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Antenna and all gnathal appendages are similarly transformed by *homothorax* knock-down in the cricket *Gryllus bimaculatus*

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Abstract

Our understanding of the developmental mechanisms underlying the vast diversity of arthropod appendages largely rests on the peculiar case of the dipteran *Drosophila melanogaster*. In this insect, *homothorax (hth)* and *extradenticle (exd)* together play a pivotal role in appendage patterning and identity. We investigated the role of the *hth* homologue in the cricket *Gryllus bimaculatus* by parental RNA interference. This species has a more generalized morphology than *Oncopeltus fasciatus*, the one other insect besides *Drosophila* where *homothorax* function has been investigated. The *Gryllus* head appendages represent the morphologically primitive state including insect-typical mandibles, maxillae and labium, structures highly modified or missing in *Oncopeltus* and *Drosophila*. We depleted *Gb'hth* function through parental RNAi to investigate its requirement for proper regulation of other appendage genes (*Gb'wingless*, *Gb'dachshund*, *Gb'aristaless* and *Gb'Distalless*) and analyzed the terminal phenotype of *Gryllus* nymphs. *Gb'hth* RNAi nymphs display homeotic and segmentation defects similar to *hth* mutants or loss-of-function clones in *Drosophila*. Intriguingly, however, we find that in *Gb'hth* RNAi nymphs not only the antennae but also all gnathal appendages are homeotically transformed, such that all head appendages differentiate distally as legs and proximally as antennae. Hence, *Gb'hth* is not specifically required for antennal fate, but fulfills a similar role in the specification of all head appendages. This suggests that the role of *hth* in the insect antenna is not fundamentally different from its function as cofactor of segment-specific homeotic genes in more posterior segments.

Keywords: *Extradenticle*; *Homothorax*; Short germ; Homeosis; Segmentation; Limb development; Proximo-distal; Antenna specification; Evolution; Systemic RNAi

Introduction

Although appendage development in different arthropods is clearly based on a common genetic tool kit (e.g. Abzhanov and Kaufman, 2000; Beermann et al., 2001; Williams and Nagy, 2001; Inoue et al., 2002; Prpic et al., 2003; Minelli, 2003; Kojima, 2004), the highly divergent morphologies and developmental mechanisms are likely to be caused by fundamental modifications and adaptations of this toolkit. In hemimetabolous insects, legs and head appendages develop in the embryo as cylindrical outgrowths of the body wall. Conversely, in the derived holometabolous insect *Drosophila*, the appendages appear only after metamorphosis, through eversion and restructuring of the imaginal discs, flattened sacs of epidermal cells that invaginate during embryogenesis into the body cavity (Cohen, 1993; Fristrom and Fristrom, 1993). These differences in geometry and timing suggest deviations in the patterning process. However, at this point we have a fair understanding only of the genetic pathways underlying the growth and patterning of the proximal–distal axis in *Drosophila* imaginal discs. While expression data for appendage genes are now available for quite a few arthropod embryos, including beetles, bugs, crickets, grasshoppers, centipedes, millipedes, spiders and several crustaceans, functional data in non-dipteran taxa only exist for the beetle *Tribolium castaneum* (e.g. Beerman et al.,

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2001) and the bug *Oncopeltus fasciatus* (e.g. Angelini and Kaufman, 2004).

In *Drosophila*, the synergistic activity of the secreted morphogens Wingless (Wg) and Decapentaplegic (Dpp) regulates growth and patterning along the proximal–distal axis in imaginal discs (see Martinez Arias and Stewart, 2002 for review). Distally, Wg+Dpp induce the expression of *Distalless* (*Dll*). Proximally, Wg+Dpp repress *homothorax* (*hth*) and *teashirt* (*tsh*), which are thus restricted to the periphery of the disc (Lecuit and Cohen, 1997; Wu and Cohen, 2000; Azpiazu and Morata, 2002). Hth exerts a pivotal role in the development of proximal fates in all appendages (Wu and Cohen, 2000). In addition, larvae lacking zygotic and maternal Hth display homeotic transformation of thoracic and abdominal segments, as well as segmentation and head defects (Rieckhof et al., 1997). Moreover, Dm'Hth is thought to act as an antenna selector gene since loss-of-function clones in the antenna result in antenna-to-leg transformations. Hth exerts its function through close interaction with the *extradenticle (exd)* gene. Both genes encode proteins of the homeodomain TALE class, and binding of Hth to the Exd protein is required for the latter's nuclear localization. The close interaction of Hth and Exd is reflected by identical loss-of-function phenotypes (Rieckhof et al., 1997). The Hth/Exd heterodimer functions as cofactor for other homeodomain proteins, including Hox genes (Kurant et al., 1998; Pai et al., 1998; Rauskolb et al., 1995; Rieckhof et al., 1997; Ryoo and Mann, 1999). It is thought that the target DNA binding specificity of Hox proteins is crucially enhanced by their interaction with these two TALE proteins. Hox genes by themselves have similar binding specificities (Dessain et al., 1992; Ekker et al., 1992) and several Hox target promoters have been shown to require Hth binding (Chan et al. 1994; Pinsonneault et al., 1997; Ryoo and Mann, 1999). Loss of *hth* activity in *Drosophila* leads to partial transformation of thoracic segments towards abdominal and of anterior abdominal segments towards posterior abdominal fates while Hox expression remains unaffected. To some degree, the function of Exd/Hth appears to be conserved even in vertebrates (Mercader et al., 1999; Shanmugam et al., 1999).

