment.

Water mediated: Cysts were bleach treated as above and washed with copious amounts of aged tap water. Embryos were transferred to the micro-titer plate where the medium used to transfer the embryos was removed and replaced with 50 μl of either dsRNA (500 ng/μl) dissolved in aged tap water or a control solution. *Triops* were allowed to hatch and develop as in the soaking buffer mediated experiment and subsequently preserved as stated above.

Feeding dsRNA expressing bacteria

Tldac cDNA was PCR amplified, purified, digested with restriction endonucleases, and ligated into the double T7 vector L4440 (Timmons et al., 2001). Recombinant plasmids were transformed into DH5α max efficiency cells, mini-prepped, and verified via PCR utilizing T7 primers as well as dideoxy sequencing. Recombinant clone was transformed into competent HT115(DE3) cells that lack RNase III and are capable of inducing T7 RNA polymerase expression when IPTG is added to the culture medium. Feeding regimen took two approaches, dsRNA expressing bacteria were centrifuged and resuspended in 1) aged tap water or 2) artificial food mix consisting of ground algae, ultra-low melt agarose, and food coloring (Newmark et al., 2003). Embryos hatched in the water containing dsRNA-expressing bacteria and the media was changed every day for 2-4 days after hatching. Animals fed with the artificial food mix were not provided with the food mix until after hatching. Animals were given fresh food mix everyday for 2-4 days.

dsRNA microinjection

Triops (Ward's or Fawcett's-bags of detritus and cysts were dumped into a small aquarium and kept at room temperature) and *Artemia* (Ward's) larvae were placed on the surface of a petri dish containing 2.5% agarose in aged tap water for *Triops* and filtered artificial seawater for *Artemia*, immobilized by wicking away excess water with a kim-wipe, and injected into the body cavity using a Narishige micromanipulator. Injection solution was prepared by adding an equal volume of Phenol Red (Sigma) to a solution containing dsRNA dissolved in water ($[dsRNA]_f$ = 300-4500 ng/μl). Injected *Triops*

were cultured individually for 5-7 days in 12-well tissue culture dishes containing aged tap water conditioned with tea bags of detritus. Half of the water volume was replaced daily and the animals were fed thawed frozen brine shrimp. *Artemia* were cultured 7-14 days in 12-well tissue culture dishes containing filtered artificial seawater (FASW). Larvae were transferred to wells containing fresh FASW everyday for the first 5 days post injection and were fed a concoction of ground algae and yeast resuspended in FASW. Beyond the fifth day, half of the water volume was replaced and they were fed daily. Animals were harvested and preserved at the end of the experiment.

In Vivo GeneShuttle (IVGS) (Q-bio): Two separate solutions were prepared: 1) IVGS: 5% dextrose (D5W) in a 2:3 ratio, and 2) dsRNA added to D5W solution in a volume equal to IVGS+D5W. Combine both solutions and mix ($[dsRNA]_f = 300$ ng/µl). Solution was combined 1:1 with a Phenol Red solution and subsequently inject into 1st and 2nd larvae. *Triops* cultured and preserved as in standard microinjection.

Results

Soaking mediated

Various permutations on soaking embryos/larvae in a medium supplemented with dsRNA were explored. Initial studies involved hatching and maintaining *Triops* in a buffer formulated for *C. elegans* (Maeda et al., 2001; Tabara et al., 1998) with ds*dac*RNA. Worms were soaked in small volumes with high dsRNA concentrations, therefore, clutches of *Triops* embryos had to be cultured in small volumes to keep ds*dac*RNA concentrations high. Initially, several larvae had legs with a variety of defects (Fig. 3.5). Legs from ds*dac*RNA soaked animals were smaller than control legs and had

extremely reduced or missing medial branches (Fig. 3.5-arrows). Inability to reproduce initial phenotypes prompted the exploration of other genes known to participate in limb patterning (Fig. 3.6). After further soaking buffer trials with dsRNAs for additional leg patterning genes produced animals wild type in appearance, I set out to ascertain whether non-reproducibility was due to dsRNA penetrance into the animal. Fluorescein labeled dextran was placed in the water during *Triops* incubation and hatching. The dye was not detected within the larva until the second naupliar stage (~2h post-hatching), which follows the first molt (not shown). Animals were monitored through the fourth larval stage and the dye was only detected in the gut and what may be tubules of the digestive gland. This suggests that the dsRNA was being ingested but not spreading beyond the digestive system. There was no indication that the dsRNA was entering epithelial cells directly through the medium. This finding suggested that dsRNA penetration was an issue, therefore I used liposome additives with the dsRNAs to help facilitate systemic uptake (Fig. 3.6; Templeton et al., 1997). However, these additional soaking permutations were incapable of inducing phenotypes.

Feeding mediated

Since soaking animals in a medium containing fluorescein dextran led to visible fluorescein dextran in the digestive system I sought to increase the concentration of dsRNA in the digestive system, hypothesizing that this might increase the likelihood of uptake by other cells. I explored supplementing the diet (Newmark et al., 2003; Timmons et al., 2001; Timmons and Fire, 1998) with ds*dac*RNA expressing bacteria. Bacteria were provided as either a suspension or gelatinous paste. This approach offered

two obvious benefits; 1) ds*dac*RNA concentration would not be an issue, therefore larvae were cultured individually with ample volumes of water fortified with food in microtiter wells, and 2) cost associated with *in vitro* dsRNA synthesis was eliminated. However, cultured animals fed on bacteria demonstrated limited survivability. This may have been due to starvation because appropriate food sources/nutrients were not provided (n=57, larvae rarely survived >24h).

Injection based for Triops and Artemia

Hemolymph injections eliminate the need for continued exposure of *Triops* embryos/larvae to an outside source of dsRNA. Additionally, only small amounts of dsRNA were required to inject a large number of animals. Injecting dsRNA into the hemolymph of larval and adult insects, as well as *Artemia* larvae, have proven beneficial in learning about gene function (Angelini and Kaufman, 2004; Bucher et al., 2002; Copf et al., 2006; Copf et al., 2004; Liu and Kaufman, 2004a; Liu and Kaufman, 2004b; Ober and Jockusch, 2006; Tomoyasu and Denell, 2004). This approach appeared the most promising in its potential to produce phenotypes for various limb patterning genes in *Triops* due to its reported success in *Artemia* (with *caudal*), which I was able to reproduce (Fig. 3.4). The injection of *Triops* (Fig. 3.7) with ds*dac*RNA initially produced mutant phenotypes that corresponded well with its expression pattern (n=3; Fig. 3.8). The polarity of the primary leg axis and medial branches was affected (Fig. 3.8). In addition, the legs were missing the gnathobase (Fig. 3.8-arrow). However, in no case was I able to repeat the phenotype. It was possible that DAC protein levels were indeed being knocked down, but that the level of reduction was insufficient to generate a

phenotypic response. Using the *Triops* DAC antibody that I made (described in Chapter 2) I examined DAC protein expression in ds*dac*RNA-injected animals in 24h intervals post-injection to determine if DAC expression was disturbed at any point during the course of the experiment. Each animal pictured is representative of all cohorts preserved at that particular time point. DAC was expressed in the posterior trunk at all time points examined and staining of ds*dac*RNA injected animals is virtually as intense as the control (Fig. 3.9). Thus, I conclude that there was either very little, or no reduction in protein levels in dsRNA injected animals.

In a parallel set of experiments, I investigated whether gene duplication was an underlying cause for low phenotype penetrance. The genomes of *Artemia* and *Triops* are not sequenced, and it is not known to what degree duplicated genes are present in the genome. In an unrelated project two copies of the *Triops Hox* gene *Ultrabithorax* were cloned, therefore I injected dsRNAs for each copy of *Ubx*. Early injections produced two individuals with reduced trunks and virtually no posterior limb growth-precisely what one might expect if these *Hox* genes were required for limb patterning (Fig. 3.10-arrow). As with previous injections, I was unable to induce more mutant phenotypes.

Since *Triops* was proving a challenge, I decided to use *Artemia* as a system to study *dac* function. As with *Triops*, injections of *dac* (*Afdac*) into *Artemia* yielded a promising initial result, which was the asymmetrical development of ventral neural clusters (Fig. 3.11-yellow arrow). However, subsequent experiments with ds*Afdac*RNA generated no additional phenotypes. Because it is known that the RNAi machinery is present and functional in this species (Copf et al., 2006; Copf et al., 2004), I tested

whether other limb patterning genes might generate knockdown phenotypes. I therefore also performed injections with the *Artemia wg* ortholog but no deformities were noted (Fig. 3.12). Injections with ds*Afcad*RNA were performed in parallel with each *Artemia* trial. Each ds*Afcad*RNA injection produced animals with published phenotypes.

Discussion

Even after bleach treatment removes the alveolar (outer) layer of the cyst, *Triops* embryos are still surrounded by two layers; 1) outer cuticular membrane that protects the embryo from penetration by molecules larger than the CO_2 and 2) a transparent and highly elastic embryonic cuticle that develops into the hatching membrane during hatching incubation. Although I speculated that if the dsRNA were present in the medium during the time when the cysts were rehydrating and hatching, I might be able to penetrate through these layers to the epithelial cells, this was not the case.

Furthermore, due to its cannibalistic nature, *Triops* requires special culturing conditions. Once *Triops* reach larval stage 3-4 (~12-20h post-hatching) they have depleted their yolk stores and begin to feed. Consequently, other newly hatched *Triops* within the vicinity provide a ready food source. This particular behavior coupled with limiting dsRNA quantities necessitated short soaking incubation times or culturing each animal separately.

Why is Triops refractory to RNAi?

In *Artemia*, RNAi has only been demonstrated to work on a small percentage of the genes investigated and sometimes they produce unexpected and subtle phenotypes

(Averof, personal communication; Table 3.1). Failure of genes other than *caudal* to generate mutant phenotypes in *Artemia* may stem from a variety of sources, which include the following: redundant genes either through gene duplications or different genes, perhaps from a common gene family, performing a similar function; phenotypes may be too subtle to detect; mRNA molecules may not be accessible to RNAi machinery due to interactions with RNA binding proteins; the temporal window of gene function might be small and easily missed; RNAi not capable of reducing gene dosage below effective levels; or dsRNA molecules do not possess qualities, such as size or sequence composition, for optimal efficiency. Two pieces of evidence arguing against quality considerations comes from *Oncopeltus* where they functionally dissected numerous genes involved in development (Angelini and Kaufman, 2004; Angelini and Kaufman, 2005a; Angelini et al., 2005; Hughes and Kaufman, 2000). First, clones for the *Hox* genes *proboscipedia*, *Deformed*, and *Sex combs reduced* used in successful functional studies ranged from 258-358 bp in length and second, most ESTs used as templates for dsRNA synthesis were cloned using standard degenerate PCR for gene capture. Finally, *Triops* larvae undergo 3-4 molts within the first 24h of life and this rapid growth and development may provide unfavorable conditions for RNAi.

