The function of appendage patterning genes in mandible development of the sexually dimorphic stag beetle

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ABSTRACT

One of the defining features of the evolutionary success of insects is the morphological diversification of their appendages, especially mouthparts. Although most insects share a common mouthpart ground plan, there is remarkable diversity in the relative size and shapes of these appendages among different insect lineages. One of the most prominent examples of mouthpart modification can be found in the enlargement of mandibles in stag beetles (Coleoptera, Insecta). In order to understand the proximate mechanisms of mouthpart modification, we investigated the function of appendage-patterning genes in mandibular enlargement during extreme growth of the sexually dimorphic mandibles of the stag beetle *Cyclommatus metallifer*. Based on knowledge from *Drosophila* and *Tribolium* studies, we focused on seven appendage patterning genes (*Distal-less* (*Dll*), *aristaless* (*al*), *dachshund* (*dac*), *homothorax* (*hth*), *Epidermal growth factor receptor* (*Egfr*), *escargot* (*esg*), and *Keren* (*Krn*). In order to characterize the developmental function of these genes, we performed functional analyses by using RNA interference (RNAi). Importantly, we found that RNAi knockdown of *dac* resulted in a significant mandible size reduction in males but not in female mandibles. In addition to reducing the size of mandibles, *dac* knockdown also resulted in a loss of the serrate teeth structures on the mandibles of males and females. We found that *al* and *hth* play a significant role during morphogenesis of the large male-specific inner mandibular tooth. On the other hand, knockdown of the distal selector gene *Dll* did not affect mandible development, supporting the hypothesis that mandibles likely do not contain the distal-most region of the ancestral appendage and therefore co-option of *Dll* expression is unlikely to be involved in mandible enlargement in stag beetles. In addition to mandible development, we explored possible roles of these genes in controlling the divergent antennal morphology of Coleoptera.

Keywords; appendage patterning; exaggerated trait; stag beetle; sexual dimorphism; RNAi

INTRODUCTION

Insects are arguably one of the most successful groups of organisms in their evolution, ecology, and overall diversity. One of the defining features of their success is the morphological diversification of appendages, particularly the morphological diversification of mouthparts (Grimaldi & Engel 2005, Simonnet & Moczek 2011). The insect mouthpart ground plan is composed of three paired post-oral appendages known as the labia, maxillae, and mandibles (Fig. 1A, B, Snodgrass 1935, Labandeira 1997, Jockusch et al. 2004, Grimaldi & Engel 2005, Angelini et al. 2012). Although most insects share this ground plan, there is remarkable diversity in homologous mouthpart appendages among the different insect groups. Striking examples include highly modified mouthparts such as the hinged, mask-like labia of dragonfly larvae, the straw-like maxillae of Lepidoptera, and the knife-like mandibles of horseflies (Grimaldi & Engel 2005).

One of the most extreme examples of mouthpart modification can be found in the enlargement of mandibles (Fig. 1C, D). Mandible enlargement has evolved multiple times in insects; for example, in the sickle-like mandibles of many Neuroptera, (Contreras-Ramos 2011), the enlarged biting mandibles of soldiers in Isoptera (Miura 2005), and the spectacularly enlarged mandibles of males of many species of Coleoptera (Lucanidae: Kawano 2000, Hosoya & Araya 2005, Staphylinidae: Hanley 2001, Cerambycidae: Kawano 2006, Eberhard 2009, Meloidae: Yamamoto 2010). In these extreme cases, the mandibles are no longer used for feeding. Instead, they have assumed a novel function as weapons for combat with rival males or for defending their colonies from enemies. Yet, the molecular mechanisms responsible for mandible enlargement (or any other mouthpart enlargement) in insects remain largely unknown (Simonnet & Moczek 2011, Angelini et al. 2012).

Recently, the male-specific mandible enlargement in the sexually dimorphic stag beetle *Cyclommatus metallifer* was found to be controlled by both nutrition-dependent juvenile hormone (JH) effects and through the sex-specific sex determination cascade (Gotoh et al. 2011, 2014, 2016). Males of this species exhibit extremely enlarged mandibles and larger males have disproportionally larger mandibles than smaller males (Fig. 1E). These size differences between males are caused by differential JH titers during the prepupa period, suggesting that it is the critical period for mandible growth, Fig. 1F (Gotoh et al. 2011). Females also have mandibles but they are not modified or enlarged compared to the mandibles of other beetles (Fig. 1E). In females, the female-specific isoform of the sex-determination gene *doublesex* (*dsx*) decreases the mandibular sensitivity to JH, which results in undeveloped small mandibles in females (Gotoh et al. 2014).

Given that the insect mandible is a modified appendage, members of the canonical insect appendage-patterning pathway might be involved in the postembryonic mandibular enlargement alongside JH and sex-specific genes. Here, in order to more fully understand proximate mechanisms of extreme mouthpart growth, we investigated the functions of appendage-patterning genes in developing

mandibles of the stag beetle *Cyclommatus metallifer*. In this species, the male mandible has serrate teeth structures in the most apical region and a pair of inner teeth structures located in the middle to proximal region (Fig. 1E, S1). Serrate teeth appear in all males, but the inner teeth only occur in large males (Fig. 1E, S1). In each of the female mandibles, there are two teeth, which are homologous to the male serrate teeth (Fig. S1). We predicted that genes involved in mandible patterning would also be necessary for developing these teeth structures.

Organization of the Insect Appendage

Insect limbs, including mandibles, are outgrowths from the body wall. Analysis of imaginal disc patterning in *Drosophila* has led to the identification of three main regions of the limb disc, with corresponding suites of genes that regulate each region: proximal (closest to the body wall), medial (the middle of the appendage), and distal (the farthest from the body wall; Kojima 2004, Angelini & Kaufman 2005). The developmental patterning of this proximal-distal axis in insect appendages is highly conserved and regulated by many genes (Kojima 2004, Angelini & Kaufman 2005). Based on previous studies of appendage morphogenesis in *Drosophila* and *Tribolium* (Lecuit & Cohen 1997, Kojima 2004, Angelini et al. 2009, Angelini et al. 2012), we focused on seven of these appendage-patterning genes, *Distal-less* (*Dll*), *aristaless* (*al*), *Epidermal growth factor receptor* (*Egfr*), *Keren* (*Krn*), *dachshund* (*dac*), *homothorax* (*hth*) and *escargot* (*esg*), as likely candidates for the regulation of mandible growth.

Distal Region Patterning

Distal-less (*Dll*)

A homeodomain transcription factor *Dll* plays significant roles in establishing the identity of distal regions in appendages during embryonic and post-embryonic development across the insects (Cohen et al. 1989, Panganiban et al. 1994, Cohen et al. 1989, Popadic et al. 1998, Scholtz et al. 1998, Kojima 2004). However, the insect mandible is thought to have lost expression of the distal portion of the appendage pattering pathway, including *Dll*, resulting in outgrowths that are homologous with the proximal, or basal, regions of other appendages (i.e., insect mandibles are gnathobasal; Popadic et al. 1998, Scholtz et al. 1998, Coulcher & Telford 2012). Most of the recent expression and functional analyses on *Dll*'s role in mandible development collectively suggest that *Dll* does not play a significant role in the mandibular development of insects (Panganiban et al. 1994, Popadic et al. 1998, Beermann et al. 2001, Angelini & Kaufman 2004, Angelini & Kaufman 2005, Simonnet & Moczek 2011, Coulcher & Telford 2013), and our *a priori* assumption was that *Dll* would not be not expressed in stag beetle mandibles. However, it is known that *Dll* has been independently co-opted during the development of novel traits, especially epidermal outgrowths in insects (Panganiban et al. 1994, Moczek & Nagy 2005, Moczek & Rose 2009, Toga et al. 2012), raising the possibility that distal patterning, including *Dll* expression, might have been

restored in the evolution of extreme mandible size in these beetles. Thus, we suggest two hypotheses for the role of *Dll* during extreme mandibular enlargement in stag beetles. The first is that *Dll* is evolutionarily co-opted during postembryonic development of the enlarged mandibles similar to other novel outgrowth traits; whereas the second hypothesis is that *Dll* serves no function during postembryonic mandibular enlargement, as in the mandibles of other studied insects and, instead, other appendage patterning genes have been recruited for mandibular enlargement.

aristaless (*al*)

The homeobox transcription factor *aristaless* (*al*) is known to be an essential gene for the development of distal appendage tips in both *Drosophila* (Campbell & Tomlinson 1998, Kojima et al. 2005) and *Tribolium* (Beermann & Schroder 2004). Similar to *Dll*, *al* expression is found only in the distal tip of leg appendages, and is therefore not expected to play a role in insect mandible development. Although there have been no reports of *al* function in mandible development in any insects, previous work has shown that *al* has been co-opted during the development of horns in *Onthopagus* dung beetles (Moczek & Nagy 2005, Moczek 2006). Thus, *al* is another potential candidate for regulating the extreme growth of male stag beetle mandibles, if the distal portion of the appendage pathway was co-opted in the evolution of extreme mandible size.

