

Conservation and diversification of gene function during mouthpart development in *Onthophagus* beetles

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SUMMARY The evolutionary success of insects is in part attributable to the tremendous diversification of their mouthparts, which permitted insects to radiate into novel food niches. The developmental genetic basis of mouthpart development has been well studied in at least two insect taxa possessing derived mouthparts, the hemipteran *Oncopeltus fasciatus* and *Drosophila*. However, much less is known about the regulation of mouthpart differentiation of the presumed ancestral mandibulate type. Here we aim to extend current insights into the patterning of mandibulate mouthparts through a functional genetic analysis of three leg gap genes, *homothorax* (*hth*), *dachshund* (*dac*), and *Distal-less* (*Dll*), in the dung beetle *Onthophagus taurus*, a species whose mouthpart arrangement has in part retained, as well as diverged form, the ancestral mandibulate mouthpart type. We specifically include in this study a first functional genetic

analysis of the adult labrum, an enigmatic mouthpart whose appendicular origin has been the subject of a long-standing debate. Our results support a functional role of all three patterning genes in the development of the labium, maxilla, as well as the labrum. In contrast, mandible development appeared to rely only on the patterning functions of *hth* and *dac*, but not *Dll*. Here, our results raise the possibility that evolutionary changes in the *dac*-patterning may have played an important role in the evolutionary transition from a short, triangular mandible adapted for chewing to the elongated, flat, and blade-like mandible of modern filter-feeding scarabaeine beetles. In general, our results contribute to a growing body of studies that suggest that basic patterning genes can contribute to morphological evolution of adult features while maintaining traditional patterning responsibilities at earlier developmental stages or in other body regions.

INTRODUCTION

One of the key features accounting for the evolutionary success of insects is the diversification of their appendages (Grimaldi and Engel 2005). Evolutionary diversification of the mouthparts in particular, has played a significant role in permitting insects to diversify their food sources and radiate into novel food niches. Here, a mandibulate mouthpart type still found, for instance, in extant grasshoppers, beetles, and earwigs is believed to have given rise, among many others, to the sucking mouthparts of the true bugs, the sponging mouthparts of higher flies, or the extendable proboscis of butterflies and moths (Labandeira 1997). The developmental genetic basis of mouthpart development has been well studied in at least two taxa possessing derived mouthparts, the hemipteran *Oncopeltus fasciatus* (Angelini and Kaufman 2004) and *Drosophila* (Jouliat et al. 2005, 2006). However, much less is known about the regulation of mouthpart differentiation of the presumed ancestral mandibulate type, in particular with respect to the development of adult, rather than larval or nymphal mouthparts. A recent study on *Tribolium* beetles has begun to fill this gap (Angelini et al. unpublished data). Here

we aim to extend current insights into the patterning of mandibulate mouthparts through a functional genetic analysis of three leg gap genes, *homothorax* (*hth*), *dachshund* (*dac*), and *Distal-less* (*Dll*), in the dung beetle *Onthophagus taurus*, a species whose mouthpart arrangement has in part retained, as well as diverged form, the ancestral mandibulate mouthpart type (for details see below and Fig. 1). We specifically include in this study, a first functional genetic analysis of the adult labrum, an enigmatic mouthpart whose appendicular origin has been the subject of a long-standing debate (Haas et al. 2001; Boyan et al. 2003; Kimm and Prpic 2006; Posnien et al. 2009).

Onthophagus is an extremely species-rich genus within the subfamily Scarabaeinae, or true dung beetles, which rely on dung as a principal food source (Halffter and Edmonds 1982; Lopez-Guerrero and Zunino 2007). Scarabaeine larvae feed primarily on the fibrous portion of dung and feature mandibulate mouthparts very similar to those found in most other beetles. Adults, in contrast, are specialized to consume the fluid portions of fresh dung and dissolved small particles and cannot chew fiber (Miller 1961; Hata and Edmonds 1983). To aid in this process, adult dung beetle mouthparts, in particular

the mandible, have become adapted for sifting and filter-feeding (Edmonds 1972; Hata and Edmonds 1983; Lopez-Guerrero and Zunino 2007). Here we provide a detailed description of the mouthparts of the dung beetle *O. taurus*, and investigate the role of the leg gap genes *hth*, *dac*, and *Dll* in patterning conserved and diverged aspects of mouthpart morphology.

The functions of the leg gap genes *hth*, *dac*, and *Dll* have been investigated in great detail during *Drosophila* leg development. Here, *hth*, *dac*, and *Dll* are expressed in antagonistic domains, which help establish the proximal–distal axis of the adult leg (reviewed in Kojima 2004). Specifically, the combinatorial interaction between *hth* and its cofactor *Extradenticle* determines where developing appendages will anchor into the body wall (Rieckhof et al. 1997), whereas *dac* expression is required for the formation of medial leg regions (Lecuit and Cohen 1997). Lastly, *Dll* expression is critical for the establishment of the identity of the most distal leg regions such as the tarsal segments and claw (Campbell and Tomlinson 1998). Despite the highly derived mode of appendage development found in higher flies such as *Drosophila*, there remain many striking similarities in the proximo-distal patterning of appendages across holo- and hemimetabolous insect orders as well as across appendage types (Inoue et al. 2002; Prpic et al. 2003; Angelini and Kaufman 2004). Specifically, studies on mouthpart morphogenesis in arthropods in general and insects in particular, confirm in large part that mouthparts rely on the same or similar patterning mechanisms as other appendages, including the leg (reviewed in Angelini and Kaufman 2005). For example, in the *Tribolium* embryo, *hth* is expressed in the base of the maxillary and labial mouthparts (Prpic et al. 2003), *dac* transcripts are detected in proximal and intermediate domains in the maxilla (Prpic et al. 2001) and the distal maxillary and labial palps express *Dll* (Beermann et al. 2001). A major exception is the mandible, a nonsegmented appendage of presumed gnathobasic origin (Popadic et al. 1998; Scholtz et al. 1998; Prpic et al. 2001), which lacks a corresponding *Dll* expression domain. However, the nature and the evolution of the most anterior mouthpart, the labrum, are less clear. Molecular approaches support contradictory hypotheses regarding both the segmental origin (apical vs. intercalary segment) (Haas et al. 2001; Boyan et al. 2002; Posnien et al. 2009), and presumed appendicular nature of the labrum (serial homology vs. convergence) (Prpic et al. 2001; Boyan et al. 2002; Posnien et al. 2009).