Future directions

Is RNAi an option in *Triops*? Further exploration of the components of the RNAi pathway present in *Triops* is needed. Are dsRNAs capable of being transported systemically into cells? This may be verified by tracking labeled dsRNA molecules post hemolymph injection. Also, existence and viability of the cellular components necessary

to carryout the destruction of particular mRNAs could be ascertained by testing the ability of *Triops* extracts to degrade target mRNAs with addition of dsRNA or siRNA as previously demonstrated for *Drosophila* (Elbashir et al., 2001; Tuschl et al., 1999). Another way to enhance the chances of establishing RNAi as a means of investigating gene function is to hone *Triops* husbandry in combination with a viral or transposable element system for delivery (Lewis et al., 1999; Oppenheimer et al., 1999; Pavlopoulos and Averof, 2005).

Alternatively, gene function may be ascertained indirectly by cloning the entire coding region of *Triops* limb patterning genes and subsequently transforming *Drosophila* with these genes. One could not only assay their abilities to rescue mutant *Drosophila* strains, but other phenotypic consequences of expressing an exogenously provided ortholog might be found as well (Galant and Carroll, 2002; Ronshaugen et al., 2002).

Group	Species	RNAi friendly	Genes (+) phenotype	Genes (-) phenotype	Methods of introduction	Source
Hexapods						
	Drosophila melanogaster	yes	*GM06434*	?	injection	Dzitoyeva et al., 2001
	Manduca sexta	yes	recognition protein mediated immune responses	?	injection	Eleftherianos et al., 2006
	Tribolium castaneum	yes	*caudal, Distal-less, decapentaplegic, even-skipped, odd-skipped, paired, runt, sloppy-paired, Ultrabithorax, wingless, zerknullt*	?	injection	Tomoyasu and Denell, 2004; Copf et al., 2004; Tomoyasu et al., 2005; van der Zee et al., 2005; Choe et al., 2006; Ober and Jockusch, 2006
	Onthophagus taurus	yes	*dachshund*		injection	Sewell thesis, Appendix A
	Blattella germanica (cockroach)	yes	*rxr*	?	injection	Martin et al., 2006
	Oncopeltus fasciatus	yes	*Abdominal-A, Abdominal-B, Antennapedia, dachshund, Deformed, Distal-less, decapentaplegic, engrailed, even-skipped, homothorax, hunchback, Kruppel, pangolin, proboscipedia, Sex combs reduced, tiptop, Ultrabithorax, wnt-1, zerknullt*	?	injection	Hughes and Kaufman, 2000; Angelini and Kaufman, 2004; Lui and Kaufman, 2004a; Lui and Kaufman, 2004b; Angelini et al., 2005; Angelini and Kaufman, 2005; Herke et al., 2005; Lui and Kaufman, 2005; Panfilio et al., 2006
	Gryllus bimaculatus	yes	*armadillo, caudal, hunchback, Kruppel*	*hedgehog, wingless*	injection	Miyawaki et al., 2004; Shinmyo et al., 2005; Mito et al., 2005; Mito et al., 2006
	Schistocerca gregaria	no		*engrailed*	injection	Averof and Akam, personal comminication
	Thermobia	no		?	?	Kaufman, personal communication
Crustaceans						
	Artemia franciscana	yes	*Abdominal-B, caudal, even-skipped, spalt, X, Y*	*Antennapedia, dachshund, Ultrabithorax, wingless*	injection	Copf et al., 2004; Copf et al., 2006; Copf and Averof, personal communcations; Sewell thesis, Chapter 3
	Triops longicaudatus	no		*caudal, dachshund, decapentaplegic, Distal-less, Ultrabithorax, wnt-1*	soaking, feeding, injection	Sewell thesis, Chapter 3
	Litopenaeus vannamei (white shrimp)	yes	antiviral silencing	?	injection	Robalino et al., 2005
Myriapods						
	--------	--------	--------	--------	--------	
Chelicerates						
	Cupiennius salei	yes	*Delta, Distal-less, Notch, Presenilin, Suppressor of Hairless*	?	injection	Schoppmeier and Damen, 2001; Stollewerk et al., 2003; Schoppmeier and Damen, 2005
	Achaearanea tepidariorum	yes	*decapentaplegic, short gastrulation*	?	injection	Akiyama-Oda and Oda, 2006
	Amblyomma americanum	yes	histamine binding protein	?	injection	Aljamali et al., 2002
	Ixodes scapularis	yes	anticomplement gene	?	feeding	Soares et al., 2005

Table 3.1. Survey of RNAi in Arthropods. (Legend follows.)

Table 3.1. Survey of RNAi in Arthropods. This table provides a brief overview of RNA interference attempts in various arthropod species. Whether RNAi has been successfully employed is indicated in the column labeled RNAi friendly. Columns designated Genes (+) phenotype and Genes (-) phenotype list genes capable or incapable, respectively, of eliciting a phenotype. Column six states method used to introduce dsRNA into the animal and the last column lists the information sources. A (?) denotes that particular piece of information is unknown. Yellow cells represent personal communiqués whereas information for species in white cells was retrieved from the literature. *Drosophila*, *Manduca*, *Blattella*, *Litopenaeus*, *Amblyomma*, and *Ixodes* have only one gene or gene product listed merely to demonstrate that RNAi was a viable approach to study gene function. Note: this is not an exhaustive list.

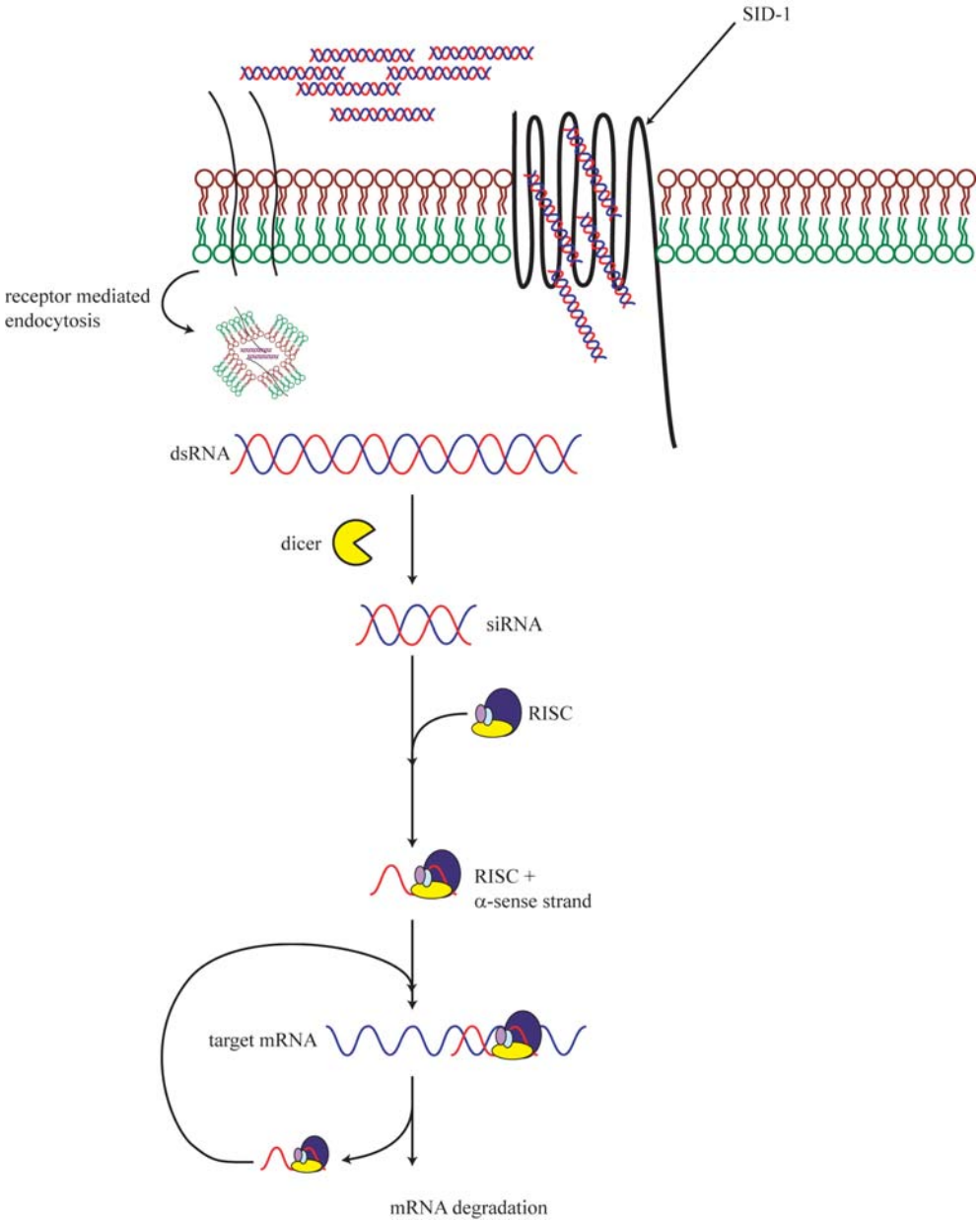


Fig. 3.1. RNA interference pathway. dsRNA is transported in a non-ATP dependent fashion into the cell via SID-1, a transmembrane receptor, or via active receptor mediated endocytosis. Once inside the cell, dsRNA is cleaved into small dsRNA fragments by Dicer. RISC binds the diced dsRNA, degrades one strand, uses the other strand to locate cellular RNAs with complementary sequences, and digests the target RNA.