***E**pidermal growth factor receptor* (*Egfr*) and *Keren* (*Krn*)

Epidermal growth factor (EGF) signaling is a well-conserved morphogenetic pathway involved in distal appendage development in *Drosophila* (Campbell 2002, Galindo et al. 2002) and *Tribolium* (Angelini et al. 2012). *Keren* (*Krn*) encodes the EGF ligand and *Egfr* encodes the EGF receptor. Moreover, in *Tribolium*, *Krn* is the only gene yet examined whose knockdown affects mandible development (Angelini et al. 2012). Therefore, *Krn* is a strong candidate gene for the mandible patterning and evolution of mandible enlargement.

Medial region patterning

dachshund

A transcriptional regulator *dachshund* (*dac*) is also an important appendage patterning gene which provides positional cues for cells in the developing appendage in a diversity of insect species including *Drosophila*, *Tribolium*, and *Onthophagus* (Mardon et al. 1994, Abzhanov & Kaufman 2000, Prpic et al. 2001, Kojima 2004, Moczek & Rose 2009), as well as crustacean species (Sewell et al. 2008). Mutation or experimental silencing of *dac* leads to the loss or reduction of medial regions in the antenna and leg in *Drosophila*, *Tribolium*, and *Onthophagus* (Mardon et al. 1994, Angelini et al. 2009, Moczek & Rose 2009). Two previous studies have also investigated *dac* function in adult beetle mandible development. Angelini et al. (2012) reported that knockdown of *dac* via RNAi did not affect mandible morphology in

Tribolium, whereas Simmonet & Moczek (2011) showed that RNAi knockdown of *dac* resulted in abnormal mandible formation through a deletion of the medial mandible region in *Onthophagus* (Simmonet & Moczek 2011). These conflicting results point to a potentially divergent role for *dac* in mandible evolution. Thus, *dac* is likely to play a significant role in mandible morphogenesis and enlargement.

Proximal region patterning

homothorax (*hth*) and *esgargot* (*esg*)

hth encodes a homeodomain protein which is necessary for normal development of the proximal region of appendages in *Drosophila* (Kurant et al. 1998, Ryoo & Mann 1999, Wu & Cohen 1999). During embryonic and post-embryonic appendage development, *hth* is expressed proximally in the developing discs and if deleted the resulting phenotypes show defects in these regions of the appendage (Wu & Cohen 1999, Kojima 2004). The proximal selector role of *hth* is also conserved in other insects including *Tribolium* (Angelini et al. 2009, 2012). In mandibles of *Onthophagus*, Simmonet & Moczek (2011) revealed that *hth* expression is necessary for proper formation of the mandible molar. A zinc-finger protein *esg* is co-expressed with *hth* in developing leg discs in *Drosophila* (Hayashi et al. 1993, Kojima 2004). Since mandibles are considered to be a gnathobasic appendage (Fig. 1G), *hth* and *esg* are expected to have critical roles in mandible patterning and enlargement in stag beetles.

We tested for functional roles of each of these, seven candidate genes in the postembryonic development of stag beetle mandibles via RNAi. In addition to mandibles, and to provide a benchmark for comparing gene function between stag beetles and other insects, we also analyzed the effects of knockdown of these same candidate genes on postembryonic development of the legs and antennae.

MATERIALS AND METHODS

Insect Husbandry

Stag beetle adults (*Cyclommatus metallifer*) were purchased from the insect shop Hercules-Hercules, in Sapporo, Japan. Methods for rearing, breeding and inducing large and small males are described in Gotoh et al. 2011 and Gotoh et al. 2014.

C. metallifer *candidate gene cloning*

Cloning of our seven candidate genes was performed by degenerate PCR according to Gotoh et al. 2014. The primer sequences for degenerate PCR are listed in Supplementary Table 1. Database searches for homology were performed using BlastX on the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Identified partial sequences of *Cyclommatus* homologous genes were registered with DDBJ (accession number LC199492 to LC199498).

Knock-down of candidate genes by RNA interference

To silence target gene transcripts, the cloned regions of each gene were used for synthesizing dsRNA using the MEGAscript RNAi Kit (Sigma-Aldrich) following the methods of Gotoh et al. 2014 for dsRNA synthesis. dsRNA against green fluorescent protein (GFP) was also synthesized using GFP pQBI-polII (Wako, Osaka, Japan) as a control. All dsRNA was diluted with 1 X PBS. From 0.1 to 20 μg of dsRNA in five μl of PBS was injected into the dorsal prothorax of late 3rd instar larvae. Efficiency of RNAi was validated via RT-PCR (Fig. S2). Last instar larvae one week after dsRNA injection were dissected. Total RNA was extracted from whole larval heads using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). 600 ng (*al*^{RNAi}, *Dll*^{RNAi}, *egfr*^{RNAi}, *dac*^{RNAi}, *esg*^{RNAi}, *Krn*^{RNAi} and *GFP*^{RNAi}) or 100 ng (*hth*^{RNAi} and *GFP*^{RNAi}) of total RNA were reverse transcribed with SuperScript III (Invitrogen). PCR was performed with AmpliTaq 360 DNA Polymerase (Applied Biosystems) using gene specific primers listed in Supplementary Table 2. We used *GAPDH* (accession number: BAO 23812) as an endogenous reference gene according to our previous study (Gotoh et al. 2014).

Effects of RNAi on mandible length were analyzed separately for each candidate gene using analysis of covariance (ANCOVA), with body size as a covariate, using R 3.0.2 software. The equality of the slopes of regression lines was tested as a first step. If there were no significant differences in regression slope, then the intercepts were compared.

RESULTS

Functional analysis of Dll, al, dac, Egfr, Krn, dac, hth *and* esg *in stag beetle mandibles during development*

In order to investigate the gene functions of seven candidate genes on mandible patterning and mandible enlargement, we performed functional analyses using RNAi in both females and males. Sample sizes and percent penetrance of the RNAi experiments are summarized in Supplementary Table 3. We used *GFP* dsRNA as negative control in our RNAi experiments. None of the *GFP* dsRNA injected individuals showed specific phenotypes in either males or females (Fig. 2A-C).

Distal identity gene functional analysis

Distal-less (*Dll*)

Knockdown of the *Dll* gene by RNAi in males and females induced clear and significant phenotypic effects on stag beetle antennae and legs, as predicted from its function in other insects (Fig. 2D-F). In all Dll^{RNAi} individuals regardless of sex, distal regions of antennae and legs were completely or partially absent (Fig. 2E, F). In contrast to legs and antenna, mandible development was not affected (Fig. 2D-F). The scaling relationship between mandible size against body size was not affected in these Dll^{RNAi} individuals, as they were not different from the control GFP^{RNAi} individuals (ANCOVA, slope: p = 0.10379; intercept: p = 0.3733; Fig. 3). We increased the concentration of dsRNA that we injected (up to 20 µg per larvae), but this also had no effect on the mandibular phenotype (not shown).

aristaless (*al*)

Male stag beetle al^{RNAi} individuals showed defective tarsal claws (Fig. 2G-I). Tarsal claws became slightly shorter and ectopic wart-like projections formed at the base of the claw (Fig. 2I, black arrowhead). In antennae, although pigmentation of the distal club structure was disrupted in some newly eclosed al^{RNAi} individuals (data not shown), overall morphology was not affected (Fig. 2H). Those phenotypes were also seen in eclosed females (data not shown). The scaling relationships of male mandibles against body size did not differ between al^{RNAi} and GFP^{RNAi} individuals (ANCOVA, slope: P = 0.76686; intercept: P = 0.8710). Although al^{RNAi} did not affect mandible length against body size (Fig. 2G, Fig. 3), knockdown of *al* slightly altered the mandible shape. Typically, the mandibles of large males contain two sets of “inner teeth”, but in the eclosed large al^{RNAi} males, the inner set of teeth was missing (Fig. 2G, black asterisk). On the other hand, the serrate teeth were not affected. Also, no mandible defects were detected in female al^{RNAi} individuals.

