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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

diethylether for 1 min and then injected at the basis of a metathoracic leg with a borosilicate glass capillary (Kwik-Fil™, World Precision Instruments Inc., USA) affixed to a mouth pipette. In *Gb'hth* RNAi experiments, three different amounts of dsRNA were injected per female: 2.5 μg (2.0 μl of 1.3 μg/μl=2.2 μM) was injected in 8 females each; 10 μg (2.0 μl of 4.6 μg/μl=8.2 μM) was injected in 15 females each; and 40 μg (8.0 μl of 5.6 μg/μl=10 μM) in 6 females. For each case, three independent experiments (from template PCR to injection) were performed. Controls were 6 females injected with buffer (1× Ringer's medium), 9 females injected with *DsRed* dsRNA (700 bp, 40 μg, i.e. 15.3 μl of 2.5 μg/μl), 2 females injected with a mixture of dsRNAs from *Tribolium* cDNAs (*Tc'hunchback*, *Tc'fushi tarazu*, *Tc'giant*; 17.2 μg, i.e. 8 μl of 2.2 μg/μl). Eggs laid by control females developed into morphologically normal crickets and never showed phenotypes resembling those resulting from *Gb'hth* dsRNA injection. Interference with *Gb'exd* gave similar results as *Gb'hth*.

In situ hybridization

Digoxigenin (dig-) and fluorescein (flu-) labeled (Roche) antisense RNAs were synthetized in vitro using T7 or Sp6 polymerases (Roche). For single staining, the alkaline phosphatase color reaction (anti-DIG-AP, Roche, 1:1000) was developed with NBT/BCIP and 5% polyvinyl alcohol (Fluka, Sigma). For double stainings, the flu-labeled probe was detected via a POD reaction (anti-flu-POD, Roche 1:1000) and biotin amplification (TSA Biotin System, Perkin Elmer; Streptavidin-β-Gal, Roche) followed by β-galactosidase color reaction. The protocols were adapted from established protocols for *Tribolium* (e.g. Prpic et al., 2003) and are available on request. Data in Fig. 7 were obtained according to protocols in Mito et al. (2005). Probes were generated from previously published cDNAs (Inoue et al. 2002, Niwa et al. 2000, Miyawaki et al. 2002, Zhang et al. 2005).

Antibody staining

After rehydration to PTw (PBS containing 0.05% Tween-20), extensive washing and blocking (3% BSA, Sigma, in PTw for 30 min), embryos were incubated with the primary antibody (rabbit polyclonal anti-Dll antibody, kindly provided by Grace Panganiban), diluted 1:200 in 3% BSA/PTw and detected via secondary antibody (FITC conjugated goat-anti-rabbit, Jackson Immuno Research) diluted 1:500. DAPI (1 μg/ml in PTw) was added to the last wash for nuclear counterstaining.

Cuticle preparation

RNAi nymphs were cleared in lactic acid at 65 °C overnight. For flat preparations, the nymphal cuticle was sagittally cut in two halves with a razor blade. Left and right sides were mounted flat in Hoyer's medium and incubated at 65 °C o.n. Digital images were taken with a ProgRes C14 camera connected to a Zeiss Axiophot or Zeiss stereomicroscopes.

Results

Expression of Gb'hth in head appendages

Expression of *hth* in the proximal region of *Gryllus* leg primordia has been described previously (Inoue et al., 2002). Fig. 1 depicts how *Gb'hth* transcription emerges in the early embryo and in the head appendages. In the growing germ band, i.e. prior to limb outgrowth, *Gb'hth* is homogeneously expressed throughout the germ band except in and near the growth zone. Initially the mRNA abundance is quite low but significantly increases as appendages grow to their final size. Similarly to leg primordia, also in the head appendages *Gb'hth* is expressed proximally at all stages. This includes the clypeolabrum, whose segmental origin still is uncertain (Haas et al., 2001). At later stages (Figs. 1D–I) expression in all appendages except the mandible becomes uneven in that rings of increased abundance can be detected within the proximal domain. Once the appendage segments become morphologically recognizable (Figs. 1H, I), *Gb'hth* is restricted to the presumptive scapus and pedicel of the antenna whereas the flagellum does not express it. In the mandible, *Gb'hth* is eventually lost from the most distal cells (similarly to Exd protein in the cricket *Acheta domesticus*; Abzhanov and Kaufman, 2000). In maxilla and labium, the basipodite (including the two endites) continues to express *Gb'hth*, and an additional narrow ring of expression is found in the proximal portion of the palps (Fig. 1H). This is similar to the situation in the legs, where *Gb'hth* expression extends well into the telopodite, i.e. the femur (Figs. 1H, I; see also Inoue et al., 2002).

In *Drosophila*, the overlap between *hth* and *Dll* expression domains is larger in the antenna than in the leg, and this overlap appears to be required for the differentiation of antennal fates. In *Gryllus*, *Dll* remains expressed in a large domain in the antenna whereas in the legs the initial large domain breaks down into a ring-and-sock pattern (Figs. 1J, K). In contrast to *Drosophila*, however, the majority of antennal cells do not express *Gb'hth* (Figs. 1G, I). Therefore, the overlap between *Dll* and *hth* domains in the *Gryllus* antenna appears not to be significantly larger than in the leg primordia.

Gb'hth parental RNAi results in a range of severe embryonic phenotypes

The *Gb'hth* parental RNAi range of phenotypes varies with time after injection and with the amount of RNA injected (Fig. 2). Similarly as in *Dm'hth* and *Dm'exd* mutants, body segmentation, proximo-distal development and segment identity are affected in these nymphs (Fig. 3, discussed below). As expected, the severity and penetrance of RNAi phenotypes are dependent on dsRNA concentration (Fig. 2; see also Fig. S2 and Table S1). Surprisingly, the strength of the RNAi effect appears to increase at least up to the 7th day after injection (since egg production of injected females tends to cease around this time, we were not able to extend this analysis any further). The delayed materialization of strong phenotypes may relate to the slow growth of oocytes in panoistic ovarioles (after dsRNA injection into *Tribolium* adult females, the strongest larval phenotypes are produced on the second day after injection; G. Bucher and MK, unpublished).