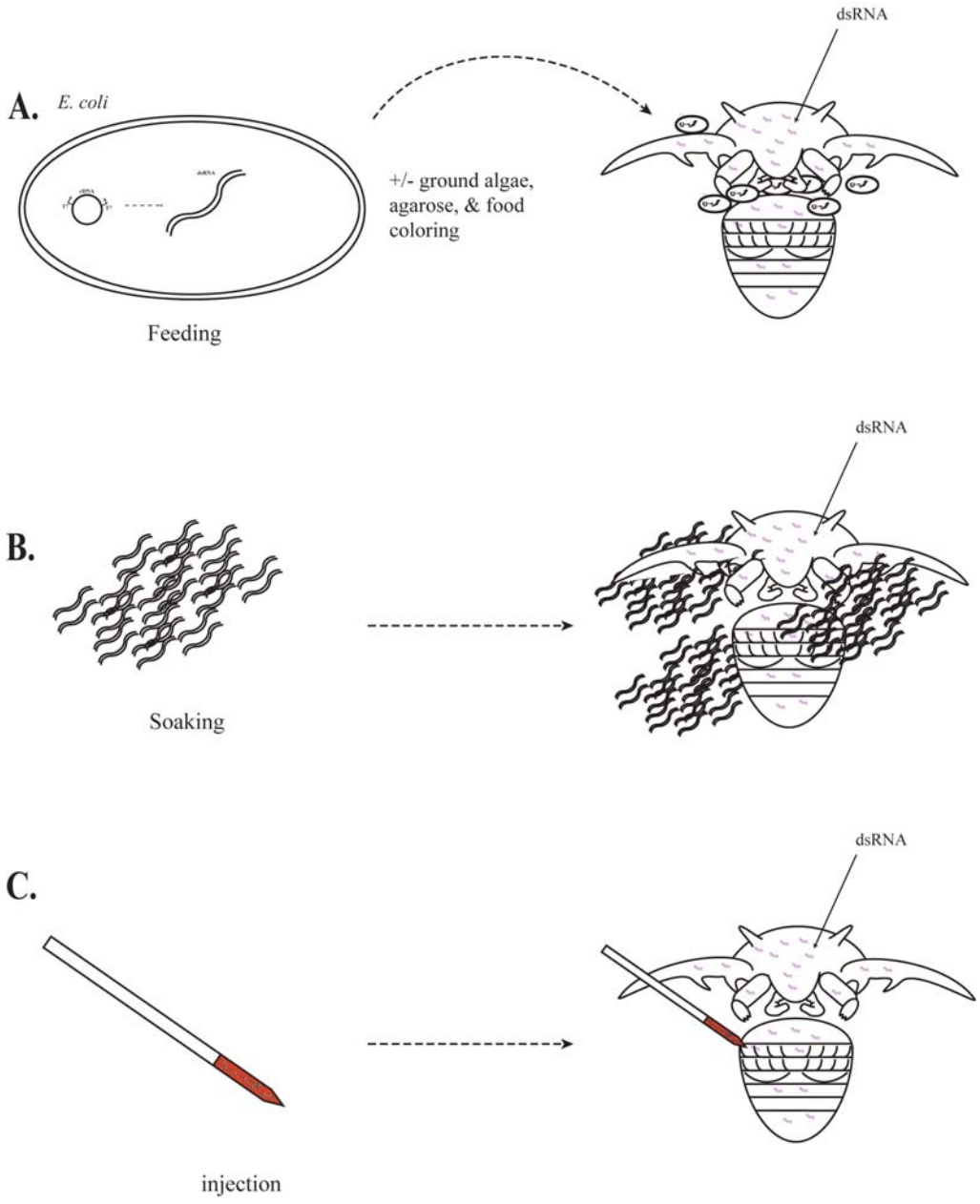


Fig. 3.2. Standard methods for introducing dsRNA. (A) Engineered bacteria are induced to express dsRNAs of the gene of interest. Bacteria are ingested and dsRNA enters animal through the gut. (B) Specimen is incubated in an aqueous medium with dsRNA, either dissolved or encapsulated by a lipid vesicle. dsRNA is uptaken by cells by an as yet poorly understood mechanism. (C) Synthesized dsRNA is delivered into the hemolymph by injection, disseminated throughout the animal, and enters cells possibly by way of a transmembrane receptor like SID-1.

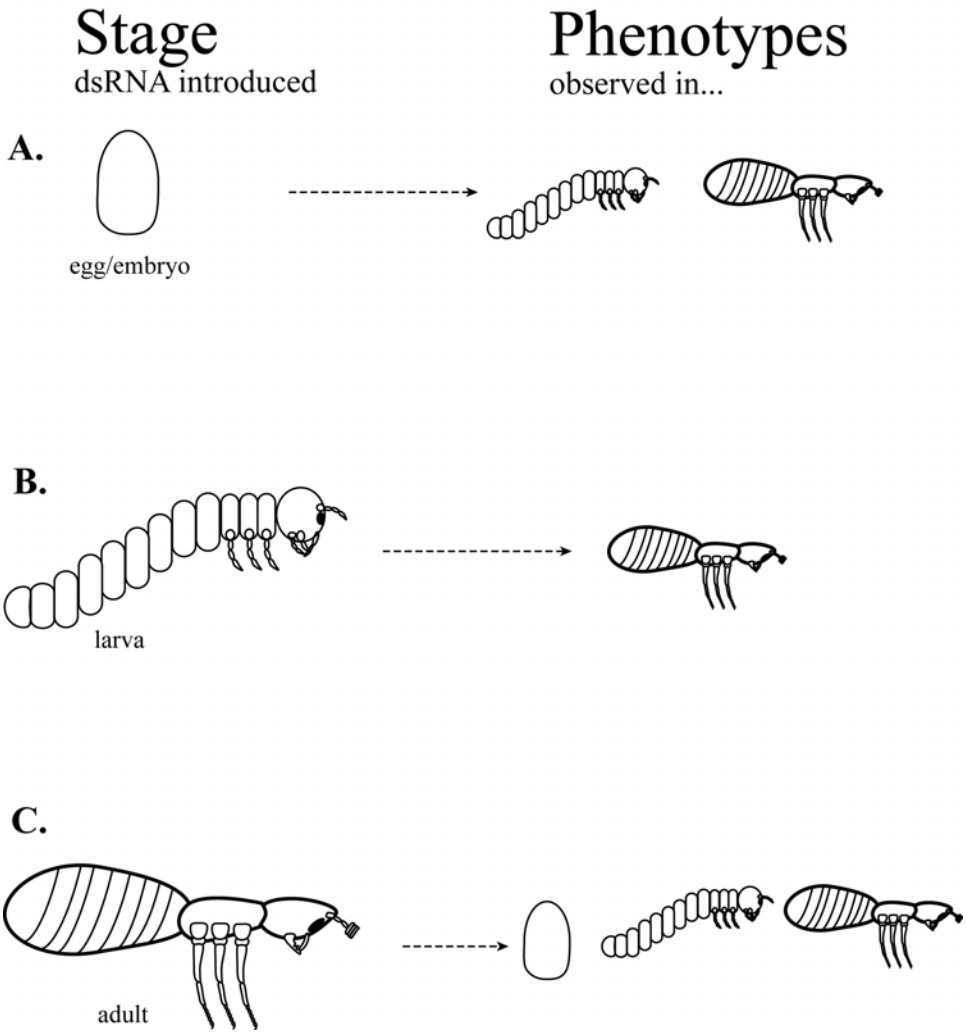


Fig. 3.3. Stages for introducing dsRNA and detecting phenotypes. Animals are exposed to dsRNA, via one of the previously mentioned methods, during one or more of the three general developmental stages: (A) egg/embryo, (B) larva, or (C) adult.

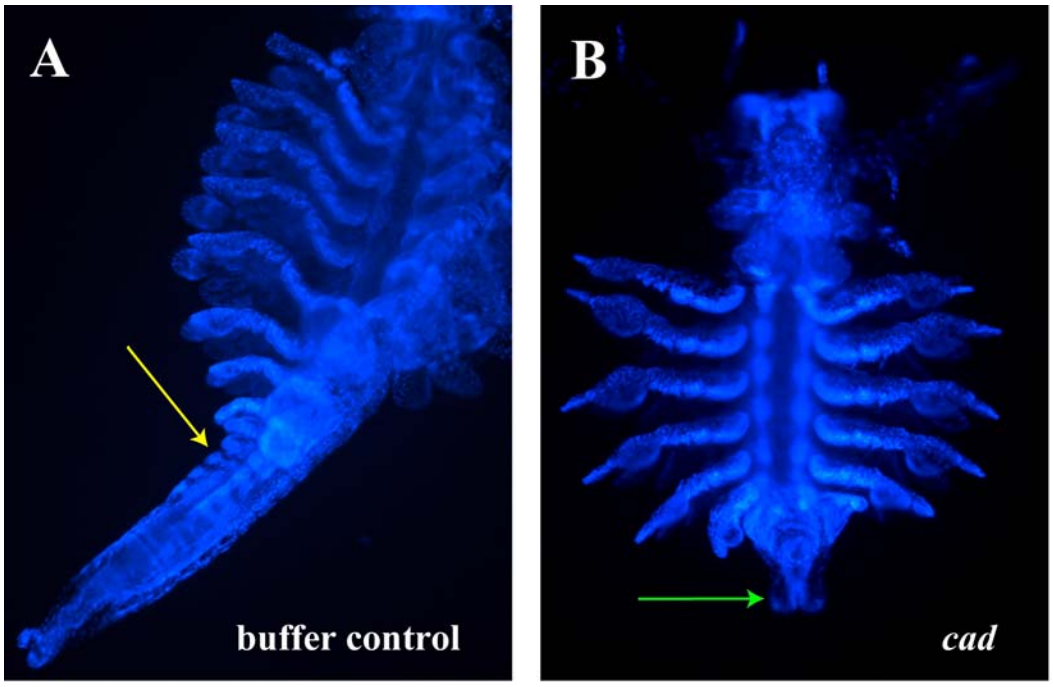


Fig. 3.4. ***Artemia caudal*** **knockdown.** Specimens (oriented anterior up) are visualized with Hoechst nuclear stain. (A) Control animal with developed thorax and abdomen (yellow arrow). (B) ds*cad*RNA injected animal with severely malformed posterior region (green arrow). This demonstrates that injection of dsRNA into early larvae is a viable method for investigating function.