Epidermal growth factor receptor (*Egfr*) and *Keren* (*Krn*)

Krn^{RNAi} individuals showed mild deformation of the distal antennal and leg regions (Fig. 2J-L). In *Krn*^{RNAi} antennae, the normal distal club did not form and did not fully pigment (Fig. 2K). The *Krn*^{RNAi} leg phenotype exhibited a loss of the hairs in each tarsal segment (Fig. 2L, open arrowheads). Also, the tarsal claw became smaller in *Krn*^{RNAi} individuals (Fig. 2L, pink asterisk). However, *Krn*^{RNAi} individuals did not show severe effects on mandible development, either in size or shape (Fig. 2J, Fig. 3). There were no significant differences in regression slope (Fig. 3, P = 0.74662) or intercept (Fig. 3, P = 0.0694) in mandible scaling relationships against body size between *Krn*^{RNAi} and *GFP*^{RNAi} males.

Egfr knockdown on appendage development could not be analyzed due to lethal effects of *Egfr*^{RNAi}. *Egfr* dsRNA injected animals survived until the prepupal stage, but all of them died before pupation.

Medial identity gene functional analysis

dachshund

Stag beetle male and female *dac*^{RNAi} individuals showed deletion of the medial regions in both antennae and legs (Fig. 4). In antennae, the distal club structures were fully formed as was the first proximal segment, although the first proximal segment was reduced in length. On the other hand, most of the medial antennal segments were fused into two or three segments (Fig. 4A, E). In legs, the distal tarsal claw and proximal coxa and trochanter were not severely affected, while the femur and tibia were fused and a few tarsal segments were lost (Fig. 4A, F). The mandibles of *dac*^{RNAi} individuals were thinner and sharper, and lacked the serrated tooth structures at the distal tips (Fig. 4A, C, asterisks). Interestingly, the inner mandible teeth never formed, even in large males (Fig. 4A, C). The scaling relationship between mandible length and body size was significantly different between *GFP*^{RNAi} and *dac*^{RNAi} males (ANCOVA slope: $P = 0.00232$; Fig. 3). Male *dac*^{RNAi} individuals possessed shorter mandibles compared to controls and this difference became larger as body size increased (Fig. 3, compare Fig.4A and 4B). Female *dac*^{RNAi} individuals exhibited mandibles that completely lacked both of the teeth which are homologous to male serrated teeth (Fig. 4D, arrowhead), but did not show a reduction in mandible length (Fig. 4D).

Proximal identity gene functional analysis

homothorax (*hth*) and *esgargot* (*esg*)

hth^{RNAi} individuals showed several defective phenotypes (Fig. 5). The most obvious effect was the ectopic formation of the forewings on the prothorax (Fig. 5E, asterisk) and the homeotic transformation of antennae to leg-like structures on the head (Fig. 5C). Leg morphology was also affected in *hth*^{RNAi} individuals. The coxa was slightly smaller, and the trochanter was larger, in *hth*^{RNAi} compared with *GFP*^{RNAi} individuals (Fig. 5D). Although size was not largely affected, the surface texture of the femur became tibia-like (Fig. 5D), which suggested that the femur underwent a homeotic transformation to a

tibia. In mandibles, mandible size seemed not to be affected by *hth* knockdown, although the sample size for this experiment is small due to the high mortality of hth^{RNAi} (Table S3). However, the inner mandible teeth disappeared in all of the pupated hth^{RNAi} large males (dotted blue line, Fig. 5A). In contrast to hth^{RNAi} individuals, esg^{RNAi} individuals did not show any detectable defect in any appendages in both sexes (data not shown).

DISCUSSION

Conservation and diversification of mandible patterning

Insect mandibles appear to have lost their distal-most region early in their evolution (Popadic et al. 1998). This is supported by the lack of expression of *Dll* in mandibles of all previously studied insects, including *Tribolium* and dung beetles (Popadic et al. 1998, Scholtz et al. 1998, Simmonet & Moczek 2011, Coulcher & Telford 2013). Our experiments are consistent with this pattern. Knockdown of *Dll* did not affect mandible growth and morphogenesis (Fig. 2). In spite of its widespread recruitment in appendages and other outgrowth structures among insects, our results suggest *Dll* does not play a significant role in growth and patterning of stag beetle mandibles.

On the other hand, *al*, which is also a distally-active appendage patterning gene, did affect mandible morphology. *al*^{RNAi} males did not form the pair of inner mandibular teeth (Fig. 2). Although expression of *al* in mandibles during embryogenesis has been reported in the cricket *Gryllus bimaculatus* (Miyawaki et al. 2002) and *Tribolium* (Beermann & Scholtz 2004), this is the first result showing *al* function during mandible development through direct functional analysis.

In *Drosophila* leg formation, *Dll* expression is required for *al* expression (Campbell & Tomlinson 1998, Kojima 2004), but in *Cyclommatus*, considering that *Dll* knockdown did not affect the formation of the inner teeth, *al* expression might be regulated by other factors. One possible factor regulating or working together with *al* for inner tooth formation is *hth*. *hth*^{RNAi} also affected inner tooth formation (Fig. 5). The structure of mandibular teeth varies widely in size, shape, number, and position within stag beetles. The role of *al* and *hth* in tooth growth we observe here suggests that these genes may be involved in the evolutionary diversification of inner tooth expression across stag beetles. Further studies, such as description of localization and determination of their regulatory hierarchy, as well as the functional analysis in different Lucanidae species, will be needed to examine their potential roles in mandible shape diversification.

dac^{RNAi} had the largest impact on mandible development of the seven genes analyzed in this study (Fig. 4). In contrast to *al*^{RNAi} and *hth*^{RNAi}, *dac*^{RNAi} affected mandible morphology not only in males but also in females (Fig. 4). Our results suggest that *dac* plays a significant role in mandible pattering, including forming serrate teeth in both of males and females, and forming inner teeth in large males. Severe effects of *dac*^{RNAi} on mandible size in males suggest that *dac* expression is required for mandible enlargement, as well as patterning. Since *dac* has no function during development of the ancestral, non-modified, mandible in *Tribolium* (Angelini et al. 2012), evolutionary gain of *dac* function in mandibles might contribute to the evolution of diverse shapes of mandibles, such as thin, layer-like mandibles in dung beetles (Simmonet & Moczek 2011) and enlarged mandibles in stag beetles (this study). Although it is clear that *dac* has an important role in the stag-beetle mandible development, additional genes and interactions are likely to be important as well, since the *dac* knockdown did not completely reduce male mandibles to the size of female mandibles.

Potential interaction of patterning genes with JH signaling and the sex-determination cascade.

We previously reported that JH promotes mandible enlargement, and that larger males have higher JH titers than smaller males during the prepupal period (Gotoh et al. 2011). The effects of *dac* knockdown on mandible size exhibited a significant interaction with body size (Fig. 3). Also, *al* and *hth* only affected inner tooth formation in large males (Fig. 4-5). These size-specific functions of *dac*, *al* and *hth* raise the possibility that these genes work together with, or regulate their function through, a body-size dependent factor. We suggest JH is the most likely candidate for such a body-size dependent factor.

For sexual dimorphism of mandible size, functional analysis for six of the examined genes (*Dll, hth, al, Egfr, esg, Krn)* did not reveal any function in the female mandible development. Further functional and expression gene screening is necessary to understand the developmental mechanisms used in differentiating male and female mandibles.