Unexpectedly, we did not find an obvious reduction of the *Gb'hth* mRNA signal relative to wild type when probing RNAi embryos by in situ hybridization (Figs. S3F, G). Our failure to observe the expected degradation of *Gb'hth* mRNA could indicate different modes of action of the RNAi pathway in *Gryllus* or, more likely, indicate that the parental RNAi effect in *Gryllus* embryos decreases over developmental time (see Discussion). Despite this unexpected result, we regard the *Gb'hth* RNAi phenotypes observed as specific for *Gb'hth* since in control RNAi experiments we obtained no effect (DsRed dsRNA), and in experiments with other developmental genes

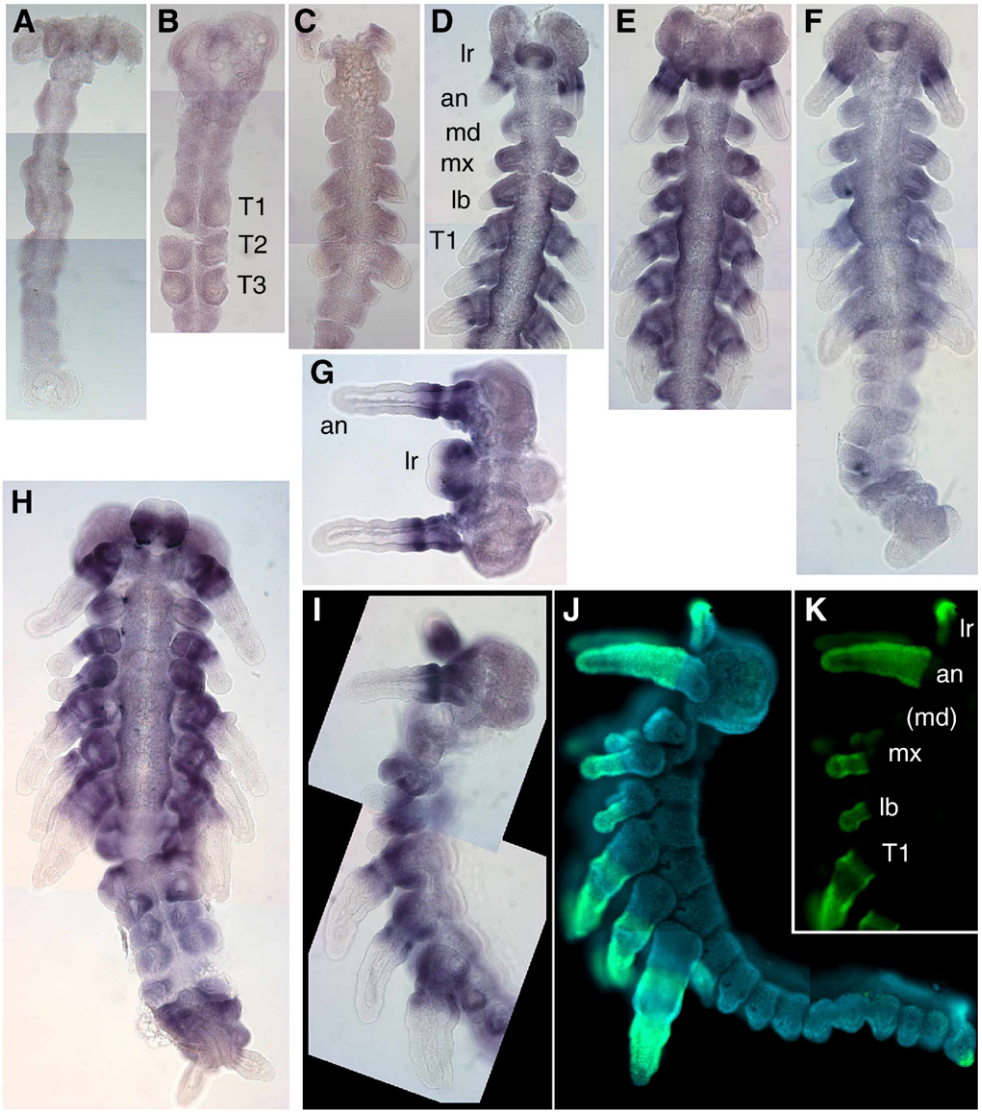

Fig. 1. Expression of *Gb'hth* and *Gb'Dll* in wild type embryos. (A–I) *Gb'hth* mRNA expression, (J, K) Gb'Dll protein expression; the embryo in J is counterstained for DNA. In early germband stages, *Gb'hth* is homogeneously expressed at a low level in all cells except the posterior growth zone (A). As appendages begin to develop, *hth* becomes excluded from the center/tip of developing legs (B, C), antennae (C, D) and gnathal palps (D). At later stages, proximal expression of *hth* intensifies in ring-like domains (E–I). (G) View of dissected pregnathal head from anterior: *Gb'hth* expression is proximally restricted in antennae and labrum. (I–K) *Gb'hth* and *Gb'Dll* expression in similarly staged embryo shows that expression overlap in the antenna is limited to a small proximal region. All embryos except the fragment in G are oriented anterior up; (A–F, H) ventral, (I–K) lateral views. Abbreviations: lr labrum, an antenna, md mandible, mx maxilla, lb labium, T1–T3 first to third thoracic segment.

(including many that contain homeodomains), we never observed similar phenotypes—except with *Gb'exd*.

Body segmentation and head defects

The most conspicuous phenotype of *Gb'hth* RNAi embryos and nymphs consists in the disruption of body segmentation and concomitant loss of head and thoracic appendages (Figs. 3A–H). Segmentation defects are more pronounced in the gnathal and thoracic region than in the abdomen. Adjacent segments often are fused, for example, labium + prothorax (lb + T1) or mesothorax + metathorax (T2 + T3; Fig. 3E). In weakly affected nymphs, partial segment fusions can manifest themselves as dorsal mismatch of corresponding hemisegments during dorsal closure. Interestingly, segmental fusions often result in the appearance of enlarged fused appendages (Fig. 3C). Frequently, these anterior–posterior appendage fusions remain incomplete such that enlarged basal podomers carry two or more distal tarsal regions. Segmental fusions are also apparent in *Gb'hth* RNAi embryos (Fig. S3) stained for the segmental marker *wingless* (*Gb'wg;* Niwa et al., 2000). In such fused appendages, the spacing of *wg* stripes is much closer than in body segments, suggesting different size regulation mechanisms.

Also the pregnathal head is affected in *Gb'hth* RNAi nymphs. In weakly affected specimen, the labrum can be reduced in size (Fig. 3H). Moreover, in such animals the nymphal complex eye can be enlarged, reminiscent of hth^{-}

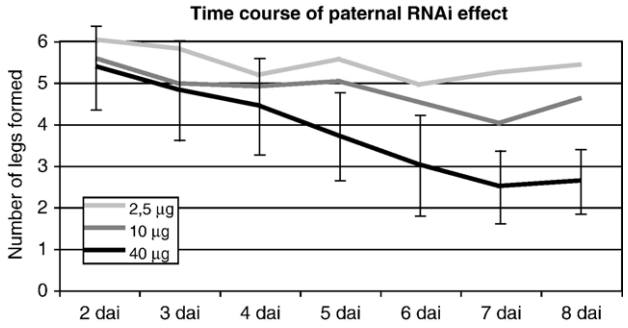

Fig. 2. The *Gb'hth* RNAi effect is time- and concentration-dependent. In this graph, as a measure for segmentation phenotype strength, the number of well-formed legs (i.e. coxae not fused with another leg) is shown as a function of time and injected amount of *Gb'hth* dsRNA. The strongest nymphal phenotypes are obtained about 7 days after injection of adult females. For the animals injected with 40 µg dsRNA, bars representing standard deviation are shown as a measure for the range of phenotypes present among animals scored for each data point (these bars do not represent variation among experiments). dai = day after injection.

clones in *Drosophila* (not shown). In strongly affected nymphs, the whole head is strongly reduced in size and appears to lack all appendages, including labrum and antennae (Fig. 3C).