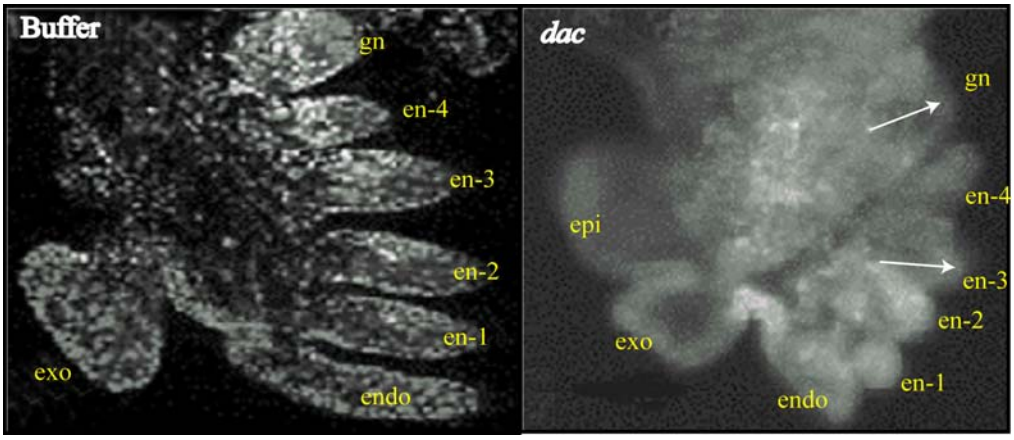


Fig. 3.5. *Triops* ds*dac*RNA soaking phenotype. Legs oriented dorsal left and distal down. ds*dac*RNA injected legs exhibit smaller size than wild type and have missing or reduce medial branches (arrows).

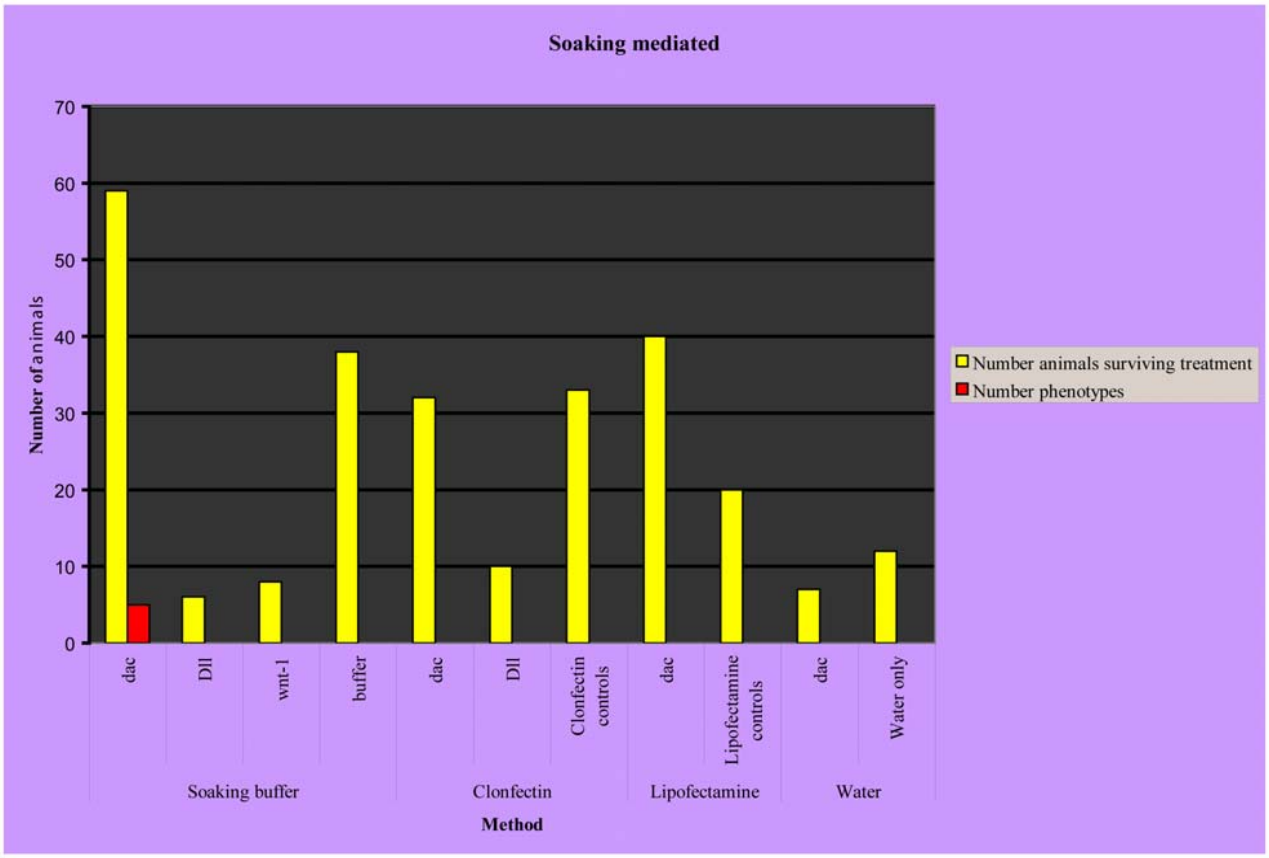


Fig. 3.6. Soaking mediated RNAi-*Triops* trials. *Triops* embryos (and larvae subsequent to hatching) were incubated with dsRNA for a variety of limb patterning genes using either soaking buffer, the liposomes Clonfectin and Lipofectamine, or aged tap water. Soaking buffer generated a few phenotypes when initially exploring this method. Subsequent trials were unable to reproduce any phenotype.

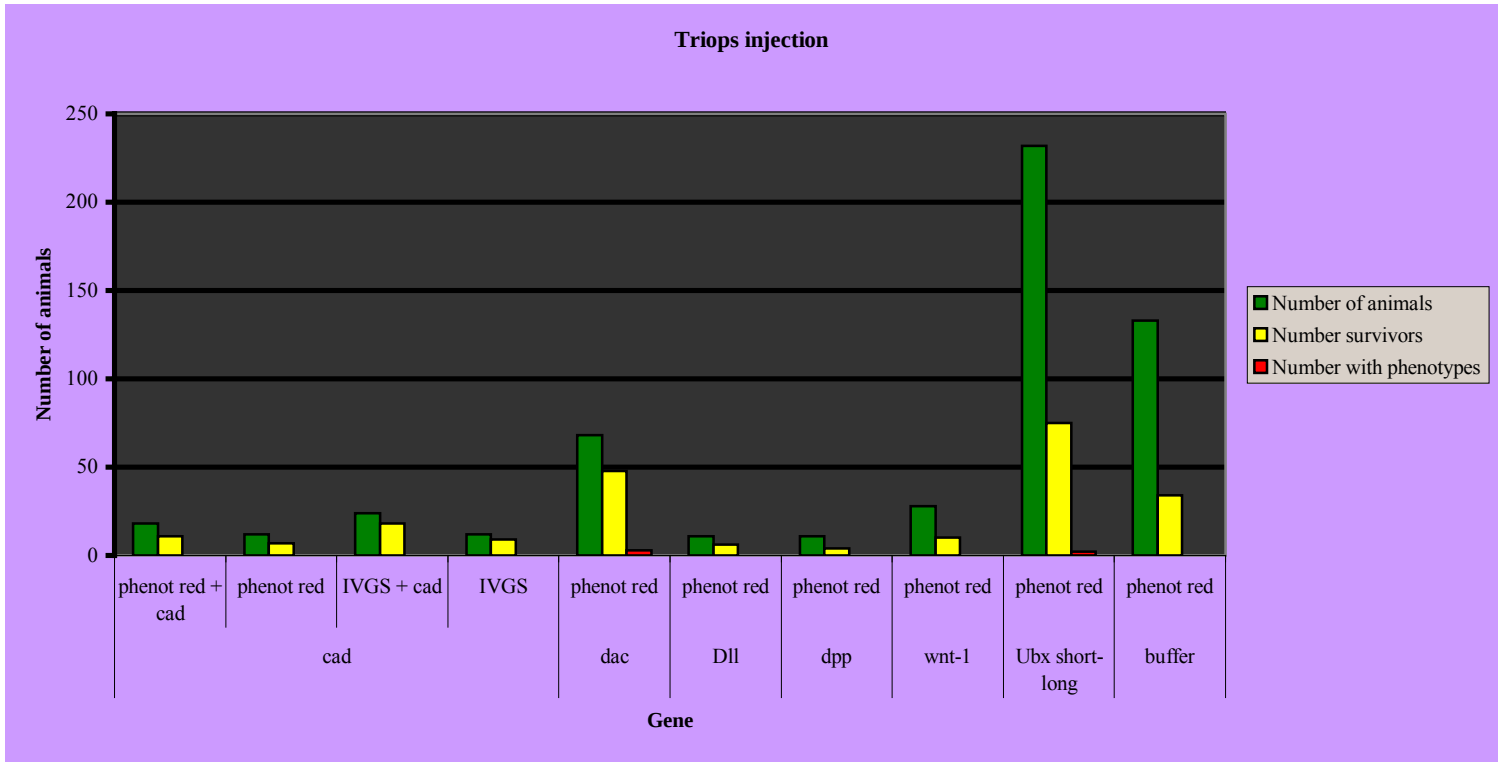


Fig. 3.7. Injection mediated RNAi-*Triops* trials. *Triops* 1st and 2nd stage nauplii were injected with dsRNAs for a variety of genes known to participate in limb specification, patterning, or modification as well as continued development of the entire animal. A handful of animals showed non-reproducible deformities.

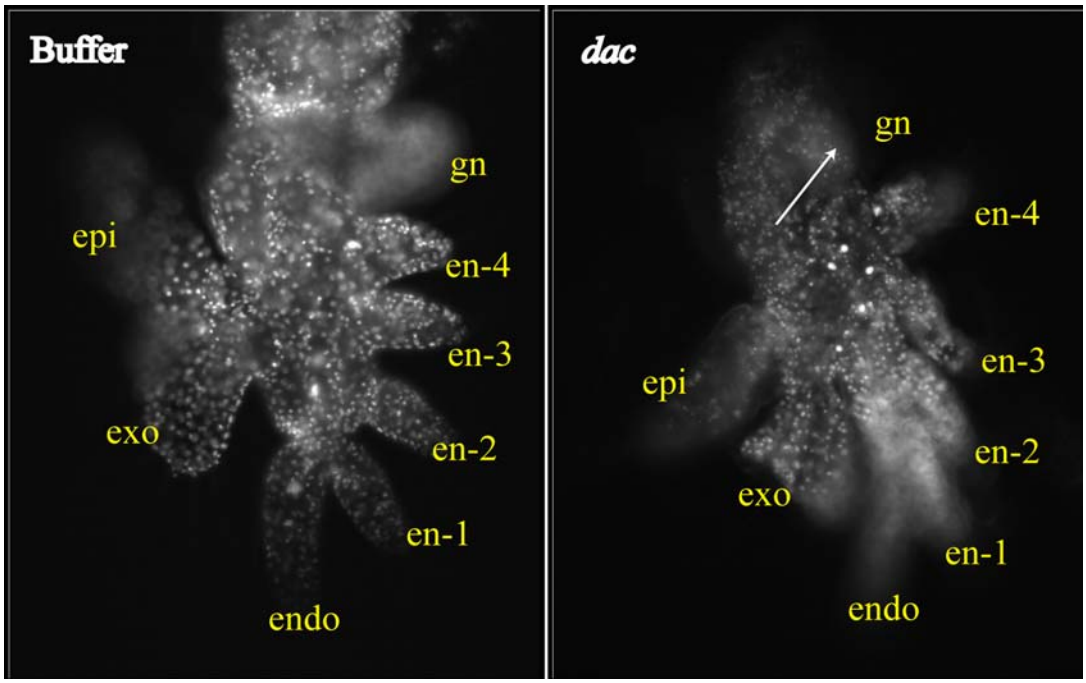


Fig. 3.8. *Triops* ds*dac*RNA injected phenotype. The medial branches of *Triops* larvae injected with ds*dac*RNA have an unusual curvature. Note the missing gnathobase (arrow). Branches are labeled for reference.