Evolutionary conservation of leg patterning pathways among insects

Our functional analyses via RNAi also gave insight to distal-proximal patterning of stag beetle legs (Fig. 6). Most of the observed RNAi knockdown phenotypes were consistent with the patterning functions of each gene reported in other previously studied insects such as *Drosophila* (Kojima 2004), *Tribolium* (Angelini et al. 2012), *Onthophagus* (Moczek and Rose 2009) and *Oncopeltus* (Angelini and Kaufman 2004). That is, the conservation of the patterning roles of the limb-gap-genes (Angelini et al. 2012) *Dll*, *dac*, and *hth* for distal, medial, and proximal identity, respectively, the distal selector roles of EGF signaling and the claw development role of *al*, were all supported by RNAi phenotypes observed in *Cyclommatus* (Fig. 2, 4, 5). The consistency of these results with previous studies provides validation for the RNAi method in *Cyclommatus*. Specifically, RNAi phenotypes of most of these genes in *Cyclommatus* showed high similarity with those found in *Tribolium*. For example, hth^{RNAi} in *Drosophila* reduced the proximal segment number of the leg (Casares and Mann 2001), while hth^{RNAi} in *Tribolium* affected the size and shape of proximal segment without changing the segment number (Angelini et al. 2012). In *Cyclommatus*, hth^{RNAi} affected the size and shape of coxa and trochanter without affecting segment number (Fig. 5), just as in *Tribolium*.

One minor difference in RNAi phenotypes of legs between *Tribolium* and *Cyclommatus* was found in *Krn*. In *Tribolium*, *Krn* knockdown induced the loss of the pretarsus and/or reduced tarsal segments (Angelini et al. 2012). In *Cyclommatus*, *Krn* knockdown caused the abnormal development of tarsal structures (loss of tarsal hairs, incomplete pigmentation and small tarsal craw), but segment number and their identity was not affected (Fig. 2). Overall, these similarities of RNAi phenotype in most candidate genes between *Tribolium* and *Cyclommatus* suggest that the leg patterning mechanism is highly conserved across the Coleoptera.

In this study, we were unable to detect any morphological defects in esg^{RNAi} in *Cyclommatus*. This result suggested that *esg* function in appendage formation in *Cyclommatus* is different from that of *Drosophila*. In contrast to other appendage patterning genes such as *Dll*, *dac* and *hth*, functional analyses of *esg* function in non-Drosophilan insects is lacking. Further comparative studies are needed to reveal

any the functional conservation of the *esg* gene in appendage formation among insects.

Possible role of patterning genes in the morphological diversification of antennae

In contrast to the legs, which in general are similar, the antennal morphologies of *Cyclommatus* and *Tribolium* are very different (Fig. 6). *Tribolium* has a capitate antenna with 11 segments (Angelini et al. 2009). The size of the scape (first segment) and pedicel (second segment) are similar to the flagellum segments (3rd to 11th segment) (Angelini et al. 2009). In contrast, *Cyclommatus* (and most other Lucanidae species as well (Ratcliffe 1991)) has a geniculate antenna with a long scape (proximal first segment) and relatively short pedicel/flagellum segments (2nd to 10th segments). In both species, three distal segments (9-11th in *Tribolium*, 8-10th in *Cyclommatus*) form a club structure (Angelini et al. 2009, Fig. 2, 6). As in the legs, putative gene functions suggested from RNAi phenotypes were generally conserved between the two species. That is, *Dll*, EGF signaling, and *al*, are required for proper development of distal structures, and *dac* is required for medial region development (Fig. 2, 4, 6, Angelini et al. 2009).

However, the specific details of developmental function in these patterning genes were different between the two species. In *Cyclommatus*, the most severe effect on antenna was observed in hth^{RNAi}- that is, distal antennal segments transformed to leg-like structures (Fig. 5). The middle region of the flagellum (2-5th segmensts) became larger and gained bristles similar to tarsae (Fig. 5C). In *Drosophila*, *hth* function is required for antennal identity by activating *spineless* (*ss*). Loss of *hth* results in homeotic transformation of antennae to an indistinguishable leg-like appendage with tarsal structure (Casares and Mann 2001). In this respect, *hth* function in *Cyclommatus* appears to be more similar to *Drosophila* than to *Tribolium* (Angelini et al. 2009).

Other differences in the effects of gene knockdown between *Tribolium* and *Cyclommatus* were observed in dac^{RNAi} and Krn^{RNAi}. In both species, dac^{RNAi} deletes the medial region of antenna segments (Fig. 4E, Angelini et al. 2009). However, in *Tribolium*, dac^{RNAi} also caused homeotic transformation of funicle segments to larger, club-like segments with club-specific bristle patterns (Angelini et al. 2009). Also, in *Tribolium*, dac^{RNAi} did not affect the first and second segment morphology (Angelini et al. 2009). In contrast, in *Cyclommatus*, dac^{RNAi} did not transform funicle segments to club segments, but the size of the first proximal segment was reduced (Fig. 4E), suggesting that the region regulated by *dac* might have expanded proximally in *Cyclommatus* relative to *Tribolium*. In both species, Krn^{RNAi} affected the distal structures of antennae, but the details of these effects were different (Fig. 2, Angelini et al. 2009). In *Tribolium*, Krn^{RNAi} resulted in reduction of flagellum length, often accompanied with reductions of segment number (Angelini et al. 2009). In contrast, Krn^{RNAi} in *Cyclommatus* did not reduce the number of antennal segments (Fig. 2K). However, the distal three segments lost their club structure identity and transformed to smaller, funicle-like segments (Fig. 2K).

These data suggest that, although they are generally conserved between the two species, some parts of the appendage patterning gene function have diversified, perhaps contributing to species-specific differences in antennal morphology. Expansion of the region regulated by *dac* in *Cyclommatus* may be related to scape elongation. Further analyses on *dac* transcript localization by *in situ* hybridization are

necessary to test whether the *dac* expression region has truly expanded to cover the scape.

Conclusion

This is the first study to investigate the role of appendage patterning genes in insects with sexually-dimorphic enlarged mandibles. Our results suggest that male stag beetle mandibles incorporate *dac* regulation for enlargement, and are patterned through new functions of *al* and *hth* to generate the morph-specific inner mandible teeth. Sex and morph specific functions of *dac*, *hth* and *al* suggest that these patterning genes might cooperate with JH signaling and the sex-determination cascade to generate diverse shapes within species (Fig. 7). Although gene expression dynamics and localization in developing mandibles should be addressed in future studies, the results of this study provide important new developmental data about the function of conserved developmental patterning genes in the evolution of mouthpart modification and exaggerated traits.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: HG, TM. Performed the experiments: HG. Analyzed the data: HG, RAZ, HM, AI, YI, YS, LCL, DJE, TM. Contributed reagents/materials/analysis tools: HG, RAZ, HM, AI, YI, YS. Wrote the paper: HG, RAZ, HM, AI, YI, YS, LCL, DJE, TM.