The role of *hth* and *exd* in antenna specification has received special attention. While *exd* is expressed in all epidermal cells, *hth* is proximally restricted in the legs, thereby providing the spatial specificity of Exd+Hth function. *hth* and *Dll* domains hardly overlap in the leg discs, but these genes are extensively coexpressed in the antennal disc. Loss of Dll or Hth (or Exd) results in antenna-to-leg transformations. Moreover, in clones ectopically expressing posterior Hox genes like *Scr*, *Antp*, *Ubx* and *abd-A* in the antennal imaginal disc, which results in similar phenotypes, *hth* transcription is downregulated (Casares and Mann, 1998; Yao et al., 1999; Dong et al., 2000). The exact mechanism by which antenna specification occurs is not clear, however, since not all cells in the antenna express Hth. It appears that the presence of Hox gene products modifies the way *hth* and *Dll* interact, which then leads to altered domain overlap and results in morphological differences between these two types of appendages. In other words, a strong mutual antagonism between these two genes results in leg fate, whereas wide overlap between *hth* and *Dll* appears to result in the expression of antenna-specific genes (Dong et al., 2002; Emerald et al., 2003; Emerald and Cohen, 2004).

In this paper we aimed to understand the function of *hth* in a hemimetabolous insect representing the ancestral mode of limb development in insects. The cricket *Gryllus bimaculatus* (Orthoptera) has generalized (mandibulate) mouthparts, unlike the bug *O. fasciatus*, another hemimetabolous insect in which *hth* function has been investigated (Angelini and Kaufman, 2004). *Gryllus* is amenable to embryonic (Miyawaki et al., 2004) and parental RNAi (Mito et al., 2005; Ronco, 2004), and the *hth* gene had been isolated previously (Inoue et al., 2002). Our results show that *Gb'hth* RNAi embryos and nymphs resemble *Dm'hth*$^{-}$ mutant embryos and larvae in that they display homeotic and segmentation defects as well as head defects. *Gb'hth* RNAi nymphs also display features of *hth* loss-of-function clones in adult flies, i.e. defects in eye development, shortened legs and antenna-to-leg transformations. In addition, however, they display transformation of other head appendages which suggests that *hth* in *Gryllus* may play similar roles in the antenna and in gnathal segments.

Materials and methods

Animal husbandry and embryo fixation

G. bimaculatus adults were obtained weekly from a commercial source in Erlangen, Germany. Rearing conditions were 30 °C, 55% humidity, light:dark cycle 10:14. Oviposition occurred in humid sand, usually in the dark between 8 p.m. and 10 a.m. Eggs were washed out from the sand and allowed to develop on filter paper in humid chambers at 28–29 °C for 10–11 days until eclosion. For embryo fixation, embryos up to 20% development were dissected manually in 1× PBS (treated with 0.5 ml/l diethyl pyrocarbonate, Sigma, stirred and autoclaved) by cutting off the anterior pole and squeezing embryo and yolk out of the egg shell. Embryos from 20% development onwards were dissected by pricking the anterior pole with fine tweezers. The egg turgor then forces the embryo out of the egg case. Subsequently, embryos were cleaned from yolk and fixed on ice for 30 min in 4% formaldehyde (in PBS). To avoid clumping of embryos, 1.5 ml plastic tubes were kept horizontal during fixation. Then embryos were transferred to fresh 1× PBS on ice and fixed again as before. Fixed embryos were stored in methanol at −20 °C.

Phylogenic analysis of Gb'hth

Cloning of a *Gb'hth* fragment has been described previously (Inoue et al., 2002). In addition to the evidence provided then, we provide a phylogram of mouse, *Caenorhabditis* and arthropod *hth* genes as electronic supplement to clarify the orthology relationships.

Parental RNA interference

In order to obtain large numbers of knock-down embryos and to avoid injection artifacts, females–rather than eggs–were injected with *Gb'hth* double stranded RNA (dsRNA). A PCR template of *Gb'hth* (692 bp) was amplified using primers complementary to the T7 and Sp6 sequences of the *Gb'hth* cDNA plasmid (Inoue et al., 2002). The Sp6 primer contained T7 sequences at its 5′ end, such that sense and antisense RNAs were synthesized in the same reaction using the T7 Megascript Kit (Ambion). The in vitro transcription (20 µl) product was precipitated with LiCl according to the manufacturer's instructions and the pellet was dissolved in 50 µl DEPC-treated distilled water and kept at −20 °C. For *parental RNAi*, this dsRNA solution was mixed 1:4 with 5× Ringer's medium (1× Ringer's medium: NaCl 150 mM, KCl 9 mM, $CaCl_2$–$2H_2O$ 5 mM, $NaHCO_3$ 2 mM). For injections, selected adult females were anesthetized with