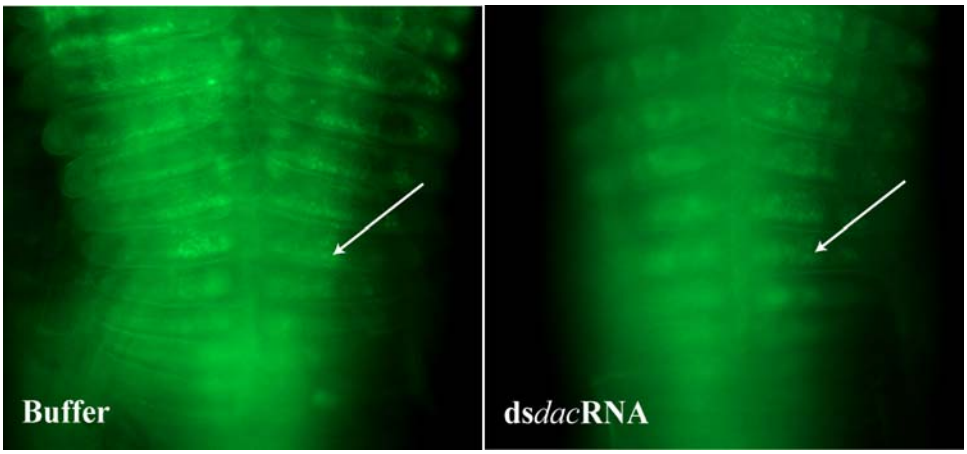


Fig. 3.9. α-DAC stained *dac* RNAi larvae. Control ds*dac*RNA injected specimens were preserved at 48h post injection and subsequently stained for the presence of DAC. DAC is detected at similar intensities in all cohorts examined (white arrows).

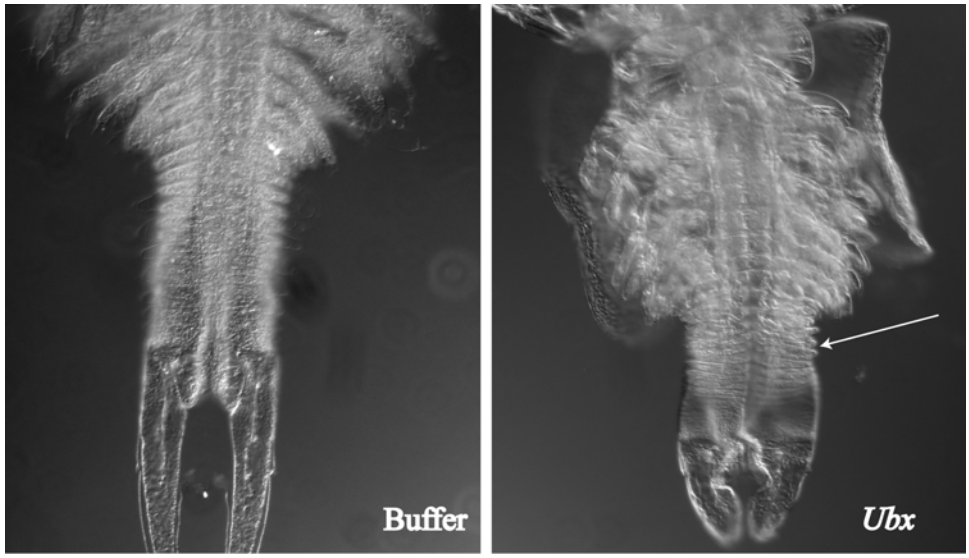


Fig. 3.10. *Triops* injection with dsRNAs for both copies of the *Triops Ubx* gene. Animals are oriented anterior up. Two animals injected with dsRNAs for both copies of the *Triops Ubx* gene possessed reduced trunk size (arrow) and posterior segments have dramatically reduce limb growth.

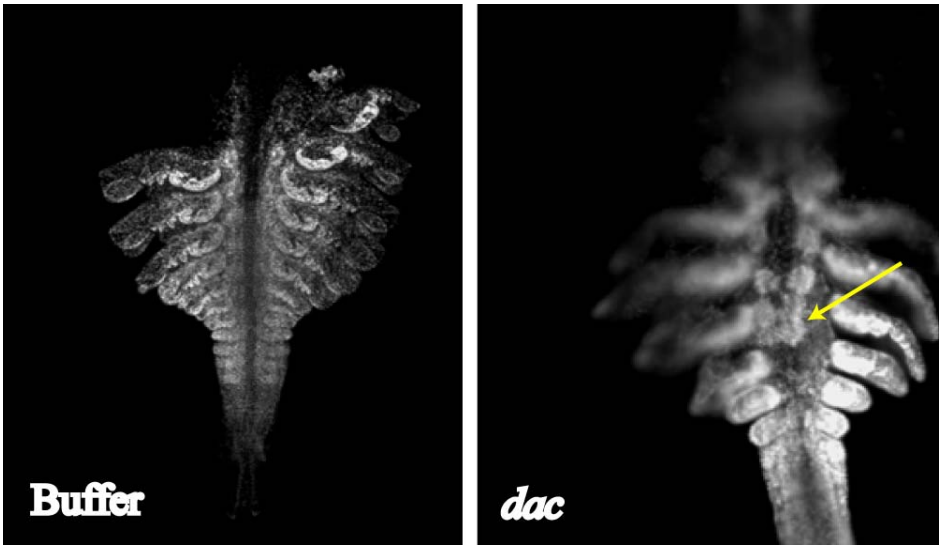


Fig. 3.11. *Artemia* ds*dac*RNA injected phenotype. Larvae are oriented anterior up. One animal injected with ds*dac*RNA had a defect in neural cluster development (yellow arrow).

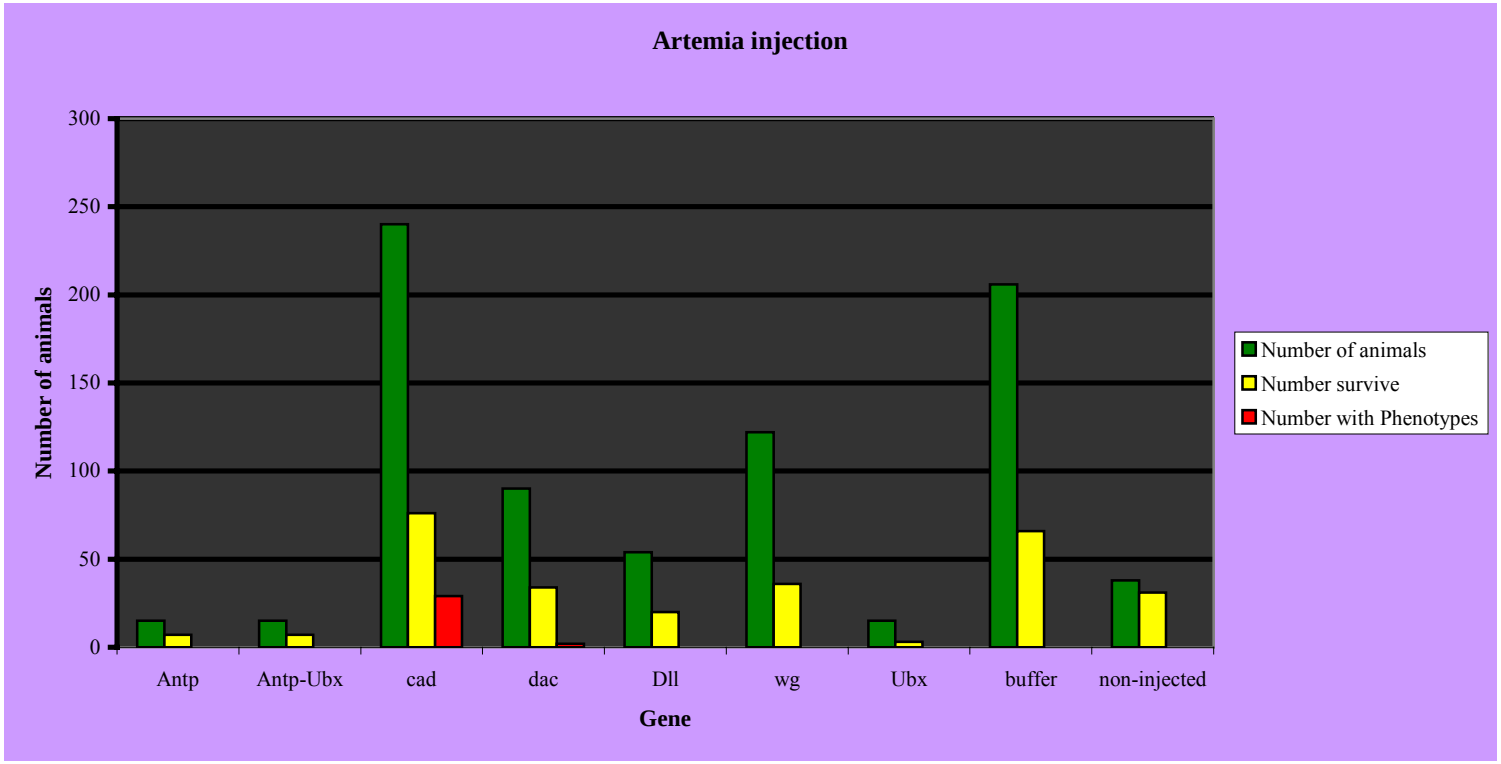


Fig. 3.12. Injection mediated RNAi-*Artemia* trials. *Artemia* 1st and 2nd stage larvae were injected with dsRNAs for a variety of genes known to participate in limb specification, patterning, or modification as well as continued development of the entire animal. *caudal* injected larvae generated repeatable phenotypes whereas trials with other genes yielded very few mutant animals. Repetition of injections, with the exception of *cad*, failed to produce phenotypes.