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FIGURES and FIGURE LEGENDS

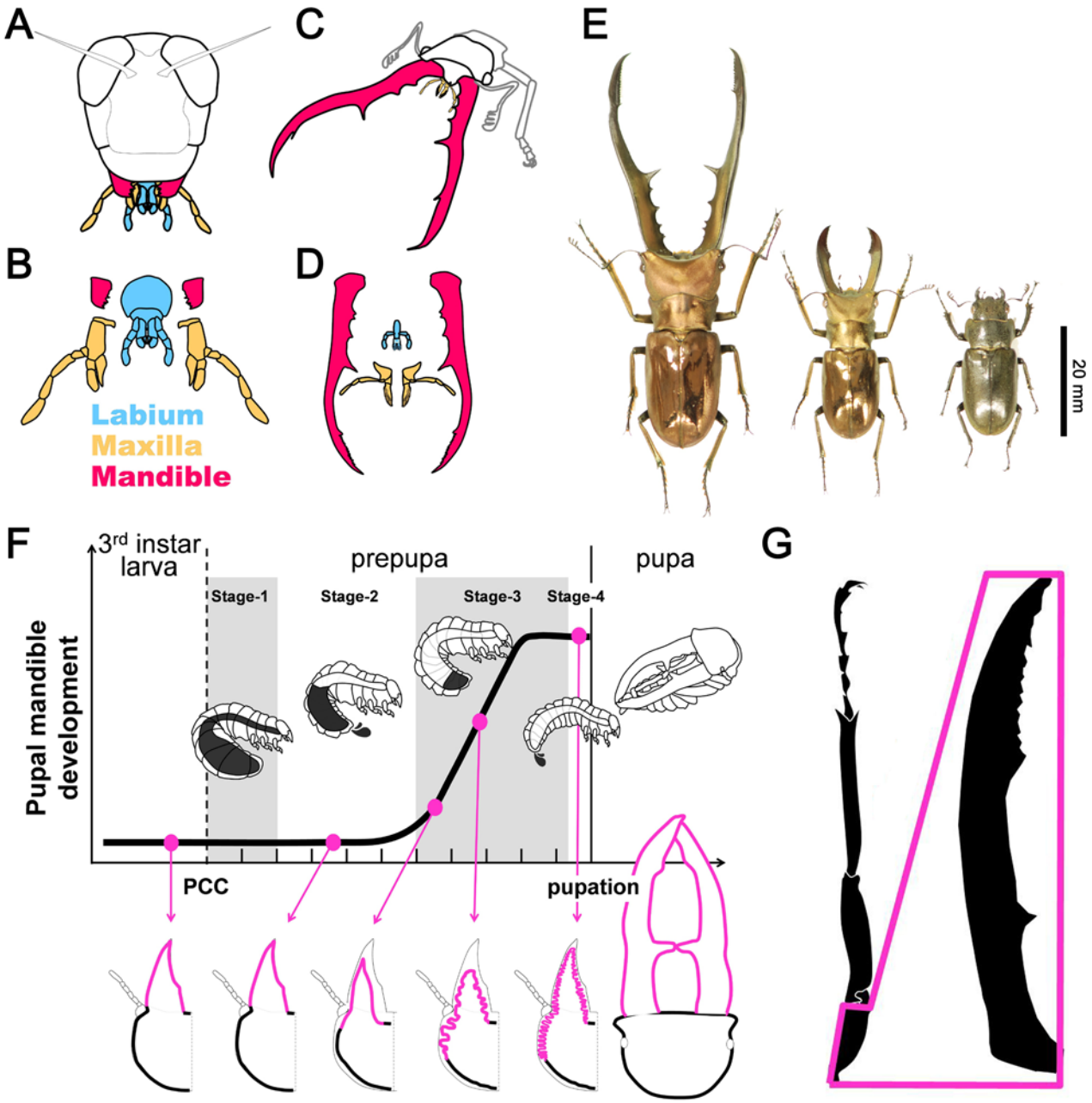

Figure 1. Morphology of insect mouthparts, material species and its developmental schedule.
(A, B) Mouthparts of a locust which has mouthparts considered to be the ancestral mandibulate state for insects. (Re-drawn from Grimaldi & Engel 2005, p122) (C, D) Mouthparts of adult male stag beetle whose mandibles are extremely elongated to combat with other males over resources. (E) The focal species of this study, *Cyclommatus metallifer* exhibits sexual dimorphism and male continuous polymorphism in mandible size. Left: Large male, Middle: small male, Right: Female. Scale bar indicates 20 mm. (F) Developmental staging chart of prepupal period modified from Gotoh et al. 2014. Y-axis indicates mandible growth along with developmental time in days on the x-axis. (G) Schematic view of homology between leg and mandible.

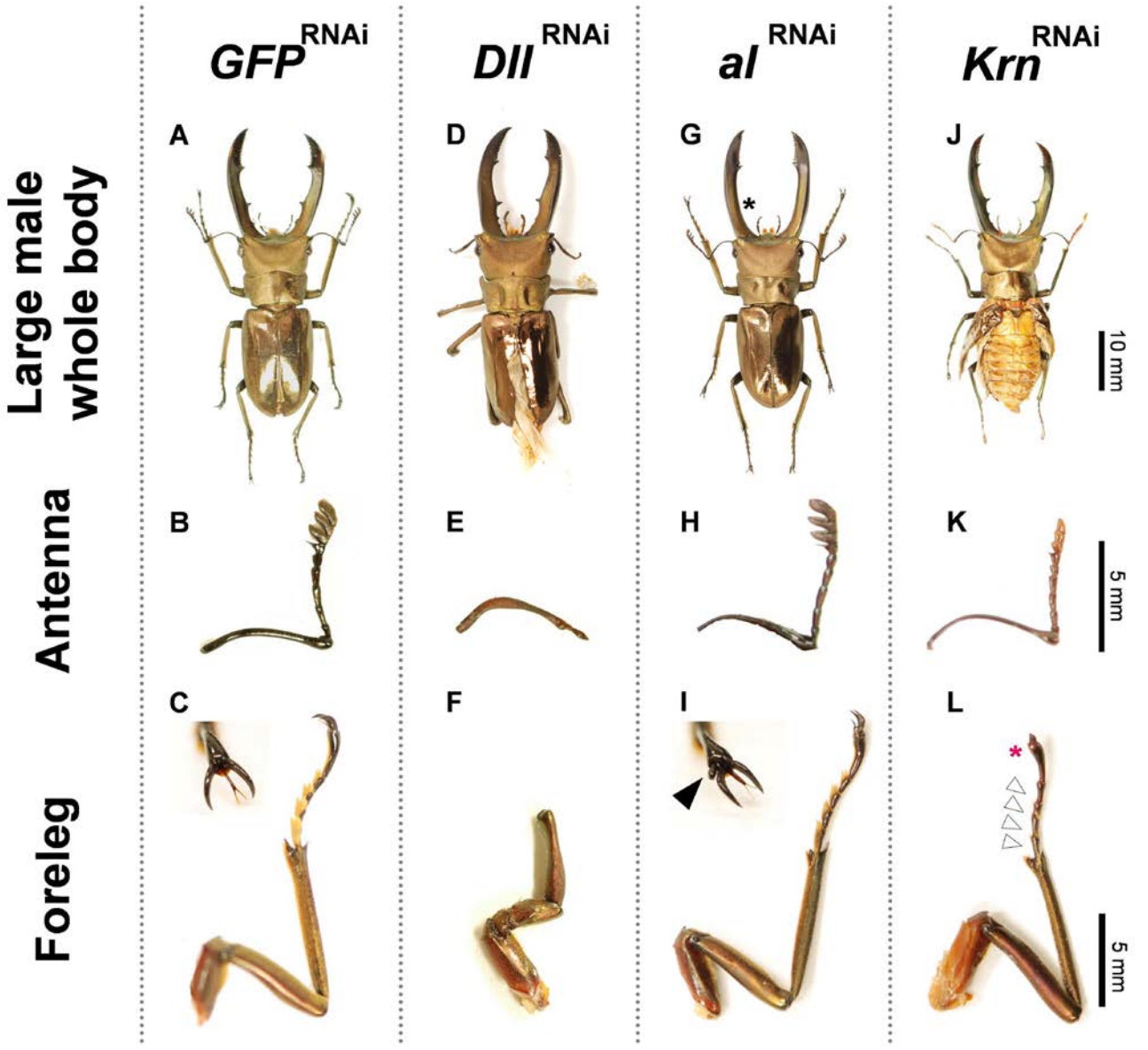

Figure 2. Phenotypes of *Dll*, *al* and *Krn* RNAi individuals.

(A-C) The adult male phenotype of the negative control *GFP*^{RNAi} individuals. (D-F) The adult male phenotype of *Dll*^{RNAi} individuals. (G-I) The adult male phenotype of *al*^{RNAi} individuals. The inner teeth of the mandibles were lost in the *al*^{RNAi} individuals (black asterisk in G). Ectopic wart-like projections were formed in the tarsal claw (black arrowhead in I). (J-L) Adult male phenotype of *Krn*^{RNAi}. Deformation of the tarsal claw is indicated by the pink asterisk. A loss of the golden-colored hairs in the tarsae compared to controls is indicated by the open arrowheads. The insets in C and I show the tarsal claw phenotype of *GFP*^{RNAi} and *al*^{RNAi}, respectively. Images illustrate typical phenotypes observed in RNAi individuals compared with *GFP*^{RNAi} phenotypes.