Disruption of proximo-distal limb patterning

Disruption of the proximo-distal axis can more easily be analyzed in the legs than in the head appendages where patterning defects are superimposed to homeotic transformations. In the legs of *Gb'hth* RNAi nymphs, femur and tibia are commonly miss-shaped (e.g. Figs. 3C, J). In nymphs displaying stronger phenotypes, the joint between these two leg segments (podomers) is lost. In extreme cases, the remaining fused femur/tibia is strongly reduced in length (Fig. 3J, Figs. S3G). The articulations adjoining the trochanter and the tarsus usually appear to be preserved. In contrast to *Drosophila*, the trochanter/femur joint appears not to be overtly sensitive to *Tb'hth* reduction.

Disruption of proximo-distal patterning is also apparent from the expression patterns of the molecular markers *Gb'dachshund* (*Gb'dac*) and *Gb'aristaless* (*Gb'al*). The dynamic expression patterns of these two genes in the wild type have been described previously (Inoue et al., 2002; Miyawaki et al., 2002). *Gb'dac* is expressed in early limb buds as a single domain of intermediate proximo-distal position. As the leg primordia grow out, this domain expands and in mature appendages it splits into two domains (Fig. 4L). In *Gb'hth* RNAi embryos the single domain in early appendages appears to arise normally. Only in mature leg primordia, where the RNAi effect is already apparent from the shortened length of the limbs, changes in the *Gb'dac* pattern become apparent. In such leg primordia the two late domains remain fused (Figs. 4M–O). This fusion of *Gb'dac* domains (and similarly of late *Gb'al* stripes, Figs. 4F, G) in *Gb'hth RNAi* embryos correlates with the loss of corresponding leg regions.

Transformation of head appendages to a mixed leg/antenna fate

Similar to mutant *Drosophila* embryos and adult clones, *Gryllus* embryos and nymphs depleted for *hth* activity display homeotic transformations. In nymphs exhibiting weak phenotypes, an additional thoracic tergite appears in the neck region: the convex, smooth and darkly pigmented posterior head capsule of wild type, possibly including portions of the intersegmental membrane, is replaced by a flat, yellowish tergite identified by its bristle pattern as an additional T1 tergite (Figs. 3A, F). Most likely, this tergite represents an incomplete transformation of the most posterior head segment to T1 identity. This effect was often accompanied by the presence of incomplete single claws on otherwise normal labial palps (not shown).

In more strongly affected nymphs, the antenna as well as maxillary and labial palps are distally transformed to leg fate. In even stronger phenotypes, all head appendages appear to be transformed into a series of morphologically similar appendages (Fig. 5A). Since in these specimens homeotic defects are superimposed to segmentation defects, individual head appendages cannot always be identified unambiguously, given their similar morphology, their often distorted arrangement, and the frequent loss of some appendages. Frequently we observed one or two leg-like appendages which basally appear to carry mandible-like sclerotizations. These structures we interpret as partially transformed mandibles that consist of a leg-like telopodite and a basipodite carrying mandible-like endites (e.g. Figs. 5B, C and 6F, G).

Partially transformed mandibles also are detected in developing embryos. Wild type mandibles express *Gb'al* distally but do not express *Gb'Dll*. In partially transformed mandibles, the *Gb'al* domain occupies an intermediate proximo-distal position (Figs. 4C, D) while *Gb'Dll* becomes strongly expressed (Figs. 4A, B). More strongly transformed gnathal appendages express *Gb'dac* in a pattern characteristic for legs (Inoue et al., 2002). In wild type, the expression of *Gb'dac* in antenna and in maxillary and labial palps is restricted to a single faint proximal domain (Figs. 4I–K). In contrast, transformed gnathal limbs of *Gb'hth RNAi* embryos display strong expression of *Gb'dac* comparable to wild type legs (compare Figs. 4I–L with M, N).

Head appendages transformed to leg-like morphology are characteristic for phenotypes of intermediate strength since strongly affected RNAi nymphs lack all head appendages. These head appendages differentiate distally as legs, i.e. they bear claws and leg-like bristles. Proximally, however, they differentiate antennal markers, i.e. rows of campaniform sensilla which in wild type unambiguously characterize the first and second antennal segments (Fig. 6). Thus, proximal and distal portions of gnathal appendages are transformed towards different fates in *Gryllus*, a phenotype combination not previously observed in gnathal appendages of other insects.

In *Drosophila*, transformation of antenna to leg in *hth*$^{-}$ embryos or adult clones does not result in anterior expression of thoracic Hox genes (e.g. Rieckhof et al., 1997). We investigated in *Gb'hth* RNAi embryos the expression of *Gryllus* homologs