Here we investigate the function of *Onthophagus hth*, *dac*, and *Dll* in adult mouthpart morphogenesis. We show that all three patterning genes are involved in the development of the labium, maxilla, as well as the labrum, whereas mandible development appeared to rely solely on the patterning functions of *hth* and *dac*, but not *Dll*. Here, *dac* function appears to be

instrumental in generating the highly derived mandible morphology typical of adult scarabaeine beetles.

MATERIALS AND METHODS

General

Animals used here have been analyzed in a previous study focused on the role of leg gap genes in beetle horn development (Moczek and Rose 2009). Cloning and sequence analysis of candidate genes, as well as RNAi methodology and validation are detailed in this earlier study. Below we briefly reiterate and summarize the methods most pertinent to the present study. We then detail methods and approaches critical to our analysis of *Onthophagus* mouthparts.

Animal husbandry

O. taurus were collected in the field and reared in the laboratory as described previously (Moczek and Nagy 2005).

Cloning, sequence analysis, and dsRNA construction

Onthophagus taurus dac was cloned in pCRII-TOPO vector (Invitrogen, Carlsbad, CA, USA) and analyzed as described in Moczek et al. (2006). *O. taurus hth* and *Dll* were cloned, in pCRII-TOPO and pSC-A vectors (StrataClone, Santa Clara, CA, USA), respectively and analyzed as described in Moczek and Rose (2009). The *dsRNA* constructs were generated via in vitro transcription using T7 and SP6 RNA polymerase (*dac*, *hth*) or T7 and T3 RNA polymerase (*Dll*) as specified by the manufacturer (MEGAscript kit, Ambion, Foster City, CA, USA) to produce both sense and anti-sense RNA strands for each of the fragments. Equimolar amounts of complementary strands were mixed and samples were heated to 95°C for 3 min then slowly cooled over 4 h to 25°C.

dsRNA injection

Larvae were injected up to 10 days after molting to the third instar (= final larval stage). Three microliter of a solution containing 0.5–5 µg of dsRNA in injection buffer (5 mM KCl, 1 mM KPO₄ pH 6.9) was loaded into a glass-tight 1801 Hamilton syringe with a 32 G needle (Hamilton, Reno, NV, USA) and injected medially behind the metanotum. After injection, larvae were returned to individual transfer plates to complete larval development and pupation. Control animals consisted of (a) untreated animals reared under the same conditions and (b) animals injected in parallel to RNAi individuals with a 167-bp portion of the cloning site of the Bluescript SK+vector in injection buffer.

Knock-down validation

Western (*dac*, *hth*) and Northern (*Dll*) blot analyses were used to evaluate the depletion of protein and mRNA levels after RNAi-mediated knockdown as detailed in Moczek and Rose (2009). Both methods documented substantial reductions in gene product for all three genes and across all tissues tested compared with wild type (leg, dorsal abdomen, thoracic horn, and head horn).

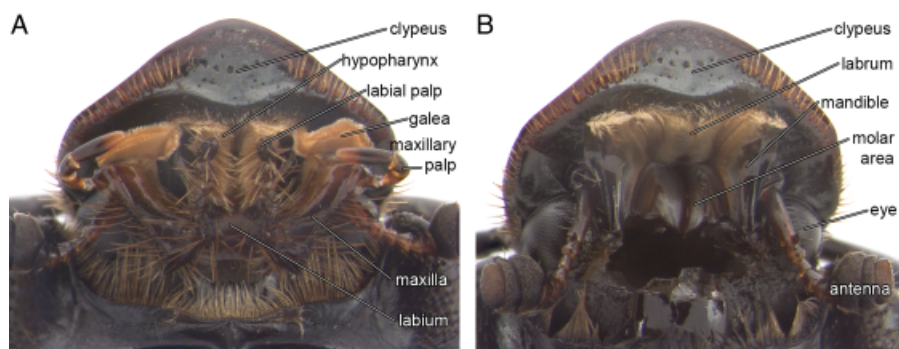


Fig. 1. *Onthophagus taurus* mouthparts in situ. (A) Ventral view of the head. The maxillae have been pulled apart slightly for visual convenience. (B) Ventral view of the head after dissection of the labium and the maxillae, revealing the mandibles and labrum underneath.

Microscopy and imaging

Adult mouthparts were dissected individually and photographed using a dissecting microscope (Leica MZ-16, Bannockburn, IL, USA) mounted with a digital camera (Scion, Frederick, MD, USA) and ImageJ software. Whenever necessary, several images were stacked using the auto-blend option in Photoshop CS4 to increase depth of field. Curve adjustments were applied to control contrast.