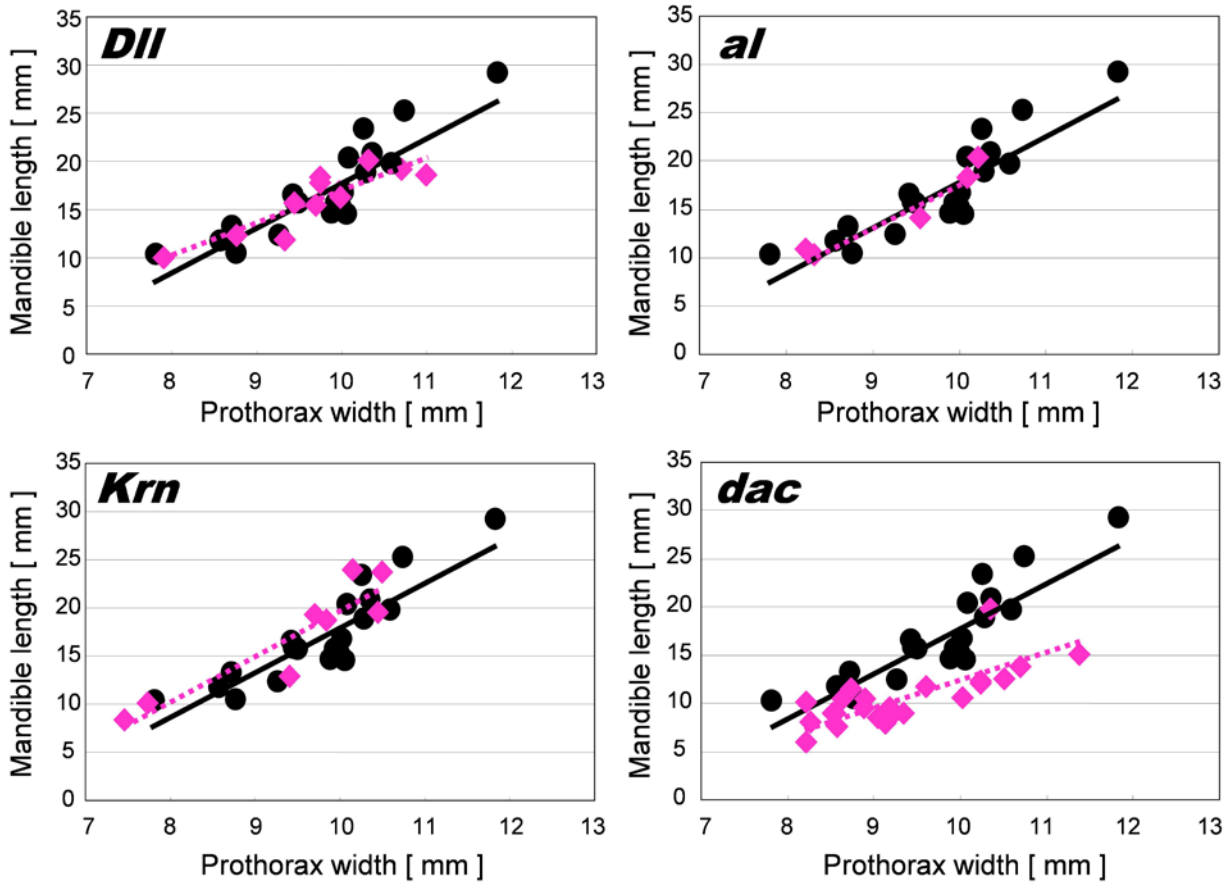

Figure 3. The relationship between prothorax width (X-axis) and mandible length (Y-axis) for *GFP*^{RNAi} individuals (black circles) and *Dll*, *al*, *Krn* and *dac* RNAi individuals (pink diamonds). The only significant difference in scaling relationships of these four target genes against the *GFP*^{RNAi} individuals was detected in the *dac*^{RNAi} treated individuals.

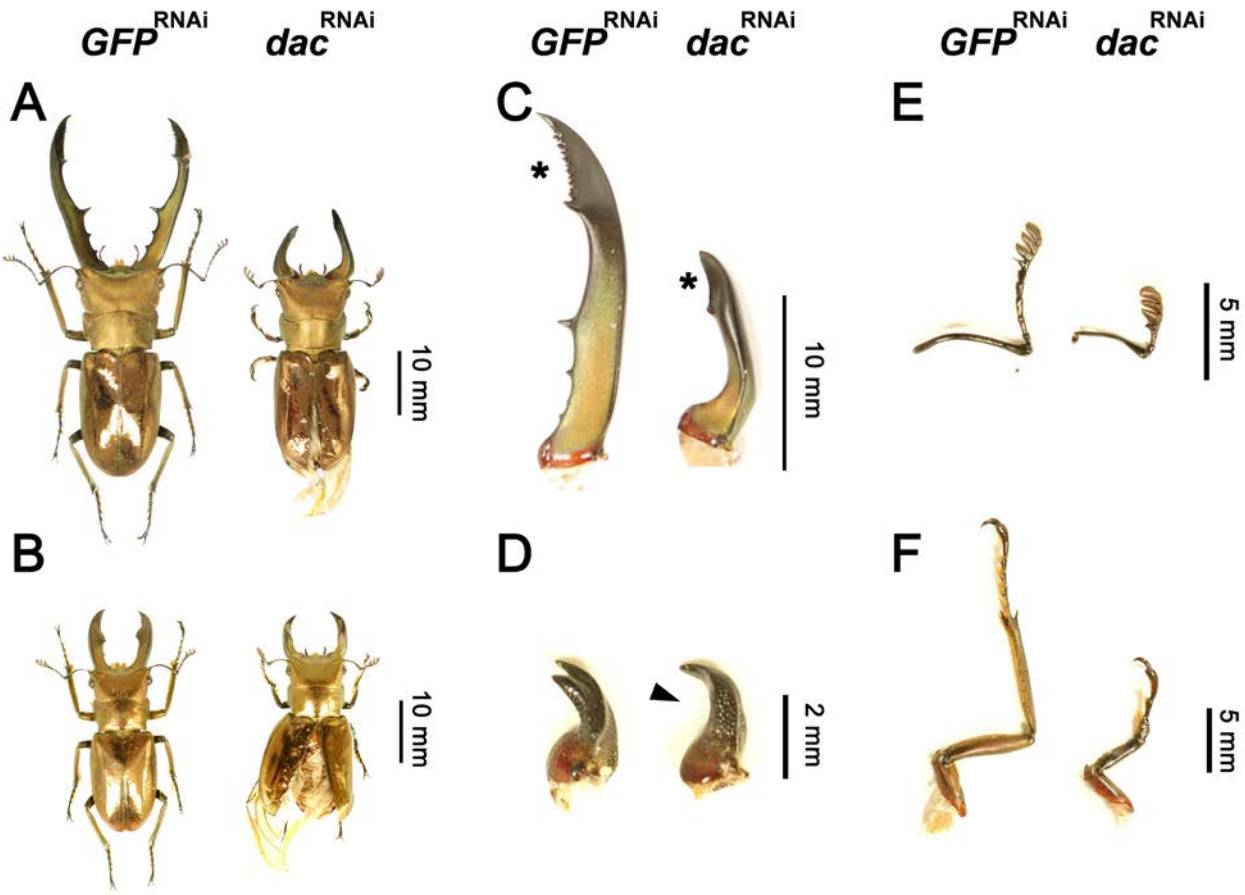

Figure 4. Phenotypes of *dac* RNAi individuals.

(A) Comparison of the large adult male phenotype of a *dac*^{RNAi} and a *GFP*^{RNAi} phenotype. (B) Comparison of the small adult male phenotype of a *dac*^{RNAi} and a *GFP*^{RNAi} phenotype. (C-F) Phenotypes of individual body parts for *GFP*^{RNAi} individuals (left) and *dac*^{RNAi} individual (right). (C) Large male mandibles. Asterisks indicate the difference of serrated teeth-like structures at the distal tips between treatments. (D) Female mandibles. The black arrowhead indicates the loss of inner teeth in *dac*^{RNAi} individual. (E) Antenna. Medial region was lost by *dac* RNAi (F) Leg. Medial region was lost by *dac* RNAi. The images shown illustrate the typical phenotypes observed in RNAi individuals compared with wild-type phenotypes.