CHAPTER 4

CONCLUSIONS

In this investigation I set out to investigate the patterning processes behind limb patterning in limb development in the tadpole shrimp *Triops longicaudatus* using genes known to effect limb patterning in *Drosophila*. Even though *Drosophila* appendages undergo a novel morphogenesis compared to most arthropods, expression and functional studies in various arthropod taxa suggest that the basic P/D positions of the leg gap genes used in *Drosophila* are conserved across taxa. However, this is based predominantly on data from species with morphologically similar legs. I have asked how are these genes are expressed in a multiramous limb. By examining the spatiotemporal expression patterns of the leg gap genes, I provide verification that a limb as morphologically distinct as the multi-branched limb of *Triops* possesses gene expression features common to uniramous legs. Additionally, I showed how deployment of these basic patterning genes in the multibranched *Triops* leg relates to uniramous leg design. My efforts at generating RNAi induced phenotypes in *Triops*, as well as non-*caudal* mutations in *Artemia*, yielded no reproducible phenotypes. As part of my work to develop RNAi methods in *Triops* I surveyed RNAi work attempted in arthropod species to date. This mined data may prove beneficial for others when choosing a species for investigating the functional aspects of diverse biological processes.

P/D characteristics of a multiramous leg

In chapter 2, I examined the role of the leg gap genes *dac* and *hth* in the development of the multiramous leg of *Triops longicaudatus*. Based on the temporal and

spatial relationships of these genes with each other and *exd* and *Dll*, I propose that early P/D patterning of the *Triops* leg is similar to arthropods with uniramous legs. *hth*/*exd* and *Dll* establish early, non-overlapping domains defining the proximal and distal domains, respectively, in the legs of all arthropods examined to date (Abu-Shaar and Mann, 1998; Gonzalez-Crespo and Morata, 1996; Inoue et al., 2002; Prpic et al., 2003; Prpic and Tautz, 2003; Williams et al., 2002). However, there is no evidence for a third, intermediate domain of *dac* expression at the earliest stages of limb development. *Triops* DAC is initially expressed in the medial regions of the leg, overlapping distally with DLL expression and proximally with EXD expression. The initial expression of both DLL and DAC proteins in the leg is virtually simultaneous. Additionally, HTH expression was detected in every cell expressing n-EXD thereby reaffirming its roles in defining the proximal domain and serving as an essential co-factor for EXD.

The *Triops dac* pattern suggests that it might serve an unexpected role in segmentation of multi-branched limbs. DAC is expressed in a reiterated pattern in the lateral portion of each endite along the ventral axis of the *Triops* leg. In *Drosophila*, a similar repeated pattern is associated with genes such as *Notch*, *Serrate*, *Delta*, and *fringe*, which are involved in forming joints at each segment boundary (de Celis et al., 1998; Rauskolb, 2001; Rauskolb and Irvine, 1999). In the fly leg, HTH and DAC are necessary for expression of *Serrate* in locations within the bounds of their discrete expression domains along the P/D axis, rather than in each segment of the leg (Rauskolb, 2001). Yet in *Triops*, the HTH pattern does not lend itself to an obvious association with segmentation. Olesen et al (2001) proposed that endites in polyramous legs were

homologous with individual leg segments in the uniramous leg. Although I do not have evidence to support a direct morphological homology between regions of the *Triops* leg and individual leg segments in an insect, my results support the idea that patterning in the *Triops* leg proceeds through a process of segmentation. The end result of this segmentation process is not a series of segments separated by joints, but a medially iterated series of branches. Interestingly, treatment of the developing *Triops* larvae with an inhibitor of Notch signaling, DAPT, leads to the loss of the medial branches in the limbs (Ho and Nagy, unpublished results). Thus, I hypothesize DAC functions upstream of a Notch/Serrate signaling pathway that determines the number and position of the medial endites in the *Triops* limb.

dac supports serial homology of Triops appendages

While similarities in gene expression patterns do not define homology, they can provide support for homologous relationships. Prpic and others (2001) showed that *dac* is expressed throughout most of the mandible and a proximal domain in the maxilla, labium, and leg of *Tribolium*. Furthermore, *dac* is detected in the distal (telopod) region of the maxilla and leg. Interestingly, the expression of *dac* in the mandible and proximal maxilla, labium, and leg were not affected by a mutation in the *Tribolium Dll* gene. Loss of *dac* expression in the medial domain of the *Dll* mutants was possibly due to a requirement for *Dll* in establishing distal tissues. Proximal *dac* expression in head and thoracic appendages of *Tribolium* was suggested to provide evidence for the serial homology of these appendages (Prpic et al., 2001). My results with *Tldac* expression provide similar evidence for the serial homology of the second antenna, mandible,

maxillule, maxilla, and leg within *Triops*. DAC is not expressed in the *Triops* first antenna, and HTH and DLL are ubiquitously expressed. As such, the expression patterns of these three genes do not support the serial homology of the first antennae with the remaining appendages.

RNAi a valid functional approach

While expression patterns provide clues and evoke endless speculation as to the role of a gene development, I sought to determine whether altering *dac* gene expression would directly impact *Triops* leg morphology. Given the observed expression data, I predicted that loss of *dac* function would result in a stubby appendage without endites. The ability of this appendage to move properly would also likely be impaired, based on the large proximal swath of DAC expression, close to where the body wall and appendage join. In chapter 3, I chronicled my efforts at employing RNAi, a common method for inducing gene knockdowns, in *Triops* and *Artemia*. Despite repeated attempts with a variety of genes essential for limb development and a variety of methods, *Triops* was refractory to RNAi. *Artemia* proved similarly challenging. Although several genes produce phenotypes in *Artemia* following injection with dsRNA, many other genes are not responsive to injection of dsRNA (Copf and Averof personal communication; Copf et al., 2006; Copf et al., 2004). Failure to yield phenotypes utilizing RNAi is by no means a *Triops/Artemia* specific phenomenon. The firebrat *Thermobia* and the grasshopper *Schistocerca* were resilient to phenotype producing gene knockdowns using RNAi, although an attempt with only one gene was reported for the grasshopper. Despite instances where RNAi was incapable of inducing mutant phenotypes, it is a valuable

method for investigating gene function in arthropods due to successful use in diverse arthropod taxa. Myriapods represent the only major arthropod group in which attempts at employing RNAi have not been reported. Given the phylogenetic distribution of arthropod species for which RNAi has been successful, it is very likely that species from virtually any taxon are viable candidates for utilization of RNAi to address functional questions.

Future Directions

I have shown that modifications in the expression domains and spatial relationships of the leg gap genes relate to changes in the morphological characters of *Triops* appendages. The expression of the leg gap genes in *Triops* has shown that the multiramous leg is initially subdivided into discrete proximal and distal domains. Interestingly, the medial DAC expression pattern is initiated at the same time as DLL expression and suggests an unexpected system for patterning the medial branches of the leg. In *Drosophila*, the signaling pathways DPP, WG, and EGFR regulate leg gap gene expression. Both *wg* (Nulsen and Nagy, 1999) and *dpp* (Sewell, unpublished) are expressed in the early *Triops* limb buds in a pattern consistent with a likely function upstream of *Dll* and *dac* expression. The expression of the *wg* mRNA in relation to *dac* expression is particularly intriguing as they are both expressed in a similar (but non-overlapping) series of reiterated medial stripes. However, an understanding of their functional roles in limb development awaits the development of additional tools for analyzing gene function. Furthermore, it would be beneficial to determine how *Hox* genes, such as *Antp*, *Ubx*, and *Abd-A* relate to trunk leg development in crustacean

species where their expression overlaps leg forming regions of the body. Are they necessary for limb development in branchiopod species? Copf and others demonstrated that derepression of *Ubx*/*Abd-A* in the post-genital zone, which lacks appendages, results in ectopic leg growth (Copf et al., 2006). Thus, in this species, *Hox* genes appear to promote trunk appendage development. Other possibilities are that *Hox* genes merely serve to modify the thoracic appendages or they have no influence on leg development in the trunk at all. An alternative to functional studies in *Triops* would be cloning orthologs of the development genes examined in this thesis from a species with limb morphologies similar to *Triops* and attempt functional studies using RNAi. Although *dac*, *wg*, and *Ubx* failed to generate reproducible phenotypes, *Artemia* might yet serve as a viable alternative.

APPENDIX A

DAC EXPRESSION AND FUNCTION IN THE DUNG BEETLE, *ONTHOPHAGUS TAURUS*

Summary

Dung beetles are common inhabitants of pastures where they occupy a special ecological niche, harvesting and utilizing manure for feeding and reproductive needs. Like many arthropods with uniramous legs, *Onthophagus* has a leg uniquely suited for its niche (Fig. A.1). What makes the *Onthophagus* leg interesting is that the intermediate region of the first thoracic leg predicted to be patterned by *dac* is modified with large tibial teeth structures. Additionally, the intermediate region on the second and third thoracic legs possesses tibial spikes. These modifications are essential for *Onthophagus'* burrowing lifestyle. Because of this, examination of limb patterning genes in this species is intriguing. How do modifications of the P/D genes correlate with these morphological changes? To begin addressing this question I explored the expression and function of *dac* in *Onthophagus*. I discovered that *dac* expression and function in the beetle leg is similar to what is seen in *Drosophila* in that it is expressed in the intermediate leg and reduction of *dac* expression results in loss of proximal tarsal segments. These observations were limited to the second thoracic leg of *Onthophagus*, which more closely resembles the *Drosophila* leg. As yet, *dac*'s role in the modified first thoracic leg is still unknown.

Materials and Methods

Animal rearing and fixation

Laboratory colonies were derived from animals collected in Durham, North Carolina. Colonies were kept in growing chambers at 25°C under a 16:8 light:dark cycle. Beetles were bred in plastic buckets (~ 35 cm in depth) filled 3/4 with a moist sand/soil concoction. An equivalent number of males and females were transferred into a breeding container and provided with a ~0.5 liters of homogenized and frozen treated cow dung. After several days, adult beetles were removed and brood balls were collected. Brood balls were carefully opened and larvae were transferred into artificial growth chambers lined with fresh, moist cow dung. Larval growth containers were housed in an incubator at 25°C in complete darkness except for brief examinations to determine developmental stage.