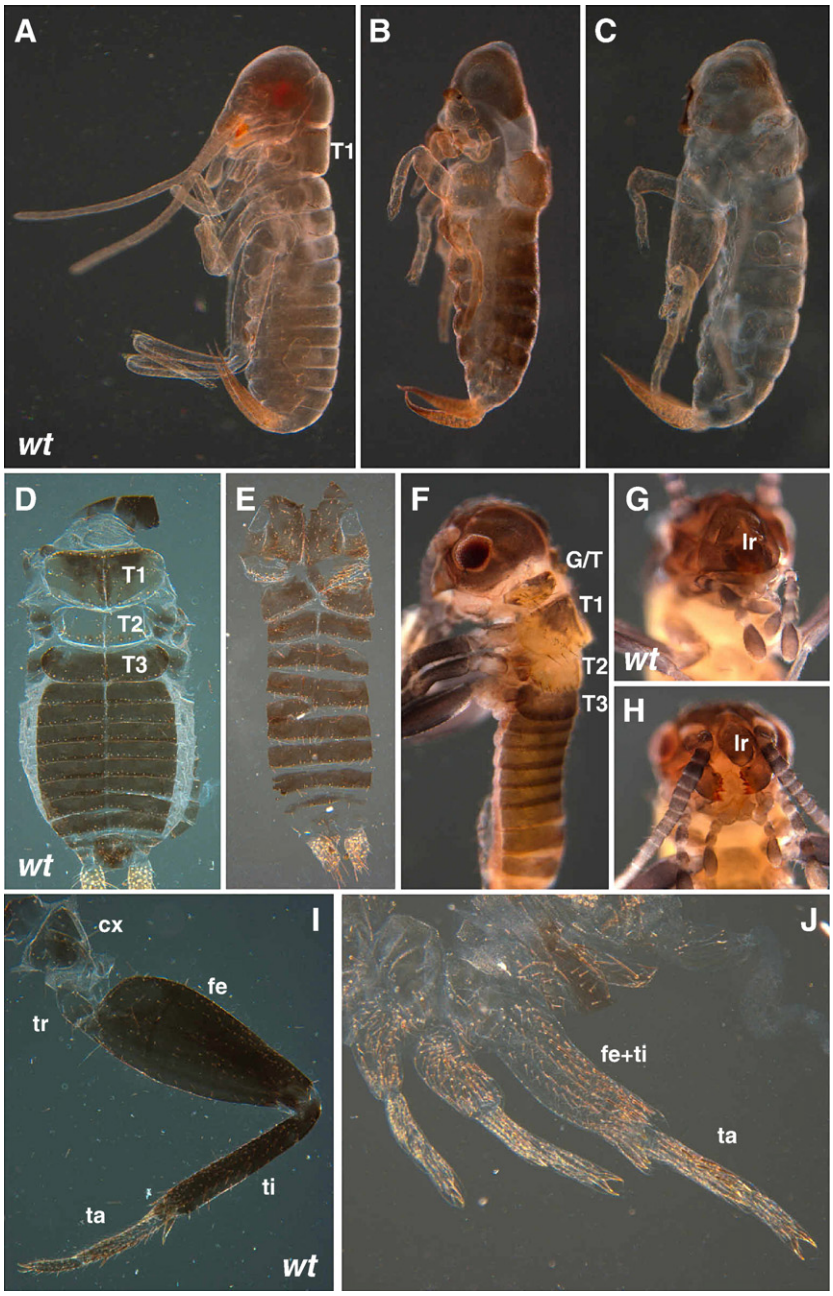

Fig. 3. *Gb'hth* RNAi phenotype of differentiated nymphs. Cuticle preparations of wild type (A, D, G, I) and RNAi nymphs (B, C, E, F, H, J). (B, C) Lateral views of intermediate and strong *Gb'hth* phenotypes with reduced number of segments and appendages. The nymph in panel C lacks all head appendages while thoracic limbs are fused proximally into a thick structure that is shortened proximo-distally and from which several tarsi emerge. (D, E) Dorsal aspect of dissected flat cuticle preparations of wt and RNAi nymphs. Several tergites of the RNAi nymph in panel E are missing or fused in one body half. (F) Weak homeotic phenotype displaying an ectopic thoracic tergite, representing a gnathum-to-thorax transformation ("G/T"). (G, H) Ventral view of wild type and weak RNAi heads/mouthparts. The labrum in panel H is reduced in size. (I) Wild type T3 leg with coxa (cx), trochanter (tr), femur (fe), tibia (ti) and tarsus (ta). (J) Dissected embryo with unfused thoracic segments. In this intermediate-strength phenotype femur and tibia are shortened and fused. Note that the specimen in panels A–C and J are still covered by the embryonic cuticle; nymphs in panels A–C and D, E are not to scale.

of *Sex combs reduced*, *Antennapedia*, *Ultrabithorax* and *abdominal-A* (Zhang et al., 2005). Similar to the situation in *Drosophila*, the pattern of these Hox genes is not expanded in RNAi embryos (Fig. 7). However, in the accompanying paper (Mito et al., 2007-this issue) the authors show that *Gb'Scr* expression is strongly reduced or abolished in *exd* RNAi embryos displaying strong phenotypes (i.e. lacking most gnathal segments). In contrast, we see normal-level *Scr* expression in *Gb'hth* RNAi embryos. This discrepancy between *Gb'exd* and *Gb'hth* RNAi effects we interprete as a quantitative difference in that the strongest *Gb'hth* phenotypes (as obtained independently in both, the Noji and Klingler laboratories) are somewhat "weaker" than the strongest *Gb'exd* phenotypes. Given the high similarity of *Gb'exd* and *Gb'hth* RNAi phenotypes in general, we deem it likely that *Scr* also might be reduced in *Gb'hth* RNAi embryos completely devoid of *hth*

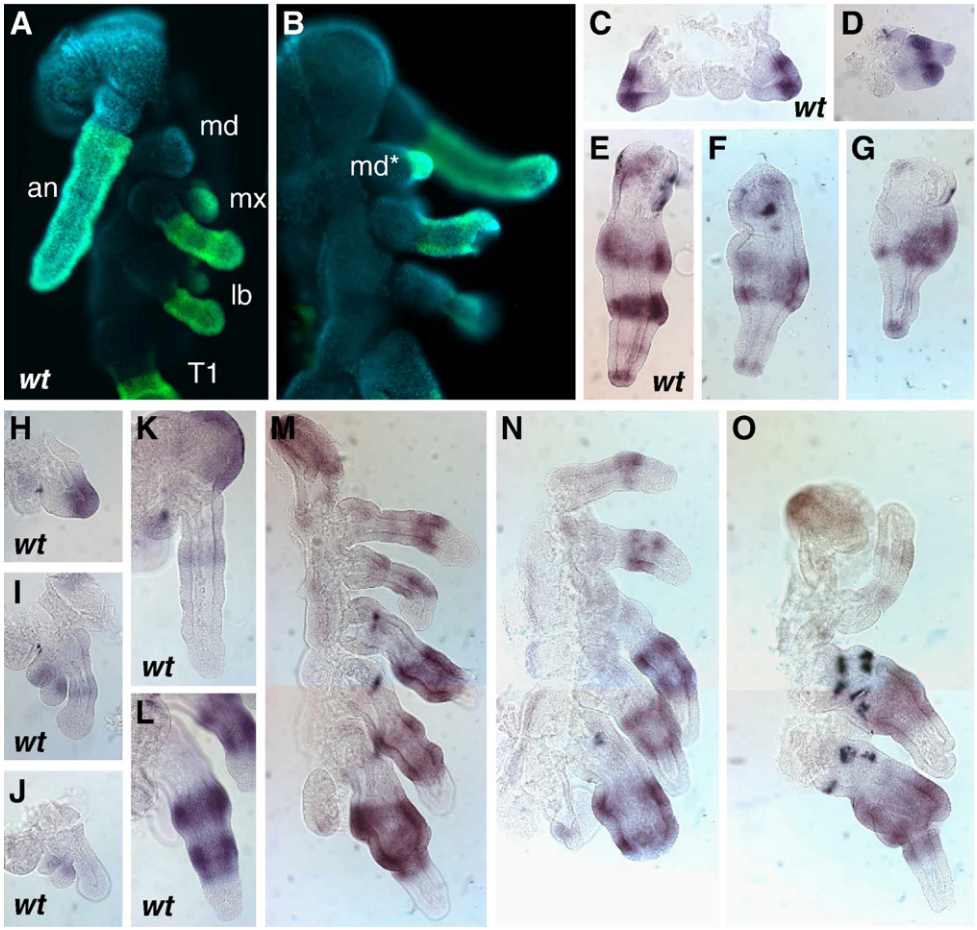

Fig. 4. Proximo-distal patterning genes in wild type and *Gb'hth* RNAi embryos. Whole and dissected *Gryllus* embryos stained for *Gb'Dll* (A, B), *Gb'al* (C–G) and *Gb'dac* (H–O) expression. (A, B) While in wild type embryos the mandible does not express Dll (A), expression in a *hth* RNAi mandible (md*) indicates partial transformation towards a thoracic appendage (B). Green fluorescence is Dll, blue fluorescence DNA; both embryos are oriented anterior up, ventral towards right, abbreviations are as in Fig. 1. (C, D) *aristaless* is expressed in a pair of dissected wild type mandibles at a dorsal/distal position. In a partially transformed *hth* RNAi mandible (D), *Gb'al* expression is unaffected, but a presumptive telopodite is extending distally from this *Gb'al* domain. (E–G) In legs, *Gb'al* is expressed in five domains. In *hth* RNAi legs (F, G), loss of intermediate proximo-distal positions is evident from fusion of the two middle domains. (H–L) Expression of *Gb'dac* in mandible, maxilla, labium, antenna and leg of wild type, respectively. In the leg, the two main *Gb'dac* domains cover the mid-distal anlage of the femur and a portion of the presumptive tibio-tarsus (Inoue et al., 2002). (M–O) In *hth* RNAi embryos, transformed head appendages express *Gb'dac* in a leg-like pattern. The two leg domains are fused in stronger phenotypes (O). Black spots in panels F, M and O are a staining artefact possibly due to nascent cuticle material.