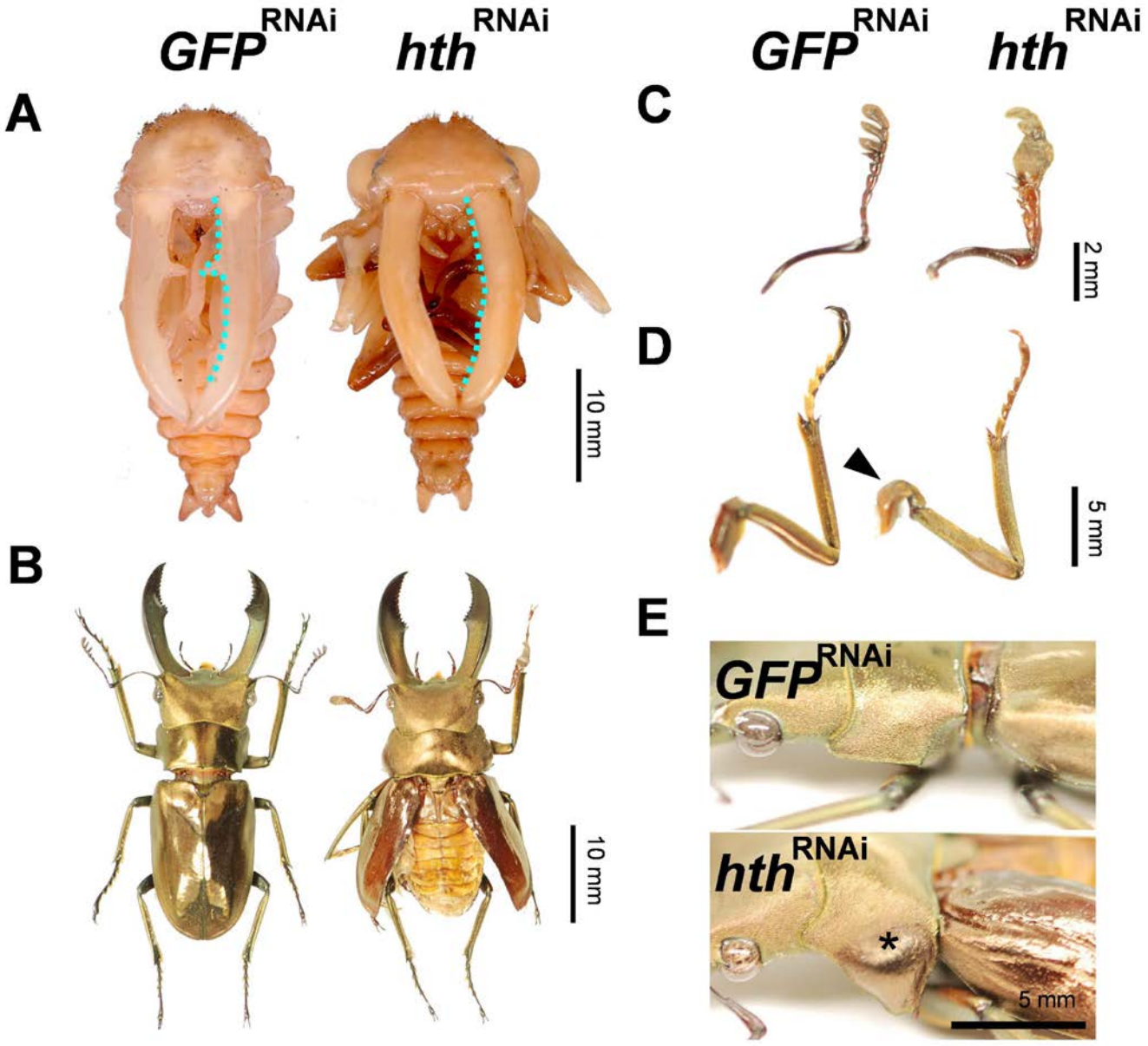

Figure 5. Phenotypes of *hth* RNAi individuals.
(A, B) Comparison of the pupal (A) and adult (B) male phenotype of a *GFP*^{RNAi} and *hth*^{RNAi} phenotype. (C-D) Antennal and leg phenotypes in *GFP*^{RNAi} (left) and *hth*^{RNAi} individual (right). An arrowhead in D indicates shortened coxa in *hth*^{RNAi} individual. (E) Ectopic elytra formation in *hth*^{RNAi} individual. The lateral part of the prothorax was transformed to an elytra-like structure which is indicated by the asterisk.

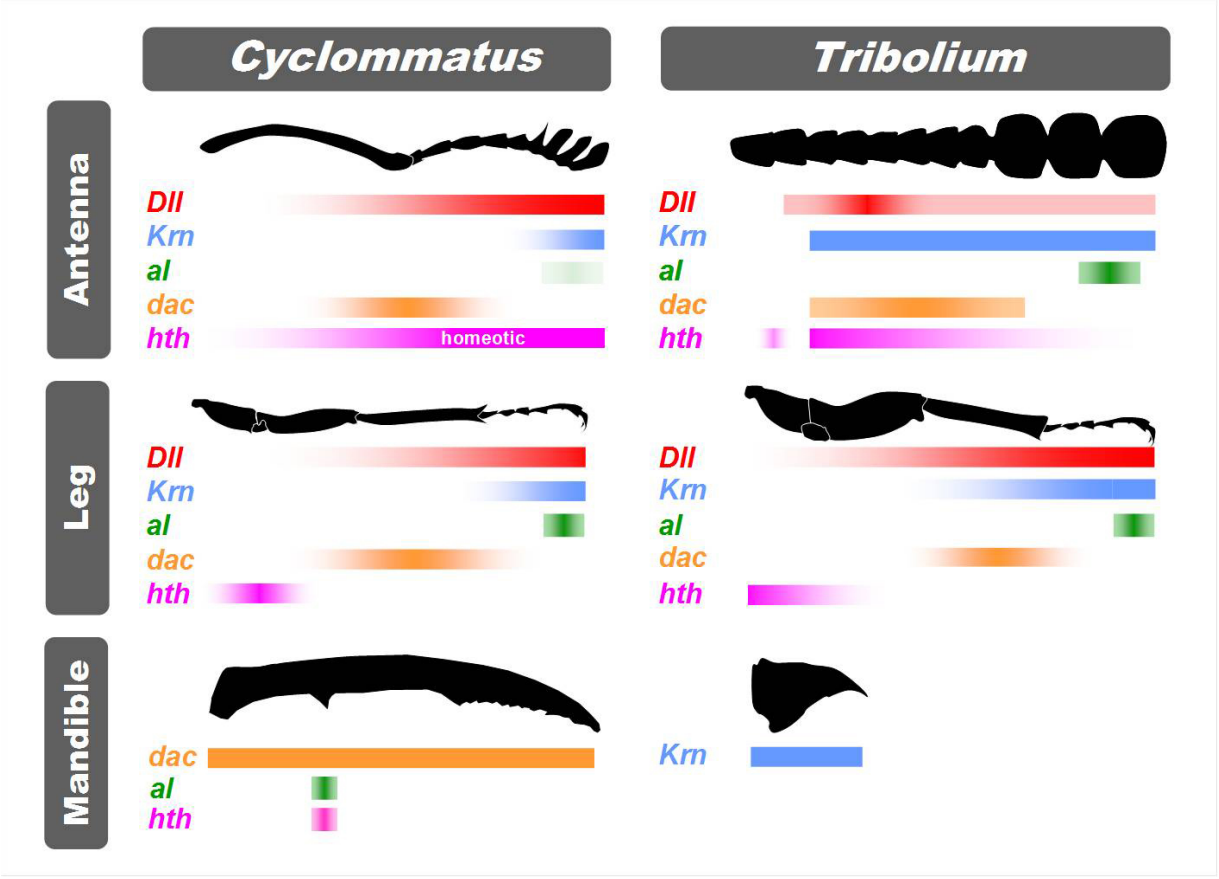

Figure 6. Summary of the regions of appendages (antenna, leg and mandible) affected by RNAi of specific genes in *Cyclommatus* (left) and *Tribolium* (right).