Prepupal abdomens were detached with a razor blade and heads and thorax were dropped into a solution containing equal volumes of 4% formaldehyde in phosphate buffered saline and heptane where they were incubated at room temperature for 1h. Preserved tissue was stored at -20°C until ready to use. Prepupae were equilibrated 24h at 4°C in a 30% sucrose solution made with phosphate buffered saline+0.1% Triton X-100 before sectioning. Specimens were freeze mounted in OCT embedding medium (Electron Microscopy Sciences) and cryosectioned at -25°C into ~20 μm thick slices using a cryostat (Microm, Heidelberg, Germany). Tissue sections were mounted on a microscope slide and stored at -20°C.

dachshund cloning

Total RNA was extracted from head and thorax tissue of *Onthophagus* prepupae by homogenization with a Polytron PT3000 (Brinkmann) in 1.0 ml TRIzol (Invitrogen).

cDNA was created using Clontech's MatchMaker 2 kit. Degenerate PCR was performed using nested degenerate primers designed by Prpic et al. (2001) and the products were cloned into pCR-II-TOPO (Invitrogen). Products were sequenced three times by automated sequencing (GATC DNA Sequencing Facility, University of Arizona) in both directions. We isolated a region of the *Otdac* (accession number DQ452570) gene containing an ORF of 966 bp (322 amino acids).

Sequence analysis

Sequence analysis and orthology assignment were performed using the NCBI BLAST [blastx] program and GCG/Seqlab (Wisconsin Package). The *Otdac* putative protein coding sequence was compared to the putative DAC sequences from *Drosophila* and *Tribolium* using the LALIGN (Pearson 1991) sequence comparison program. DAC sequences were aligned using ClustalW (Thompson et al., 1994). The alignments were generated using the BLOSUM 62 residue comparison matrix (Henikoff and Henikoff, 1992) assigning a gap-opening penalty of 10 and a gap extension penalty of 0.2. Dendrogram was generated using parsimony analysis utilizing a heuristic search, TBR with 2000 random additions, and non-parametric bootstrap with 1000 replicates (200 random additions/replicate) as implemented in PAUP 4.0 b10 (Swofford, 2003).

in situ hybridization

Place cover slip (with clay feet) over sections and wick probe solution between slide and cover slip. Incubate at 58ºC in a humid chamber, with slides elevated from moist towels, overnight. Remove cover slips and wash slides 2X with 1 ml hybridization

buffer (65ºC) by dribbling down slide. Wash in a stepwise fashion with the following ratios of Hybridization buffer: PBTriton (PBS with 0.1% Triton), 4:1--3:2--2:3--1:4 [pre-heated to 58ºC before washing slides], 58ºC, 25 minutes/step. Wash several times fast with PBTriton and then 2X, PBTriton, 5 minutes, Room temperature (RT), while agitating. Block sections 1 hour, RT, PBTriton with 2% BSA (Bovine Serum Albumin). Remove slides from Block solution and place in humidity chamber on stages. Trickle antibody solution (α-digoxigenin, 1:2000 in PBTriton with 2% BSA) over slide surface and cover with cover slip and incubate 1 hour at RT. Wash slides 3X fast with PBTriton then 6X, 10 minutes, RT while agitating. Wash slides 2X fast with 1 ml alkaline phosphatase developing solution with 0.1% Tween by dribbling down slide. Wash 2X, 10 minutes at RT in a humidity chamber elevated on a stage with alkaline phosphatase developing solution with 0.1% Tween covering tissue sections. Dribble color substrate on slides and overlay with cover slip on slide to evenly distribute substrate and keep sections submerged in color substrate. Place in humidity chamber and incubate in the dark and allow color to develop. Stop color reaction by washing several times with PBTween (PBS with 0.1% Tween). Counterstain 10 minutes at RT in a humidity chamber elevated on a stage with 1 ml Hoechst (1 μg/ml). Wash several times with PBTween.

dsRNA synthesis

cDNA was PCR amplified with M13 forward & reverse primers. Product was column purified and used as a template for the transcription reactions. Sense and α-sense

strands were transcribed in two separate reactions. *Onthophagus dac* templates were transcribed with SP6 and T7 RNA polymerases (Roche). Reactions were treated with DNase I and the RNA strands were subsequently purified using TRIzol®:chloroform extraction followed by an ethanol-LiCl precipitation. Purified/resuspended RNA strands were quantified as in chapter 3 and combined in equal quantities and annealed in a thermocycler (Perkin-Elmer) by incubating at 85°C 10 minutes and then shutting off the machine and allowing the dsRNA to slow cool for 20 minutes.

dsRNA microinjection

Onthophagus larvae were immobilized between two sponges with the dorsal thorax-abdomen exposed. *Otdac* dsRNA was injected into the body cavity between the thorax and abdominal segments using a Narishige micromanipulator. Injection solution was prepared by adding an equal volume of Phenol Red (Sigma) to a solution containing dsRNA dissolved in water ($[dsRNA]_f$ = 300-4500 ng/µl). Injected larvae were transferred into artificial growth chambers lined with fresh, moist cow dung and housed in an incubator at 25°C in complete darkness except for brief examinations to determine developmental stage. Once larvae had achieved pupation, they were transferred to a humid chamber and incubated at RT in the dark until eclosion.

Results

Dachshund sequence characteristics

Sequence alignment and phylogram were generated using amino acid data contained within the C-terminal portion of the DD1 and the N-terminus of the DD2. Two

putative Nuclear Localization Signals are present in the *Drosophila* sequence and of the two only one (indicated in the aligned sequences) is key to Dachshund nuclear transport and function (Tavsanli et al., 2004). A sequence of 16 amino acids, WENCRAAYEDIVKHLE (referred to here as the insect motif-Figure A.2), is only present in DAC from insect species and is absolutely conserved. This motif has not been previously reported and its functional significance, if any, is unknown.

Otdac in pre-pupal legs

In *Drosophila*, DAC expression extends from the distal trochanter into the basal region of the second tarsal segment. Since *Onthophagus* legs, particularly T2/T3, are morphologically similar to *Drosophila*, *Otdac* expression is expected to have parallel expression patterns in the beetle leg. *in situ* hybridization on pre-pupal tissue sections reveals *dac* expression in a region corresponding to the tibia as well as three of the tarsal segments (Fig. A.3A). *dac* is expressed in a strong striped pattern in the proximal tarsus (Fig. A.3A-black arrows) and is greatly reduced or absent from the intervening tissue. This *dac* pattern suggests an association with tarsal segment boundaries.

Otdac RNAi

Flies null for *dac* have severely condensed femur, tibia, and three proximal tarsi. Larvae injected with ds*dac*RNA produce adult beetles displaying similar defects (Fig. A.3C), as demonstrated by the loss or condensation of tarsal segments (Fig. A.3C-open arrowheads). Wild type T2 legs of *Onthophagus* possess a spiked protrusion extending off the distal ventral margin of the tibia (Fig. A.3B-red arrow). This particular

morphological feature is not present in other arthropods where *dac* expression and function have been examined. Additionally, the T2 leg has an organized array of bristles on the tibia (Fig. A.3B-red arrowhead). *dac* knockdown animals have reduced tibial spikes accompanied by disarrayed bristles (Fig. A.3C-blue arrow and arrowheads, respectively).

Summary

Otdac expression was only detected in tissue sections from two individual pre-pupal beetles. The sectioned tissues only had intermediate and distal portions of the T2 leg represented, each with patterns of expression presented in this chapter. Intermediate *dac* expression in *Drosophila* corresponds to the presumptive epithelium fated to become the femur, tibia, and proximal tarsus (Mardon et al., 1994) and in *Tribolium* it is associated with the femur and tibiotarsus (Prpic et al., 2001). These expression domains are continuous and do not exhibit the striped pattern seen in the *Onthophagus* tarsus. However, *dac* expression in *Drosophila* and *Tribolium* were restricted to the larval and embryonic stages, respectively, of development whereas *dac* expression in *Onthophagus* is shown for the pre-pupal stage. It is unknown what pre-pupal or pupal expression of *dac* looks like in *Drosophila* and *Tribolium*, although it could be speculated to resemble the striped tarsal expression of *dac* in *Onthophagus*.

Knockdown of *dac* function in *Oncopeltus* resulted in fusion of the femur-tibia joint with a reduced tibia in mild phenotypes or a loss of the tibia completely in severe phenotypes, where the two tarsal segments and claw are unaffected (Angelini and Kaufman, 2004). Interestingly, loss of *dac* function in *Drosophila* and *Onthophagus*, two

holometabolous insects, reduces the number of tarsal segments from five to two. Since *Oncopeltus* lacks *dac* expression in the presumptive tarsal tissue of embryo leg buds (Angelini and Kaufman, 2004), this suggests a role for *dac* in modulating tarsal segment number in holometabola by sub-dividing the proximal tarsus into smaller segments. This suggests two possible scenarios; 1) the tarsal segmentation function of *dac* was lost in the lineage represented by the milkweed bug, or 2) it is a derived condition in the holometabola. Further taxonomic sampling is necessary to distinguish between the two scenarios.