activity. In any case, however, the presence of *Scr* expression at least in embryos displaying intermediate phenotypes as depicted in Figs. 7B, C is relevant for our interpretation of gnathal transformations since it is exactly such intermediate-strength phenotypes where head appendage transformation is observed.

Discussion

Depletion of Gb'hth activity by parental RNAi

Upon injection of *Gb'hth* dsRNA into the body cavity, adult *Gryllus* females produce eggs displaying embryonic knock-down phenotypes. Phenotype strength increased up to about 7 days after injection, suggesting that either young oocytes take up more dsRNA than more mature oocytes, or that a continuous supply of dsRNA in the hemolymph allows growing oocytes to take up dsRNA over a prolonged time span. Our data confirm (see also Mito et al., 2005; Shinmyo et al., 2005) that the panoistic ovarioles of *G. bimaculatuss* are similarly accessible to systemic RNAi as the telotrophic meroistic ovarioles of *Tribolium* (Bucher et al., 2002) and *Oncopeltus* (Liu and Kaufman, 2004) or the polytrophic meroistic ovarioles of *Apis* and *Nasonia* (Amdam et al., 2003; Lynch et al., 2006).

Parental RNAi for *Gb'hth* results in segmentation phenotypes similarly severe as in *Drosophila* embryos that are maternally as well as zygotically mutant for *hth* null alleles. Also the appendage phenotype of these *Gryllus* embryos and nymphs is comparable to that of large clones homozygously mutant for strong *Dm'hth* alleles. Surprisingly, however, we were not able to demonstrate degradation of *Gb'hth* mRNA in RNAi embryos. *Gb'hth* RNAi embryos stained by in situ hybridization typically display *hth* expression levels similar to control embryos, even if their morphology indicates strong segmentation and proximo-distal phenotypes (Fig. S3G). We do not know the basis for this puzzling observation. Conceivably,

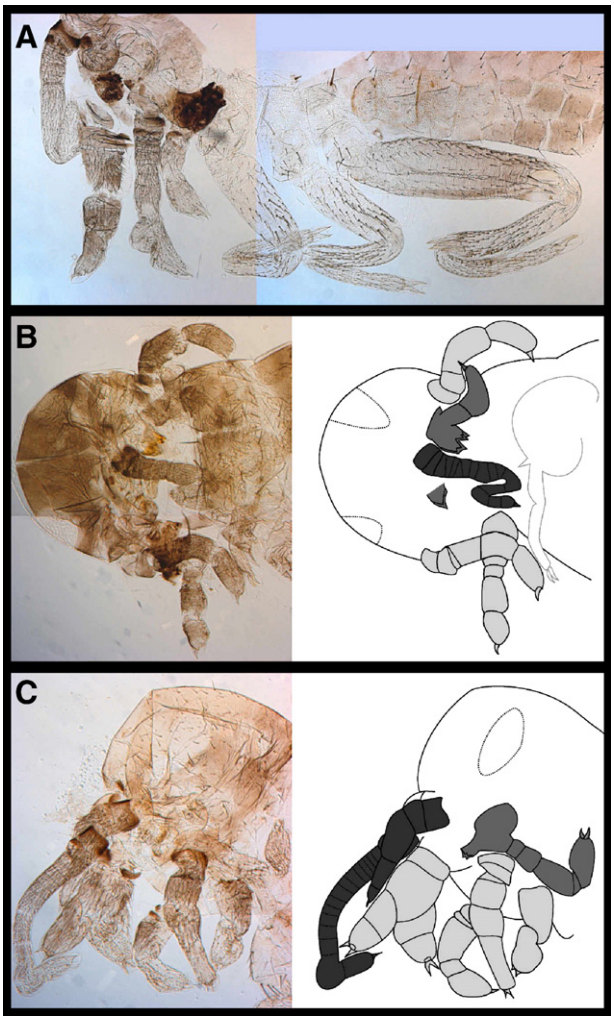

Fig. 5. Transformation of distal head appendages towards leg. Dissected and flattened cuticle preparations of *Gb'hth* RNAi nymphs. Preparations are oriented anterior to left; panels A, C are lateral views, panel B is a ventral aspect. (A) Specimen displaying transformation of all head appendages; in this preparation the right body half was removed; the left antenna and three gnathal appendages are clearly reshaped towards leg morphology. Some necrotic tissue is present in the head while the thorax in this weak *Gb'hth* phenotype is quite normal. (B, C) Flat cuticle preparations of *Gb'hth RNAi* nymphal heads; on the right side, interpretative sketches of these severely disturbed head morphologies are given. Color code: dark gray = antenna, intermediate gray = mandible, light gray = transformed gnathal appendages of uncertain segmental origin. Dotted ovals indicate the nymphal complex eyes. The gray outline in panel B probably represents a fused appendage of mixed gnathal/thoracic origin.

in our experimental animals *Gb'hth* may have been inactivated only in part, implying that the null phenotype of *hth* in *Gryllus* may be even more dramatic (this also could explain that the *Gb'exd* phenotypes are somewhat stronger). Alternatively, it could be that early embryonic stages in *Gryllus* are more accessible to parental RNAi than later stages. Since in young *Gryllus* embryos we observed only low levels of uniformly distributed *hth*, which is difficult to distinguish from background staining, our detection method may have missed changes in mRNA abundance at these stages. It is also possible that RNAi in *Gryllus* might affect gene activity by means other than mRNA degradation (however, degradation of mRNA has been observed in other *Gryllus* RNAi experiments, e.g. Miyawaki et al., 2004). No matter which explanation applies to the levels of *hth* mRNA remaining in animals displaying strong RNAi phenotypes, our control experiments, our RNAi experience with many other *Gryllus* genes and the similarity among *Gb'hth* and *Gb'exd* RNAi phenotypes clearly show that the observed phenotypes are specific and that they allow firm conclusions about *hth* function in this insect.