RESULTS

The mouthparts of adult *Onthophagus* beetles consist, from posterior to anterior, of a labium, a pair of maxillae, a pair of mandibles, and a labrum (Figs. 1 and 2A). Each part is described in further detail below. *Onthophagus* mouthparts thus share the same basic mandibulate arrangement found in other beetles such as *Tribolium*, but also differ in important aspects. For instance, although the labrum and labium of adult *Onthophagus* are overall very similar to those of *Tribolium*, the architecture of the mandible has diverged significantly (Fig. 2A). Specifically, although the mandibles of most adult beetles are heavily sclerotized and toothed, the adult *Onthophagus* mandible has evolved into a flat, thin, blade-like appendage with dense hair fringes. This novel mandible design is thought to facilitate feeding on the fluid portions of feces which constitute the principal diet of adult dung beetles (Halffter and Edmonds 1982). Interestingly, larval *Onthophagus* have retained the ancestral, triangular, heavily sclerotized, and toothed mandible identity (Fig. 2B). Thus, the derived, more membranous, and blade-like identity of the adult mandible is the product of the postembryonic modifications occurring during metamorphosis.

RNAi phenotypes

The number of adults examined and penetrance of RNAi phenotypes are summarized in Table 1. Each individual that exhibited RNAi phenotypes in one mouthpart (e.g., the labium) also exhibited corresponding phenotypes in other mouthparts (e.g., the maxillae). In general, RNAi phenotypes showed surprisingly little variability. In those cases in which a

graded effect was detected, we highlight the range of variation in the text below. RNAi phenotypes discussed below and presented in the corresponding figures illustrate typical phenotypes most commonly observed during this study. Control injections with dsRNA derived from a BlueScript plasmid

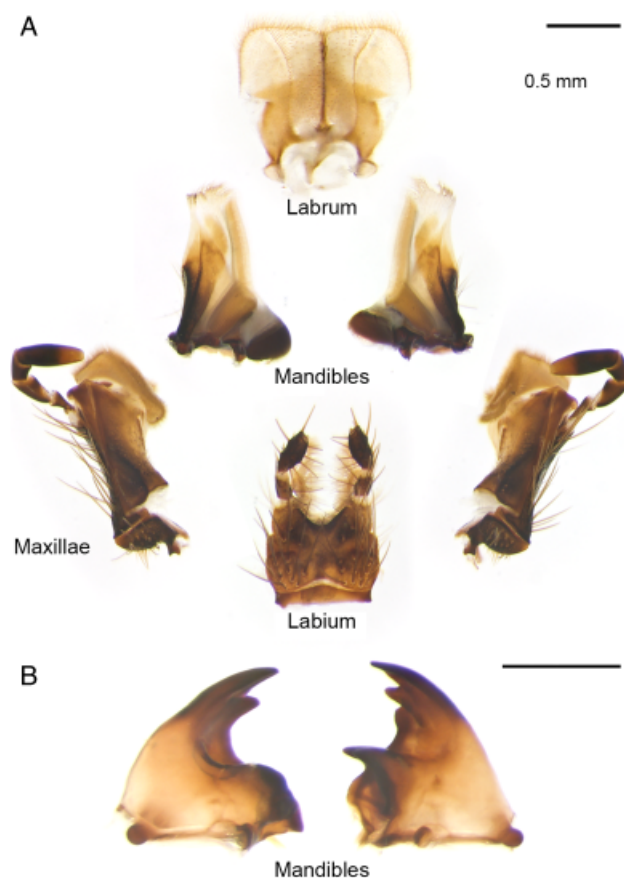


Fig. 2. *Onthophagus taurus* mouthparts. (A) Adult mouthparts. Mouthparts are arranged to highlight the position of parts relative to each other. (B) Larval mandibles. The molars are asymmetric and the distal incisors have heavily sclerotized teeth. While adult mandibles (A) maintain an asymmetric arrangement (see Fig. 5), they are transformed during metamorphosis into flat, thin, and membranous projections. Scale bar = 0.5 mm.

Table 1. Penetrance of RNAi mouthpart phenotypes for *homothorax* (*hth*), *dachshund* (*dac*) and *Distal-less* (*Dll*) in the beetle *Onthophagus taurus*

	# adults	# adults with mouthpart phenotype	Percentage
Wild type	52	0	0%
Control injected	33	0	0%
<i>hth</i> RNAi	31	23	74%
<i>dac</i> RNAi	18	16	89%
<i>Dll</i> RNAi	61	52	85%

Numbers shown are the number of individuals that survived to adulthood, and numbers and percentages of adults with RNAi phenotypes as described in the text. Control injections with dsRNA derived from a BlueScript plasmid vector sequence had no obvious phenotypic consequences.

vector sequence had no obvious phenotypic consequences and did not result in any phenotypes comparable with the RNAi-mediated transcript knockdown experiments described next.

Labium

The labium, or lower lip, is a median structure resulting from the fusion of a pair of appendages. The mentum is the most proximal structure, followed distally by the prementum (Fig. 3Ai). The latter bears two labial palps with three segments each (the larval labial palp has two segments). Dorsally attached to the labium is the hypopharynx, which includes distal portions densely covered in hair, the paraglossae (Fig. 3Aii, iii), and two strong lateral sclerites (Fig. 3Aiii).

