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. © 2007 Elsevier Inc. All rights reserved.

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Introduction

Although appendage development in different arthropods is clearly based on a common genetic toolkit (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. Beer et al., 2001).
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 (sh), 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; Rauskob 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 of Exd/Hth appears to be conserved even in vertebrates.

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 has 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.

Phylogenetic 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, CaCl2–2H2O 5 mM, NaHCO3 2 mM). For injections, selected adult females were anesthetized with
diethyl ether for 1 min and then injected at the basis of a metathoracic leg with a borosilicate glass capillary (Kwik-Fit™, 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’fashi 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’ed* 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% bovine serum albumin (BSA, 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-fluor (450–500 nm, Roche) followed by 1× galactosidase color reaction. The protocols were adapted from established protocols for *Triabolium* (e.g. Ehrman 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 PBS for 24 h at RT. The secondary antibody (FITC conjugated goat-anti-rabbit, Jackson Immunolo 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 nympha 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 (Hans 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’ed* 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 *Triabolium* 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...
(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−
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’htth 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 (podomeres) 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 overly sensitive to Tb’htth reduction.

Disruption of proximo-distal patterning is also apparent from the expression patterns of the molecular markers Gb’dachshund (Gb’dac) and Gb’aristales (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’htth 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’htth 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’htth 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’htth RNAi embryos the expression of Gryllus homologs...
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 interpret 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
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 knockdown 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. bimaculatus 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,
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 Gryllus 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 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. 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 appendages are clearly reshaped towards leg morphology. Some necrotic tissue (A) Specimen displaying transformation of all head appendages; in this oriented anterior to left; panels A, C are lateral views, panel B is a ventral aspect. (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.
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 podomeres 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. 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.
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 transform-
ation of labial palps in Oncopeltus upon reduction of hth 

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,
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'ext 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'ext 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 ext 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 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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