The pleiotropic spectrum of hth phenotypes in Gyllus is similarly broad as in Drosophila

In *Drosophila*, *hth* is involved in many developmental processes, among them formation of body segment primordia (Rieckhof et al., 1997), specification of segmental identity (Rieckhof et al., 1997), proximo-distal patterning of legs, head appendages and wings (Wu and Cohen, 2000; Casares and Mann, 2000), eye development and photoreceptor differentiation (Pai et al., 1998; Bessa et al., 2002), and patterning of the visceral mesoderm (Stultz et al., 2006). *Dm'hth* is widely expressed and is thought to interact with a number of other transcription factors, including the Hox genes and homeodomain-containing segmentation genes.

Similar as in *Dm'hth* mutations, *hth* RNAi in *Gryllus* disrupts segmentation in head, thorax, and abdomen. The strongest effects are observed in the head while the defects in the abdomen are less pronounced. This could indicate a temporal effect, i.e. the parental RNAi effect may be stronger at earlier stages when anterior segments are formed. Alternatively, some of the segmentation genes that require *hth* as cofactor may be specific for the head (i.e. head gap genes like the homeobox gene *empty spiracles*) or head gap genes may require Exd/Hth activity for their expression as suggested by Mito et al., 2007 (this issue). A specific role of *hth* in head formation is suggested by the *hth* RNAi phenotype in the hemipteran *Oncopeltus* where the antenna is particularly sensitive to *hth* knock-down, such that loss of this appendage prevented investigation of a *hth* role in antenna identity in this organism (Angelini and Kaufman, 2004). In *Drosophila* larvae, the head is also very sensitive to reduced *hth* activity. However, this could be due to disruption of head involution rather than head patterning, such that a specific role of *Dm'hth* in head patterning is uncertain.

During postembryonic development, *Dm'hth* clones result in enlarged eye primordia (Pai et al., 1998), with concomitant loss of head capsule material. Ventrally enlarged complex eyes were also observed in *Gb'hth* nymphs (not shown). Also the other two most studied functions of *Dm'hth*, proximo-distal patterning and antenna specification, are evident in the phenotype of *Gb'hth* knock-down embryos and nymphs (see below). Moreover, the similarity between *Gb'hth* RNAi (this paper) and *Gb'exd* RNAi phenotypes (see accompanying paper by Mito et al.) suggests that, as in *Drosophila*, also in *Gryllus* these genes act as a functional unit. Conservation of this molecular interaction was not unexpected, given similar data in vertebrates (Mercader et al., 1999; Shanmugam et al., 1999). However, it is remarkable that the wide pleiotropic spectrum of *hth*

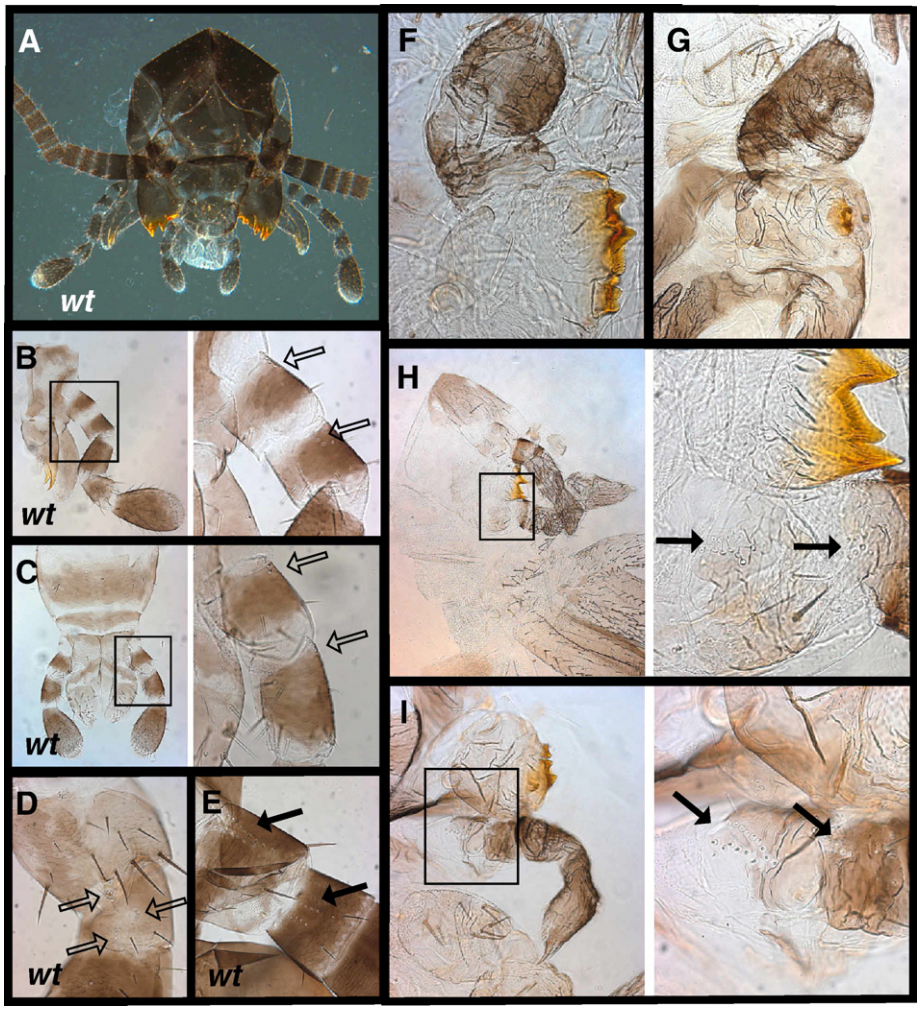

Fig. 6. Transformation of proximal gnathal appendages towards antenna. (A–E) Wild type, (F–I) *Gb'hth* RNAi animals. Magnified insets of campaniform sense organs at the basis of appendages are shown on the right in panels B, C, H and I. (A) Frontal view of a *Gryllus* nymph head with segmented antennae, maxillary and labial palps. (B–D) The basal podomeres of maxillary palps, labial palps and legs carry short single rows or groups of campaniform sensilla (open arrows). (E) The basal two antennal segments each carry two pairs of parallel rows of sensilla (filled arrows; only one pair of rows per antennomere is visible here). This pattern of sensilla unambiguously identifies the proximal antennomeres. (F, G) Partially transformed mandibles with leg-like telopodite emerging from the dorsal rim of the mandible. (H, I) Examples of more completely transformed gnathal appendages the basal podomeres of which carry antennal sensory rows. Distally they carry tarsal claws.

phenotypes is so well conserved between insect species separated by 300 Myr of independent evolution. This surprising conservation suggests that the molecular function of *hth* as cofactor acting at many homeodomain transcription factor target promoters is highly specific and cannot be replaced by other homeodomain cofactors, even over long evolutionary time spans.