RNAi depletion of *hth* resulted in a reduction in the size of the mentum, a relatively enlarged prementum, and a reduction of labial palp segment number from three to two or one (Fig. 3Bi). Furthermore, *hth* RNAi resulted in fusion of the hypopharyngeal sclerites and transformed them to a structure resembling the maxillary galea (see Fig. 4) in shape, color, and bristle patterning (Fig. 3Bii, iii). In contrast, *dac* RNAi resulted in a shortening of the medial prementum, but left the mentum and labial palps unaffected (Fig. 3C). Reduction of prementum size rendered the first labial palp segment more visible. Lastly, RNAi depletion of *Dll* resulted in an overall reduction of labium size (Fig. 3D), reduction of labial segment number from three to two, and a shortening of the hypopharyngeal sclerites.

Maxillae

The maxillae are paired mouthparts used for manipulating and directing food toward the mouth. The proximal cardo articulates the maxilla to the head. The intermediate stipes, a structure consisting of multiple sclerites, bears the lacinia, the galea, and the four-segmented maxillary palp (Fig. 4A).

hth RNAi resulted in a variable degree of fusion of the cardo and stipes (Fig. 4Bi, black arrowhead), fusion of the two ventral sclerites of the stipes (Fig. 4Bi, black arrowhead) and a reduction in the size of the parts that bear the galea and the lacinia (Fig. 4B, black dot). In contrast, *dac* RNAi resulted in a reduction of total maxillary palp length and palp segments number from four to three, apparently through fusion of the second and third palp segments (Fig. 4C, star). Lastly, *Dll* RNAi resulted in an overall reduction of maxilla size, and a complete loss of palp segments and joints (Fig. 4D), whereas galea and lacinia were unaffected.

Mandibles

The mandibles are the chewing mouthparts, articulated to the head by two condyles. From the base of the mandibles extend two lobes: the distal incisor lobe and the inner median molar lobe, which are linked by a flexible area (Fig. 5Ai to iv). The setae on the inner periphery of the incisor lobe delimit the comb and distal fringe of the incisor lobe (Fig. 5Ai). Remarkably, the molar area of the mandibles is asymmetric. The right concave molar area tightly fits the convex left molar area (Fig. 5Aii and iii), which partly mirrors an asymmetry already found in larval mandibles (Fig. 2B).

hth RNAi resulted in a reduction of the proximal molar lobe (Fig. 5Bi, ii) and a change in the shape of the molar area, most obvious in the left mandible (see Fig. 5Bii, iii). In addition, a small protrusion found in wild type between the molar lobe and the flexible area was absent in *hth* RNAi individuals (Fig. 5, apical views, black arrowhead). In contrast, *dac* RNAi resulted in a substantial shortening of the mandible due to deletion of medial mandible regions, causing mandible shape to change from an elongated blade to a short triangular cone (Fig. 5C). Specifically, the comb of the incisor lobe was deleted, as was the flexible area, causing the incisor lobe to connect directly to the molar area (Fig. 5C, white arrowhead). Lastly, the distal fringe of the incisor lobe was reduced (Fig. 5Ci, iv). *Dll* RNAi, in contrast, did not result in any mandibular phenotypes which instead were indistinguishable from wild type (not shown).

Labrum

The dorso-ventrally flattened labrum, or upper lip, is the most anterior mouthpart. Viewed from the dorsal side (Fig. 6Aii), the labrum can be divided into a proximal suspensorium and distal dulsa. The membranous ventral side of the labrum exhibits regularly distributed setae (Fig. 6Ai). High densities of setae form a median brush and two longitudinal, apically divergent, lateral files. Setae in the median area are shorter and more scattered than those in the lateral area. The dorsal side of the labrum features a medial opening and is joined dorsally to the clypeus by the articular line, which is apparent as a transverse sclerotic thickening (Fig. 6Aii).