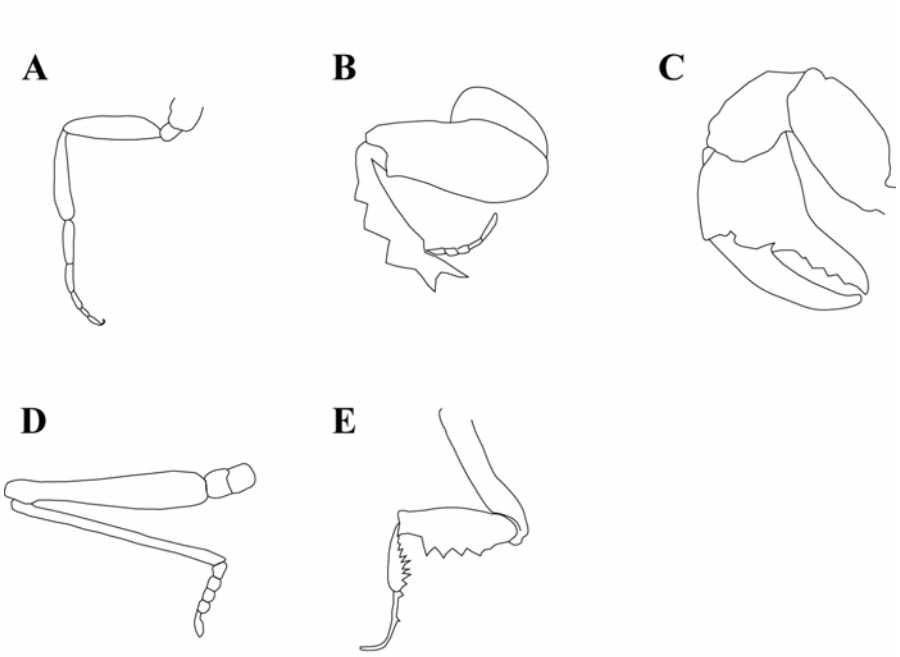
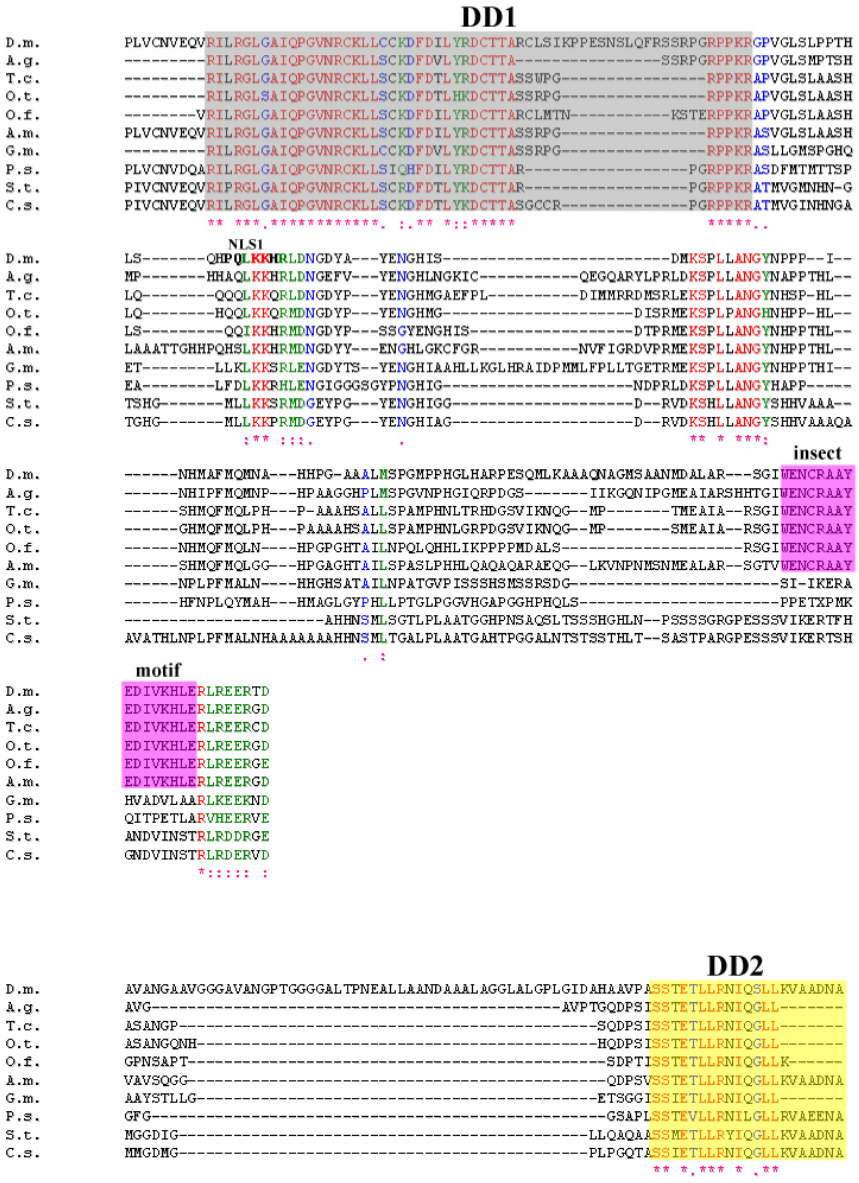


Fig. A.1. Modified uniramous appendages. (A) fruit fly thoracic leg-1, (B) dung beetle thoracic leg-1, (C) lobster cheliped, (D) grasshopper hind leg, and (E) praying mantis first thoracic leg. *Drosophila*'s leg is shown as a reference for comparison.

A.



B.

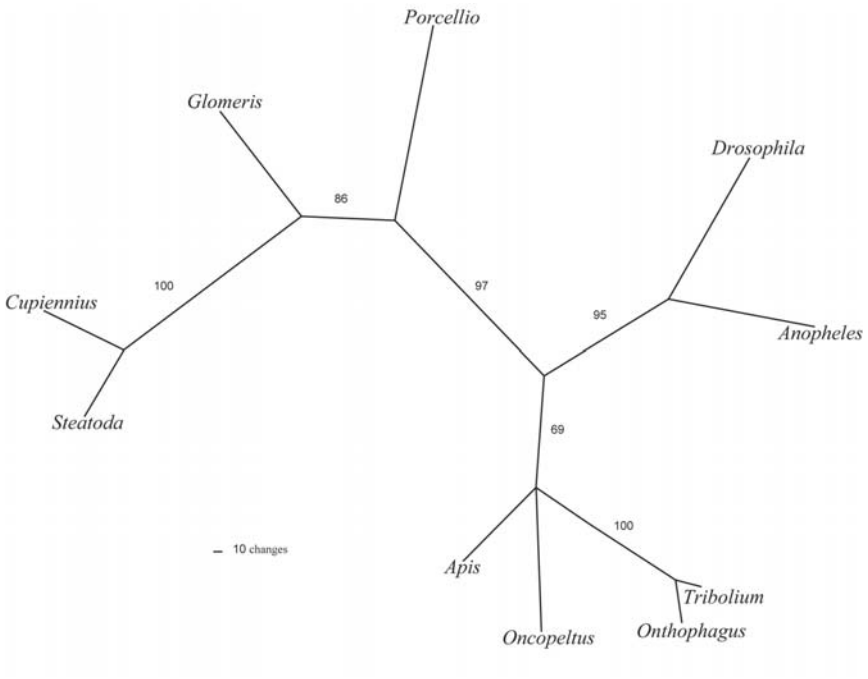


Figure A.2. Alignment and phylogram of arthropod Dachshund protein sequences. (Legend follows.)

Figure A.2. Alignment and phylogram of arthropod Dachshund protein sequences (A) Alignment of arthropod Dachshund protein sequences. Sequences shown are those lying within portions of the ascribed DD1 and DD2 domains (Davis et al., 1999). Regions of identity are represented by vertical dashes, conserved substitutions are represented by a vertical double dot, and semi-conserved substitutions represented by a single dots. Alignment was generated using ClustalW (Pôle BioInformatique Lyonnais). Amino acids 1-247 of *Ot* DAC had 53.6% amino acid identity to *Drosophila* DAC (amino acids 278-557) and amino acids 278-322 of *Ot* DAC had 45.7% amino acid identity to *Drosophila* DAC (amino acids 715-760) (Fig. 3a). The close relationship between two beetles, *Onthophagus* and *Tribolium*, was reflected in the degree of similarity of their nucleotide sequences (65.2%) as well as their putative DAC protein coding sequences, with *Ot* DAC (amino acids 1-322) sharing 78.7% amino acid identity with *Tribolium* DAC (amino acids 1-301). *Ot*DAC was compared with *Dm*DAC and *Tc*DAC using the LALIGN (Pearson 1991) sequence comparison program. The cloned portion of *Onthophagus* DAC contained 44 amino acids that constitute the C-terminal region of the DD1 (DAC domain 1) and 14 amino acids of the N-terminal portion of the DD2 (DAC domain 2). Abbreviations for animals and accession numbers: D.m. (*Drosophila melanogaster*-AAC46506.1), A.g. (*Anopheles gambiae*-XP_317545), T.c. (*Tribolium castaneum*-CAC84070.1), O.t. (*Onthophagus taurus*), A.m. (*Apis mellifera*-XP_394482.2), O.f. (*Oncopeltus fasciatus*-AAS93632.1), P.s. (*Porcellio scaber*-AAK58707.1), S.t. (*Steatoda triangulosa*-AAK58706.1), C.s. (*Cupiennius salei*-CAD57736.1), G.m. (*Glomeris marginata*-CAD82906.1). Region highlighted in gray = C-terminal region of the DD1 domain; NLS1 (amino acids bold/shadowed)=Nuclear Localization Signal 1; pink highlighted region = insect motif; region highlighted in yellow = N-terminal portion of the DD2 domain. (B) Phylogram of arthropod *Dachshund* protein sequences. Only sequences lying within portions of the ascribed DD1 and DD2 domains (Davis et al., 1999) were used for the phylogenetic analysis. A consensus tree of the 3 most parsimonious topologies (branch lengths from a single optimal topology) was used to generate a dendrogram, which is an unrooted majority rule consensus computed using parsimony analysis. Numbers along branches represent bootstrap values. Phylogenetic analysis clusters *Ot*DAC with insect DAC sequences.

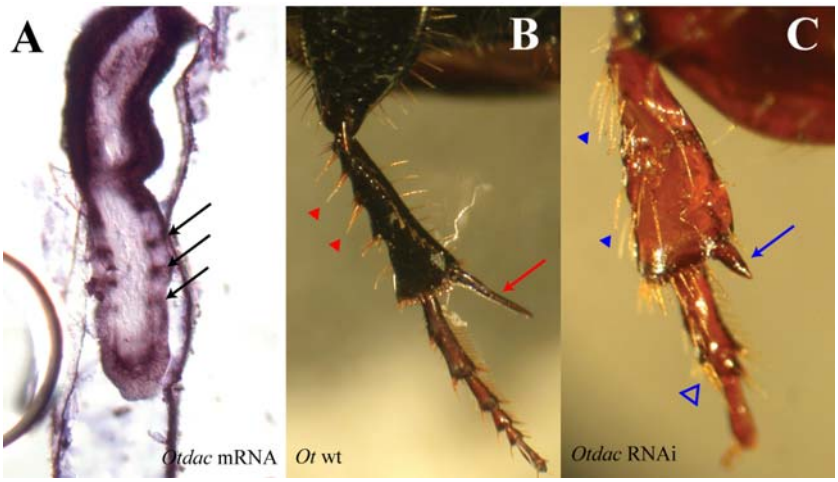


Fig. A.3. *Otdac* expression and function. (A) *dac* mRNA expression in second thoracic leg. Tibia and tarsal leg regions only are depicted in panel A. Potential tarsal segment boundaries indicated with black arrows. (B) Wild type leg has a pronounced tibial spike (red arrow) and distinct bristle (red arrowheads) pattern. (C) T2 leg of dsRNA injected animal. Tibial spike (blue arrow) and bristles (blue arrowheads) are affected. Defects are detected in the tarsus as well (open blue arrow).

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