Gb'hth function in proximo-distal patterning does not reflect its larger proximal expression domain

Using stable beta-galactosidase as reporter gene, the *Dm'hth* domain has been mapped to the leg primordia of coxa and trochanter (e.g. Dong et al., 2000). Despite this proximally restricted expression domain, large clones mutant for *Dm'hth* result in defects of coxa, trochanter and femur which become fused and greatly shortened (Wu and Cohen, 1999). Pai et al. (1998) found that even the femur/tibia joint can be affected. A very similar phenotype was observed for *Dm'exd* (González-Crespo and Morata, 1995; Rauskolb et al., 1995). To explain how *hth* can exert its effect over four podomeres, it has been proposed that an *hth*-dependent factor diffuses from the *hth* domain to pattern more distal parts of the appendage (Goto and Hayashi, 1999) or that cell migration from the *hth* expression domain into the femur primordium is affected (Wu and Cohen, 2000).

As in *Drosophila*, *hth* is proximally expressed also in *Gryllus* appendage primordia. However, *Gb'hth* differs from *Dm'hth* in the size of the proximal leg domain. At stages when the individual leg podomers become morphologically discernable, *Gb'hth* expression clearly extends well into the femur primordium (Inoue et al., 2002; see also Figs. 1H, I). Based on this difference in expression, one might have expected that the *Gb'hth* leg phenotype would differ from that of *Dm'hth*. But in *Gryllus* nymphs depleted for *hth* we observe fusion and shortening of podomers from coxa to tibia, very

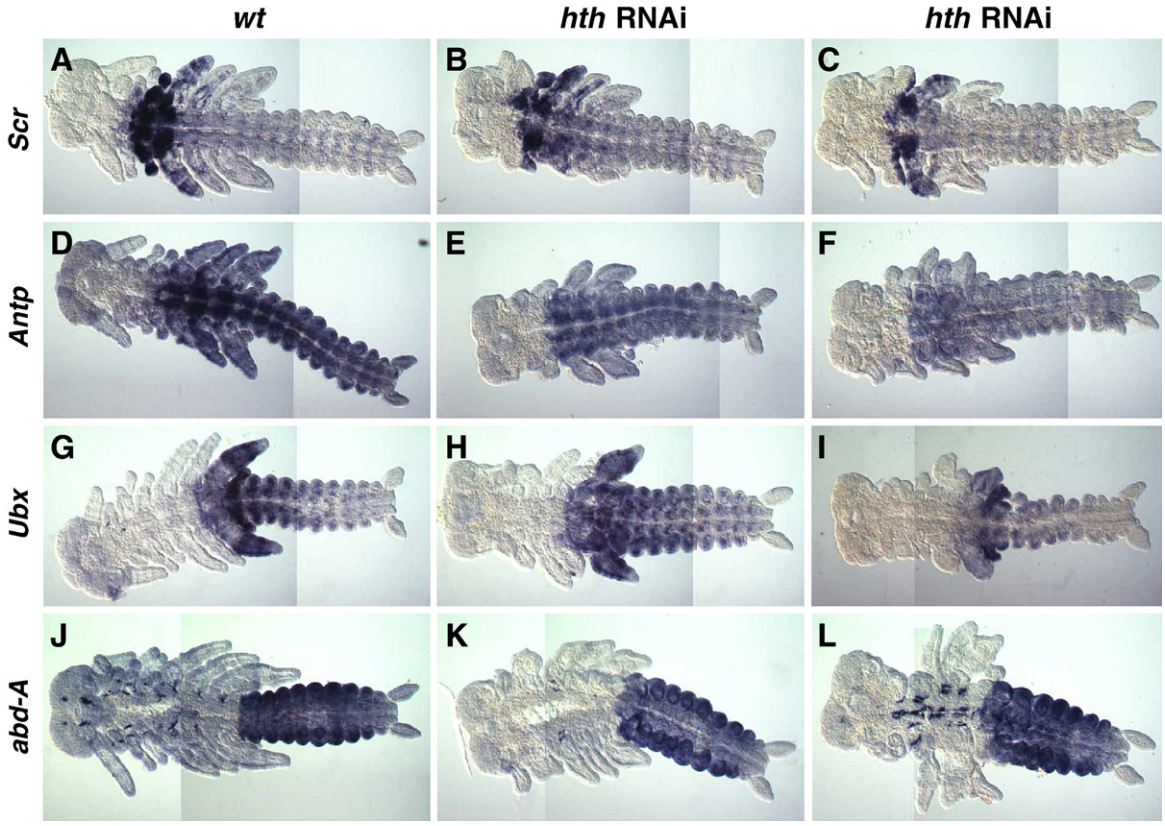

Fig. 7. Expression of Hox genes. Expression of *Gb'Scr* (A–C), *Gb'Antp* (D–F), *Gb'Ubx* (G–I) and *Gb'abd-A* (J–L) in wild type (A, D, G, J) and in *Gb'hth* RNAi embryos (right two columns). No thoracic Hox genes become expressed in head appendages upon inactivation of *Gb'hth* (note that the spots in head and thorax of the embryos in panels J and L are staining artefacts, see legend of Fig. 4). Also changes in the posterior expression domains of these genes appear to reflect secondary consequences of the severe segmentation defects.

similar to *Drosophila* (Fig. 3J). This begs the question if *Gb'hth* expression in the femur contributes to the patterning defects in this region. Nevertheless, also in *Gryllus* the defect zone extends distally beyond the expression boundary of *hth*, i.e. the puzzle of how primordia distal to its domain are affected by loss of *hth* gene activity is not peculiar to *Drosophila* imaginal discs.

While the shortened/fused expression domains of *Gb'dac* and *Gb'al* in *Gb'hth* RNAi embryos reflect the loss of intermediate proximo-distal regions (Fig. 4), we did not observe an expansion of the *dac* domain as expected if *Gb'hth* had a major role in setting the proximal limit of *Gb'dac* expression as has been observed in *Drosophila* (Wu and Cohen, 2000). In this respect our findings are similar to those in *Oncopeltus* (Angelini and Kaufman, 2004) and may represent a genuine difference in the regulatory wiring of embryonic hemimetabolous legs as compared to dipteran imaginal discs.

Gnathal appendages as well as antennae are transformed towards thoracic fate by Gb'hth knock-down

Loss of *hth* results in severe segmentation defects in *Gryllus* nymphs, which may obscure some of its homeotic function. For this reason it is not clear if *Gb'hth* plays a role in thoracic identity specification like its *Drosophila* ortholog. In the head, strong segmentation defects result in the loss of all appendages. However, in less severely affected RNAi nymphs, robust transformation of head appendages is evident. Interestingly, not only antenna but also mandible, maxilla and labium become transformed towards thoracic fates. Proximal structures seem more resistant to transformation, resulting in appendages that, for example, resemble leg distally but retain mandibular characters proximally (Fig. 5). We find that with decreasing *hth* activity all gnathal segments follow a similar phenotypic series as the antenna, with tarsal structures evident in weak phenotypes, followed by the formation of more complete legs that display antennal characters proximally, up to the complete loss of the respective segment in the most severely affected embryos. Thus, the reduction of *hth* activity in *Gryllus* head appendages results in a mixed leg/antennal fate, transformed mandible, maxilla and labium proximally displaying antenna-specific sense organs (Figs. 6, 8). While transformation of all head appendages has not been observed before in any insect, it is interesting to note that transformation of maxillary palps towards leg fate was observed in experiments involving antimorphic *Dm'hth* activity (Inbal et al., 2001) and transformation of labial palps in *Oncopeltus* upon reduction of *hth* activity (Angelini and Kaufman, 2004).