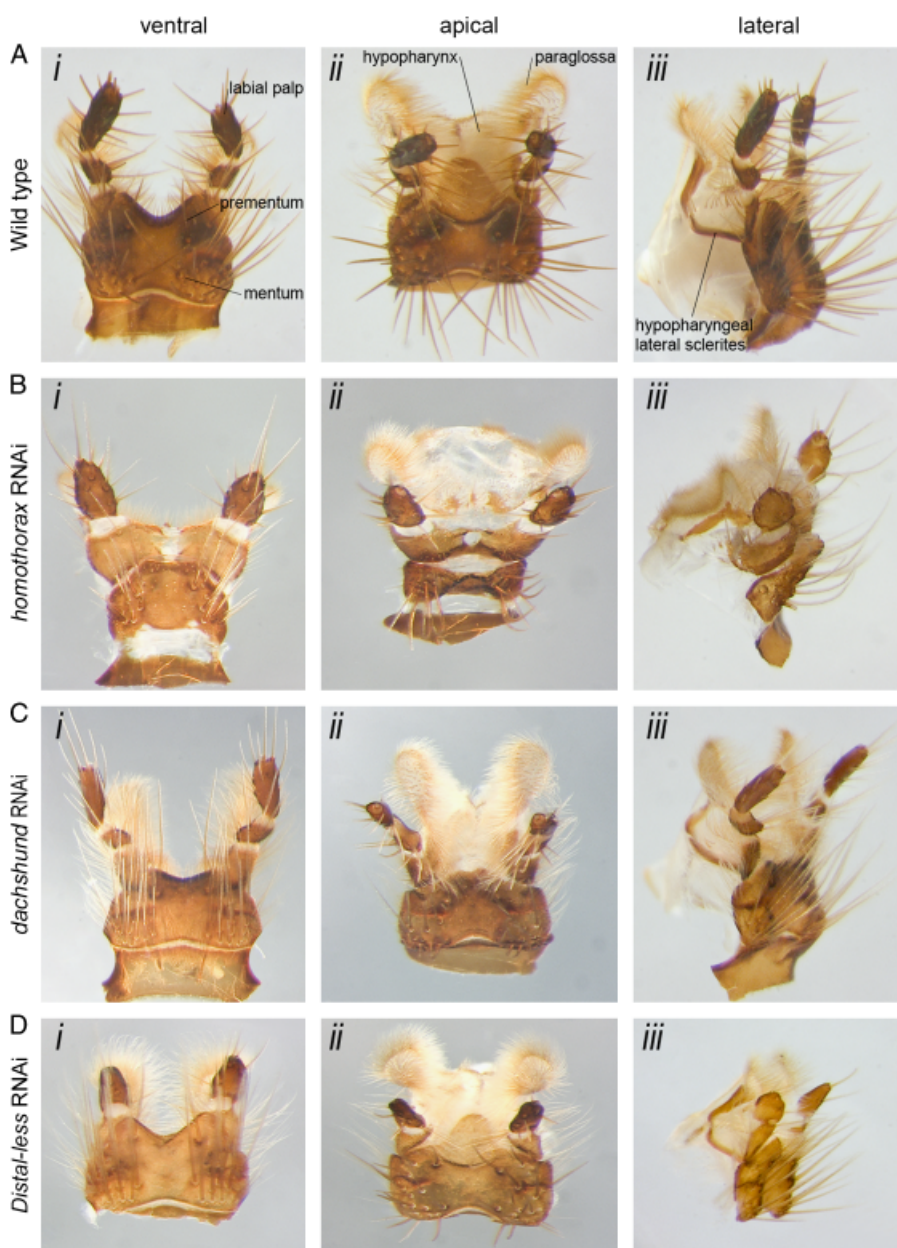


Fig. 3. *Onthophagus taurus* adult labium and hypopharynx from different views: (i) ventral view, (ii) apical view, and (iii) lateral view. (A) Wild-type labium. (B) *homothorax* RNAi phenotype. The mentum is shortened, the prementum enlarged and the labial palp unsegmented. The hypopharyngeal sclerites are transformed into a structure resembling the maxillary galea. (C) *dachshund* RNAi phenotype. The prementum is reduced, and hence the first segment of the labial palp more visible. (D) *Distal-less* RNAi phenotype. Overall, the labium is reduced in size including the hypopharynx. One labial palp segment is missing.

hth RNAi led to extensive modification of the labrum (Fig. 6B). In general, the labrum of *hth* RNAi individuals took on a more round, rather than a trapezoid shape with reduced dorso-ventral flattening, causing the labrum to appear inflated rather than flat (not shown). Specifically, the lateral files were either completely deleted, or at least lost their characteristic, linear aggregation of setae, and apical divergence (Fig. 6Bi). Long setae, normally only distributed in the distal lateral areas could now be found broadly distributed over the entire lateral areas. Lastly, although the distal dulsa region retained much of its identity and size, the proximal suspensorium appeared heavily reduced and lacked sclerotization (Fig. 6Bii).

In contrast, *dac* RNAi left distal and proximal identity of the labrum, including the distribution of setae, intact and wild-type-like, but affected the relative sizes of different labrum regions (Fig. 6C). Specifically, *dac* RNAi altered the location of the border between the proximal suspensorium and distal dulsa located in the medial labrum, resulting in a shortening of the dulsa and an enlargement of the suspensorium. (Fig. 6Ci, ii).

Finally, *Dll* RNAi had dramatic effects on labrum development and greatly reduced overall labrum size. Particularly, the distal dulsa was reduced to two highly sclerotized lobes (Fig. 6D), sometimes resulting in the fusion of the two lobes in

extreme cases (not shown). Furthermore, the median brush and lateral files including setae were absent and only a scattered apical fringe remained. The proximal suspensorium also

underwent substantial disarrangements, but overall remained more recognizable than the distal dula region (Fig. 6Dii).

DISCUSSION

We report a functional analysis of *hth*, *dac*, and *Dll* during *Onthophagus* mouthpart development. Our results support a functional role of all three patterning genes in the development of the labium, maxilla, as well as the labrum. In contrast, mandible development appeared to rely only on the patterning functions of *hth* and *dac*, but not *Dll*. Below we briefly discuss the most important implications of our results.

The origin of the labrum

The origin of the labrum has been intensely debated. Hypotheses range from an appendicular origin of the labrum, which posits that the labrum originated from a fusion of anterior appendages that also lost their segmented identity (Posnien et al. 2009), to the notion that the labrum may not be an appendage at all and instead homologous to the acron, or first segment of the arthropod head, itself (Scholtz 1997). More recent findings (summarized in Kimm and Prpic 2006), including extensive comparative gene expression data, provide support for the homology of the labrum with traditional arthropod appendages. However, with the exception of a single study on *Dll* function in the spider *Cupiennius* (Schoppmeier and Damen 2001), insights from gene function analyses have thus far been lacking. Here we provide the first RNA interference mediated gene knockdown results documenting a functional role of three leg gap genes in adult labrum formation. *hth* RNAi mostly affected the suspensorium, and thus the identity of the proximal labrum, whereas its effects on the dula, or distal labrum, were more subtle, leaving this region more wild-type like in overall architecture and bristle and pigmentation patterns. In contrast, *Dll* RNAi exerted its most dramatic effects on the distal labrum, resulting in nearly complete deletion of the dula, but leaving the suspensorium in a more recognizable, albeit still heavily malformed, state. The effect of *dac* RNAi was different yet again, and left distal and proximal identity of the labrum intact, whereas altering the location of the border between suspensorium and dula

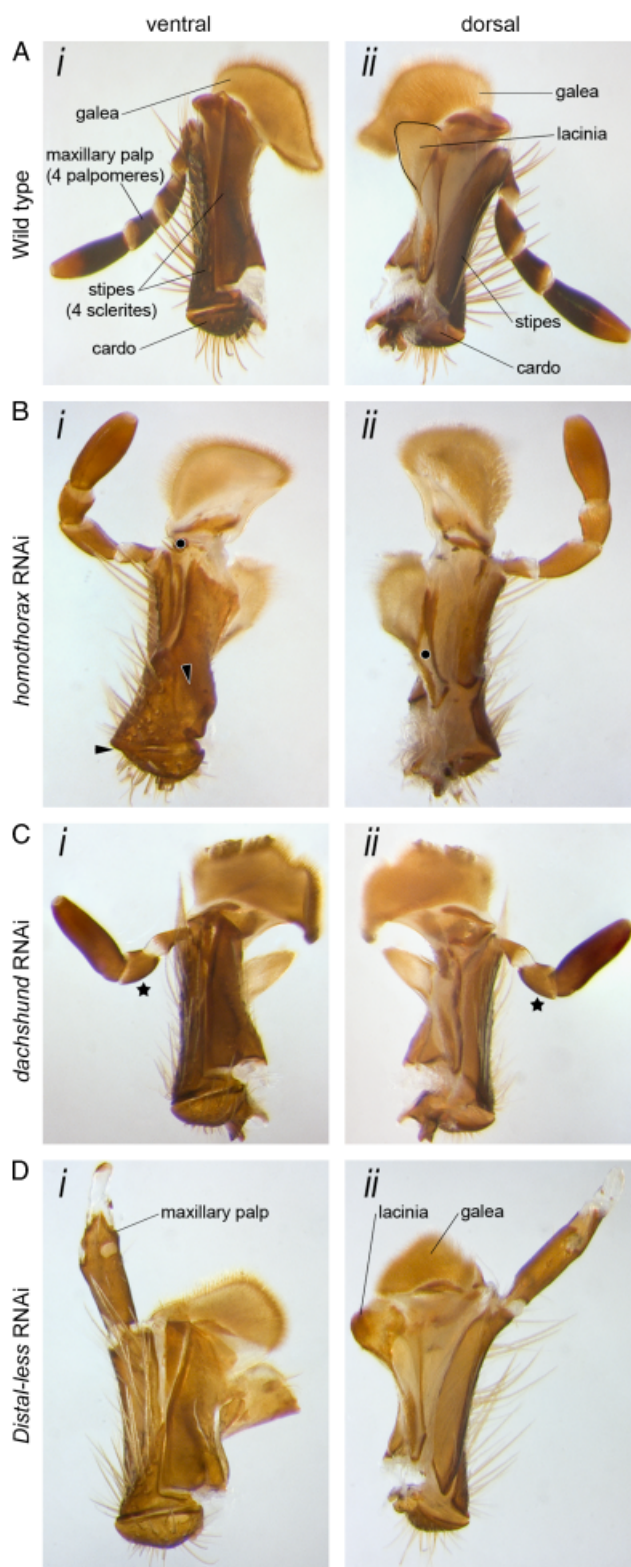


Fig. 4. *Onthophagus taurus* adult maxillae. Each column presents a different view of the maxillae: (i) ventral view, and (ii) dorsal view where the lacinia is more easily seen. (A) Wild-type maxilla. The main parts of the maxilla are (from proximal to distal): cardo, stipes, lacinia, galea, and maxillary palp. (B) *homothorax* RNAi phenotype. Cardo and stipes are fused including several stipes sclerites (black arrowhead). The parts bearing the galea and the lacinia are reduced (black dot). (C) *dachshund* RNAi phenotype. A palp segment is missing (star). (D) *Distal-less* RNAi phenotype. The maxilla is reduced in size and the maxillary palp is unsegmented.