In *Drosophila*, *hth* and *exd* are regarded as antennal selector genes since their loss results in antenna-to-leg transformation and because forced expression of *hth* can lead to ectopic antennae developing from genital discs (Casares and Mann,

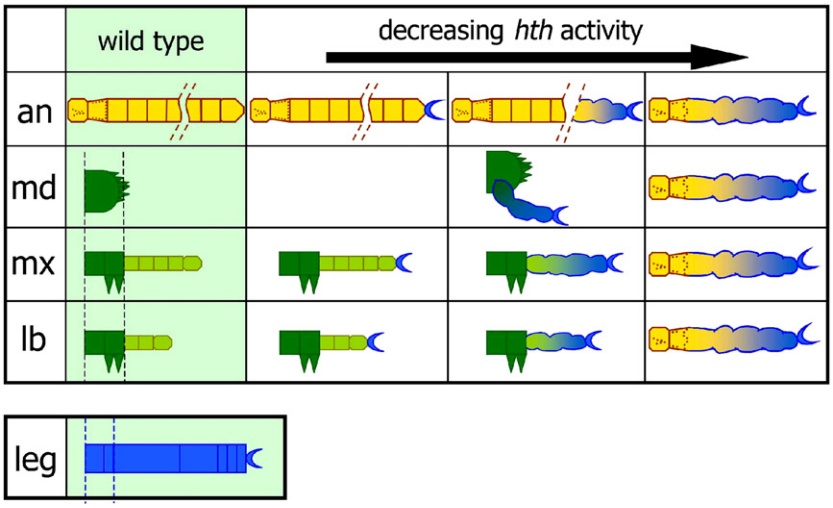

Fig. 8. Summary of head appendage transformations in *hth* deficient *Gryllus* nymphs. The left column depicts the situation in wild type: the two proximal segments of the antenna carry typical patterns of sensilla (small dots). The podomeres of mandible (md), maxilla (mx), labium (lb) and leg are grouped into basipodite and telopodite (separated by dotted lines). With increasing *hth* RNAi knock-down, head appendages progressively transform distally towards leg-like and proximally towards antenna-like morphology. Note that with decreasing *Gb'hth* activity head segmentation defects lead to the loss of appendages, such that a complete homonomous head bearing eight similar leg-like appendages is rarely observed (but see Fig. 5A).

1998; Kurant et al., 1998; Pai et al., 1998; Rieckhof et al., 1997; Yao et al., 1999). Moreover, the antenna-to-leg transformations caused by mis-expression of posterior Hox genes in the antenna have been shown to act through Hth, i.e. forced co-expression of Hth can suppress this transformation (Yao et al., 1999). Despite all that evidence, antenna specification is not fully understood. Given that all *hth* transcripts are expressed in all segments (Noro et al. 2006), how is its antenna-specifying function spatially restricted? Are the mechanisms that specify antennal fate in the embryo the same as those maintaining that fate during imaginal disc development? The additional role of *hth* in proximo-distal patterning complicates the interpretation of mutant phenotypes (Casares and Mann, 2001; Emerald and Cohen, 2001). Moreover, Exd/Hth clearly serve a different role in more posterior segments where they function as transcriptional cofactors to improve target specificity of several different Hox proteins. Our finding that in *Gryllus* all four head appendages similarly depend on *hth* to distinguish them from thoracic limbs is intriguing. In the following we discuss two alternative interpretations both of which are based on current views of *Drosophila* TALE protein function.

In *Drosophila* imaginal discs (Struhl, 1982) as well as in *Tribolium* embryos (Stuart et al. 1991), the absence of Hox gene products results in transformation of trunk segments towards antenna (note, however, that the situation in *Drosophila* embryos is somewhat different, see Struhl, 1983; Sato et al., 1985; Röder et al., 1992). Thus, it could be that the identity of the antennal segment in insects is specified by the presence of segmentation gene activity (e.g. *en*, *hh* and *wg*) combined with the absence of Hox activity. "Hth+Hox" then would specify gnathal, thoracic or abdominal segments, while "Hth alone" would specify antenna. The gnathal transformations that we observe in *hth* RNAi nymphs could in principle be explained according to this simple code, if gnathal Hox genes were not expressed in the absence of *hth*. Lack of Hox gene activation then would result in transformation towards antenna; inevitably, however, such gnathal antennae would also become transformed towards thoracic limbs due to the absence of Hth. Loss of *Dfd* and *Scr* expression has been observed in *Gb'exd* RNAi embryos displaying strong phenotypes (see accompanying paper by Mito et al.). Although we did not observe loss of *Gb'Scr* expression in *Gb'hth* RNAi embryos (Fig. 7), the overall similarity of *Gb'hth* and *Gb'exd* RNAi phenotypes suggests that head Hox genes likely also would be affected by complete knock-down of *Gb'hth*. This would be consistent with the above explanation for transformation of gnathal appendages as a two-step process caused by (1) loss of Hox expression followed by (2) transformation towards leg due to missing Hth activity.

However, it is important to note that transformation of head appendages is only observed in phenotypes of weak or intermediate strength since strongly affected embryos do not develop head appendages. In *Gb'hth* RNAi embryos displaying intermediate strength phenotypes, which would have differentiated into nymphs displaying gnathal transformations, we found *Scr* to be expressed at high levels (Figs. 7B, D). Thus, loss of head Hox gene expression does not appear to be required for gnathal transformation towards leg fate. On the other hand, our finding that all four types of head appendages in *Gryllus* similarly depend on *hth* to distinguish them from thoracic limbs could indicate that *Gb'hth* actually fulfills similar molecular functions in antenna and more posterior segments. An alternative explanation for gnathal appendage transformation would be that in the absence of Hth/Exd activity the target promoter specificity of head Hox genes is altered, resulting in the activation of target genes promoting thoracic rather than head fate. Such a mechanism is thought to transform thoracic and abdominal identities in *Drosophila exd* and *hth* mutants. This model could also explain transformation of the antenna, if Hth/Exd would interact with an antenna-

specific homeotic gene similarly as it interacts with Hox genes. It has been suggested that the *ems* gene, whose gene product contains a Hox-like homeodomain but is not located in a Hox cluster, may function like an anterior Hox gene (Macias and Morata, 1996). If Ems or another antenna-specific factor also requires Hth to exert its function, then the transformation of antenna to leg in *Gb'hth* RNAi embryos could be interpreted analogously to the transformation of gnathal (and trunk) segments in these embryos.

While we cannot distinguish between these alternative explanations at this time, investigating the interplay among head gap genes, Hox genes and Hox cofactors in *Gryllus* should unravel if the apparent differences between *Gryllus* and *Drosophila hth* functions are caused by diverged molecular mechanisms, or if a basically conserved molecular machinery has different phenotypic consequences in embryos with derived and basal modes of head development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.09.059.

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