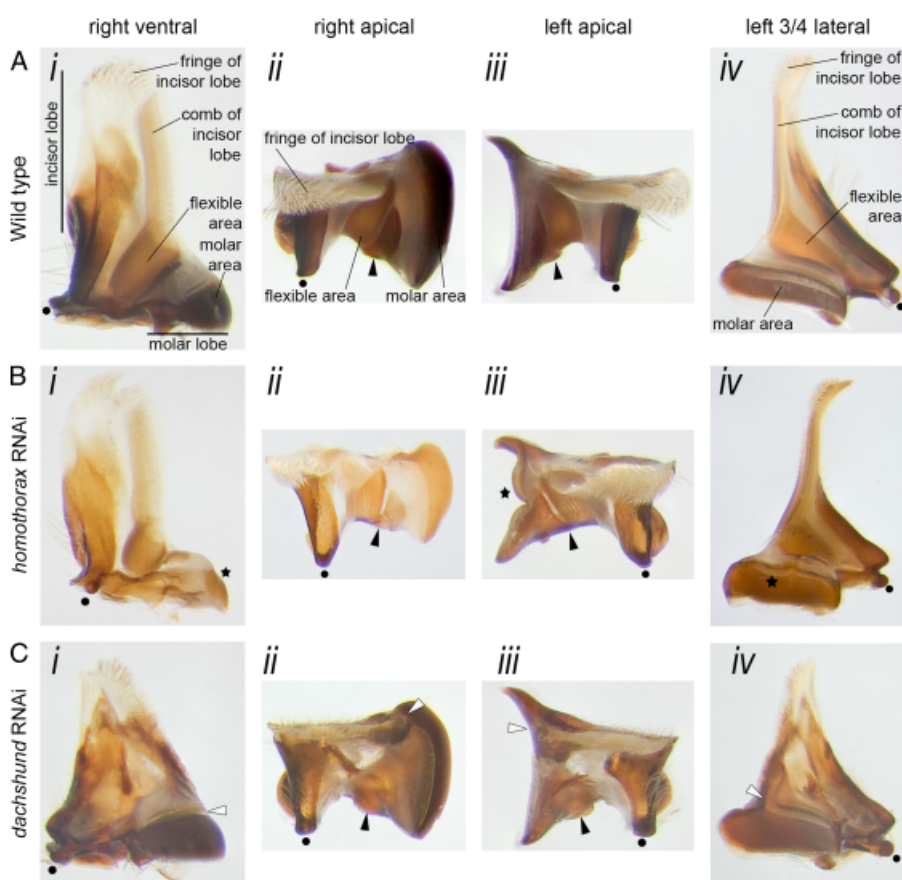


Fig. 5. *Onthophagus taurus* adult mandibles. In each image the black dot indicates the position of the ventral articular condyle to provide a common landmark for the viewer to better relate the different images to each other. Specifically, we show (i) ventral view of the right mandible, (ii) apical view of the right mandible, (iii) apical view of the left mandible, (iv) ventro-lateral inner view of the left mandible. (A) Wild-type mandibles. Note the asymmetry of the mandibles, which is clearly seen from the apical view. (B) *homothorax* RNAi mandibles. The ridge between the molar lobe and the flexible area is modified (compare black arrowheads in wild type and *dachshund* RNAi mandibles). The molar area is also misshapen (star), more obvious for the left mandible. (C) *dachshund* RNAi mandibles. The medial part of the mandible is deleted. Instead, the incisor lobe is directly connected to the molar lobe (white arrowhead) and the comb of the incisor lobe and the flexible area are not recognizable.

located in the medial labrum. Combined, RNAi phenotypes of all three patterning genes thus mapped to a considerable degree onto a proximodistal axis, consistent with the hypothesis that *hth*, *dac*, and *Dll* pattern labrum development in ways at least partly similar compared with more traditional appendages. More generally, our findings thus support the serial homology of all arthropod appendages (Prpic et al. 2001; Boyan et al. 2003), and suggest that the labrum may not only be appendicular in origin (Haas et al. 2001; Boyan et al. 2002), but also consists of proximal, medial, and distal appendage elements similar to those of all other mouthparts, with the exception of the mandible. However, our findings are not sufficient to fully exclude alternative explanations. For instance, *hth*, *dac*, and *Dll* functions could have been recruited secondarily, either separately or collectively as a developmental module, during the evolution of the labrum from a non-appendicular origin. If correct, this would suggest that the similarities in the development of the labrum and other mouthparts are the product of convergent evolution, which recruited *hth* function to pattern where the labrum attaches to the head, and *Dll* function to pattern distal labrum identity. However, the recruitment of *dac* function may be more difficult to explain, as the labrum does not really possess a

well-defined medial region. *Dac* expression and function in the labrum may thus either be the incidental product of the convergent recruitment of all three gap genes as a module, or alternatively, and perhaps more convincingly, a signature of true serial homology of the labrum. Clearly, more functional genetic analyses of labrum development across taxa are needed to further address this issue.

Conservation and diversification of the beetle mandible

The insect mandible is the only mouthpart lacking a distal domain of *Dll* expression (Popadic et al. 1998; Scholtz et al. 1998), and reduction or loss of *Dll* function does not affect mandible expression (Beermann et al. 2001; Angelini and Kaufman 2004). This has generally been interpreted as reflecting a gnathobasic origin of the mandible, that is the mandible is itself homologous only to the base of an appendage which has lost its originally distal compartment (Popadic et al. 1998; Scholtz et al. 1998). Our results further support this notion: although *Dll* RNAi resulted in severe phenotypes in all other mouthparts (Figs. 3, 4, and 6) as well as legs and antennae (not shown), it left mandible formation unaffected.

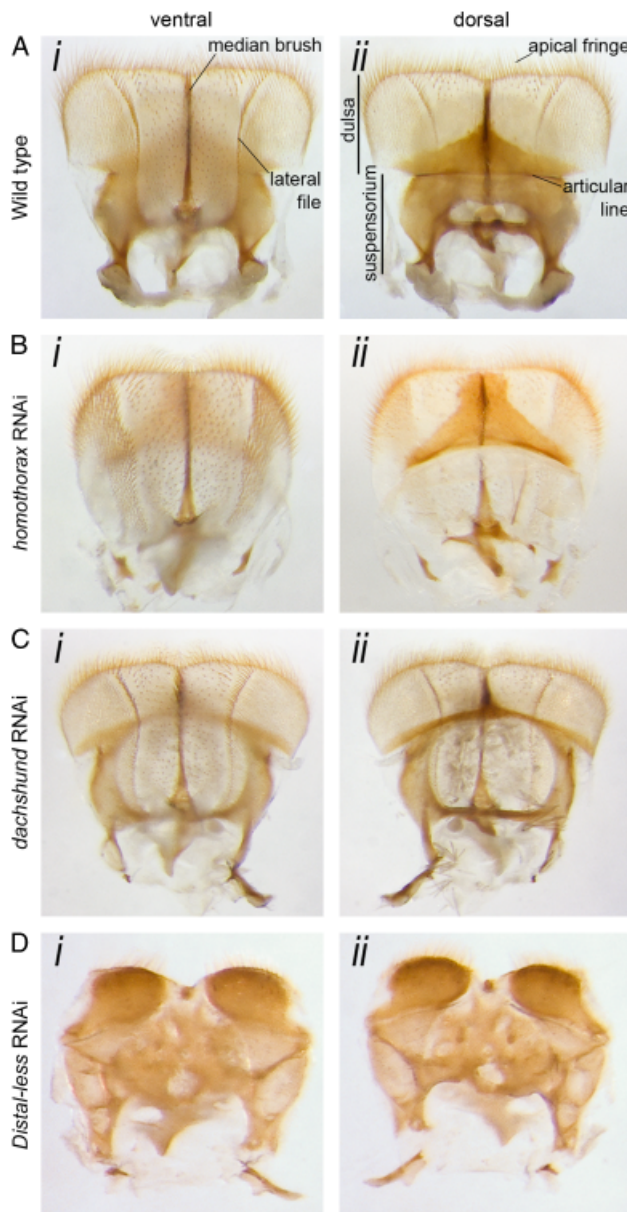


Fig. 6. *Onthophagus taurus* adult labrum. The ventral (i) and dorsal (ii) sides of the labrum are shown. (A) Wild-type labrum. (B) *homothorax* RNAi phenotype. The shape of the labrum changes from trapezoid to oval and is less dorso-ventrally flattened. The lateral files are replaced by wide column of hairs. The suspensorium is almost entirely unsclerotized. (C) *dachshund* RNAi phenotype. The proportion of the different parts of the labrum is changed. The dursa is reduced and the suspensorium extended with a wide opening. (D) *Distal-less* RNAi phenotype. The distal lobes of the labrum are reduced to small sclerotizes lobes lacking hairs and setae. The suspensorium is extensively deformed.

dac RNAi, in contrast, had a major and highly interesting effect on adult mandible formation. Specifically, *dac* RNAi resulted in the loss of the blade-like elongation of the mandible. Instead, the mandible was much reduced in length,

stout, and triangular in shape. This suggests that evolutionary changes in *dac*-patterning may have played an important role in the evolutionary transition from a short, triangular mandible adapted for chewing, similar to the one still found in larval dung beetles, to the elongated, flat, and blade-like mandible of modern filter-feeding scarabaeine adults. It is interesting to speculate that partly similar changes may have mediated the more drastic elongation of mandibles seen in the sister family to the scarabs, the stag beetles (Family Lucanidae). More generally, our data add to a growing number of studies that suggest that the role of *dac* function in mandible development may be rather labile during insect evolution. For instance, there is no evidence for *dac* function, or even expression, during mouthpart development in *Drosophila* (Abzhanov et al. 2001; Joulia et al. 2006). In contrast, *dac* is expressed in the *Tribolium* mandible, but *dac* RNAi does not appear to result in obvious mandible phenotypes (Angelini et al. unpublished data). Lastly, studies on the hemimetabolous milkweed bug *O. fasciatus* suggest that *dac* may only be required for maturation, but not initial elongation, of the stylet-like mandibles typically found in hemipteran insects (Angelini and Kaufman 2004). Future comparative studies into *dac* function, as well as into the conservation or lability of *dac* targets during appendage development, are necessary to further explore this issue.

Conservation and diversification of the labium and maxilla

All three genes studied here affected formation of the maxilla and labium in a manner consistent with a conservation of patterning function along the proximo-distal axis of appendages and largely in line with earlier gene expression and gene function analyses (e.g., Abzhanov and Kaufman 2000; Angelini and Kaufman 2004; reviewed in Prpic et al. 2001; Inoue et al. 2002; Prpic and Tautz 2003; Prpic and Damen 2004; Angelini and Kaufman 2005). However, even when compared with *Tribolium* (Angelini et al. unpublished data), the most closely related species for which gene function data are available, several interesting differences become apparent. For example, *Onthophagus hth* RNAi resulted in the fusion of the proximal parts of the maxilla, the cardo and the stipes. In contrast, in *Tribolium* the cardo and stipes did not fuse and instead were reduced (cardo) and enlarged (stipes) in size, respectively. *Onthophagus dac* RNAi had no effect on the labial palp despite severe effects in other mouthparts, whereas all labial palp joints were lost in *Tribolium*. The maxillary palp showed the same pattern, exhibiting fusion of only two palp segments in *Onthophagus* compared with complete fusion or deletion of all palp segments in *Tribolium*. Because gene function analyses in *Tribolium* (Angelini et al. unpublished data) and *Onthophagus* were both conducted using larval RNAi it appears less likely that gross differences in

methodology could account for these differences, though this cannot be fully discounted. Alternatively, differences in RNAi phenotypes across species may be reflective of evolved divergences in labial and maxillary development between the two taxa.

CONCLUSIONS

Most developmental research on mouthparts has been conducted on embryos. Here, we show that three appendage patterning genes previously implicated during embryogenesis are also involved during postembryonic development. Our results suggest that conservation as well as diversification of their functions may have played important roles in the conservation (labium, maxillae, and labrum) and divergence (mandibles) of mouthpart identities. More generally, our results contribute to a growing body of studies that suggest that basic patterning genes can contribute to morphological evolution of adult features while maintaining traditional patterning responsibilities at earlier developmental stages or in other body regions.

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