The colored bars indicate the affected (deformed or lost) regions as a result of gene knockdown. The *Tribolium* diagrams were drawn according to the results of Beerman & Schröder (2004), Angelini et al. (2009, 2012a and 2012b).

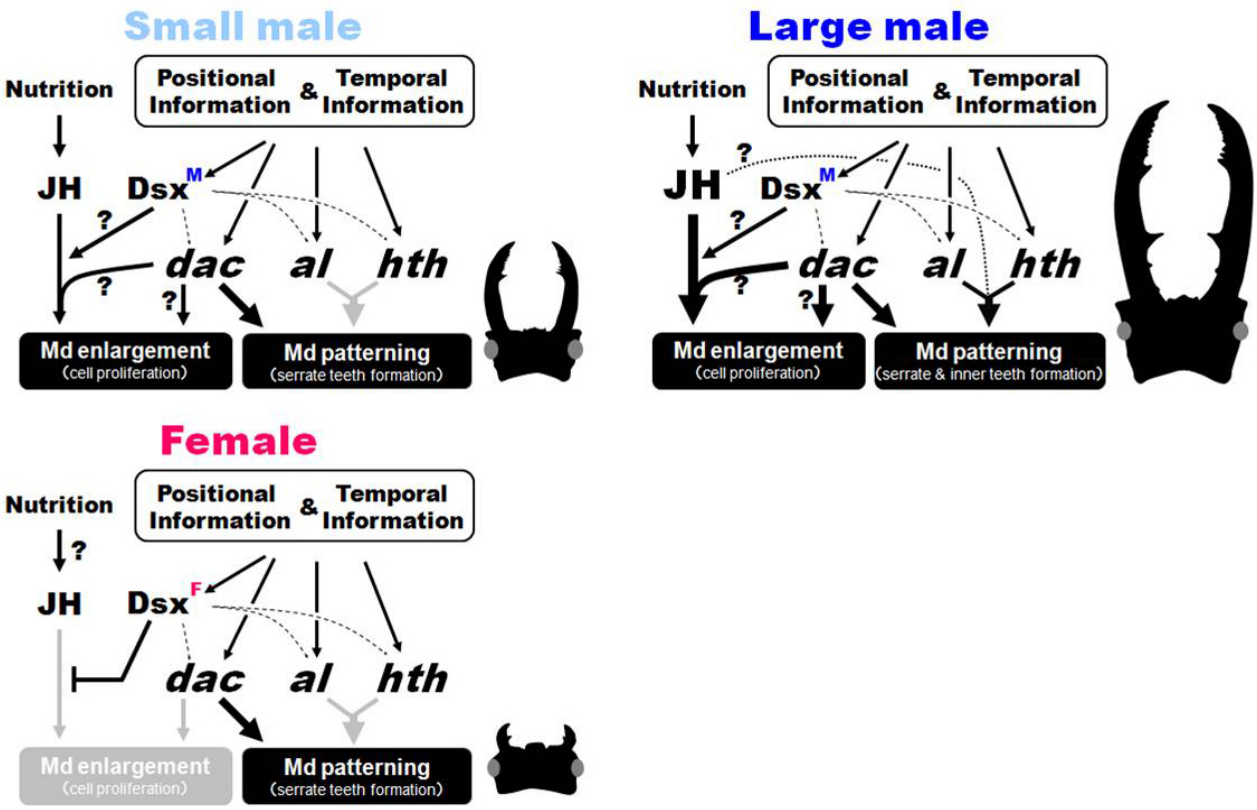

Figure 7. Schematic view of developmental cascade for sex-specific and condition-dependent mandible growth.

DsxM is the male-type Dsx isoform and DsxF indicates the female-type Dsx isoform (Gotoh et al. 2014). Our results suggest that *dac*, *al* and *hth* are expected to be under the control of the *dsx* sex-determination cascade. In addition, *al* and *hth* function in large male inner tooth formation is likely to be under the control of JH signaling. Putative positional information regulating *dsx* and appendage patterning genes might include hox and its related genes for mandible identity. Putative temporal information includes molting hormone ecdysone and its downstream signaling genes.

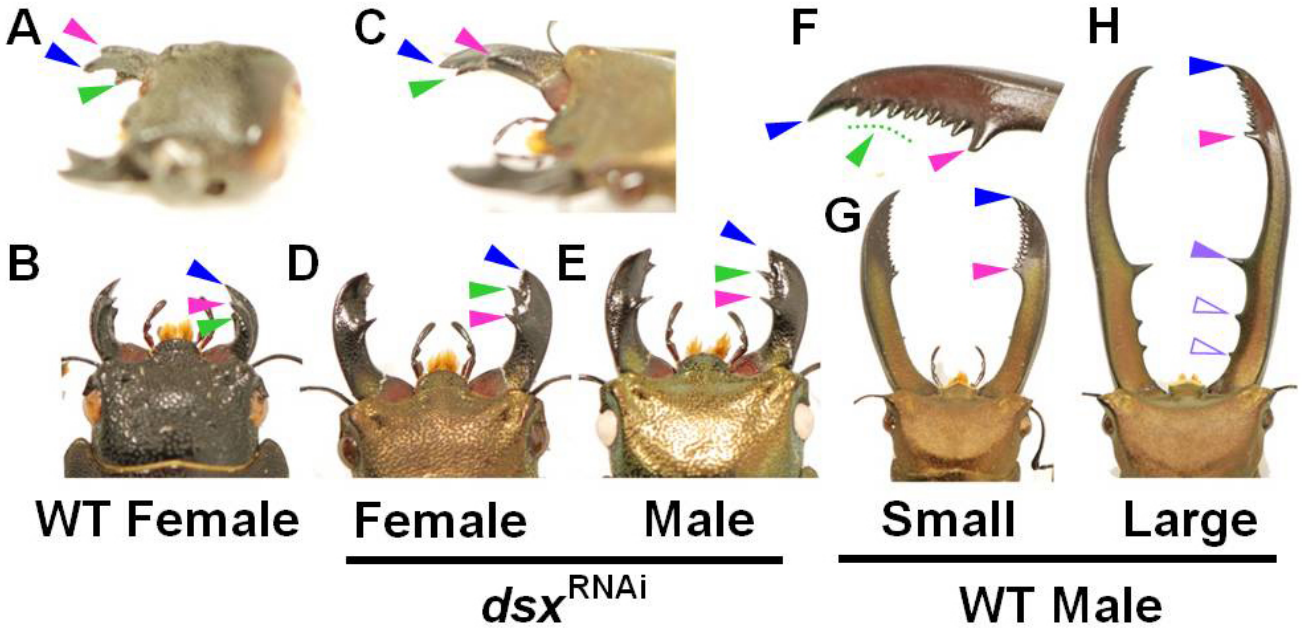

Figure S1. Homology of mandible teeth between males and females

By observing the teeth structures in *dsx*^{RNAi} individuals expressing intersex phenotypes (from Gotoh et al. 2014, 2016), we can determine the homology of these structures between sexes. Predicted homologous teeth are indicated by arrowheads with same color. (A, B) In female mandibles, in addition to the mandible apex (blue arrowhead), two teeth (pink and green arrowheads) are recognized in mandible. (C, D, E) In *dsx*^{RNAi} females and males, these teeth structures become intermediate between wild-type female and wild-type males. We can recognize two teeth (pink and green arrowheads) which are likely to be homologous to female two teeth. (F) Serrate teeth structure in male mandibles. The mandible apex (blue arrowhead) is homologous to the mandible apex in females. According to its position, one of the serrate teeth (green dotted line and green arrowhead in F) might be homologous to one of the female teeth (green arrowhead in A and B). The most proximal tooth of the serrate teeth in males (pink arrowhead in F) might be homologous to a different tooth in female (pink arrowhead in A and B). (G, H) In addition to the apical serrate tooth structure, a pair of big teeth, which we refer to as “inner teeth”, appears in the middle of the mandible only in large males (H, purple closed arrowhead) but not in small males. Larger males also have smaller inner teeth (open purple arrowhead) between these major inner teeth and the base of mandibles.

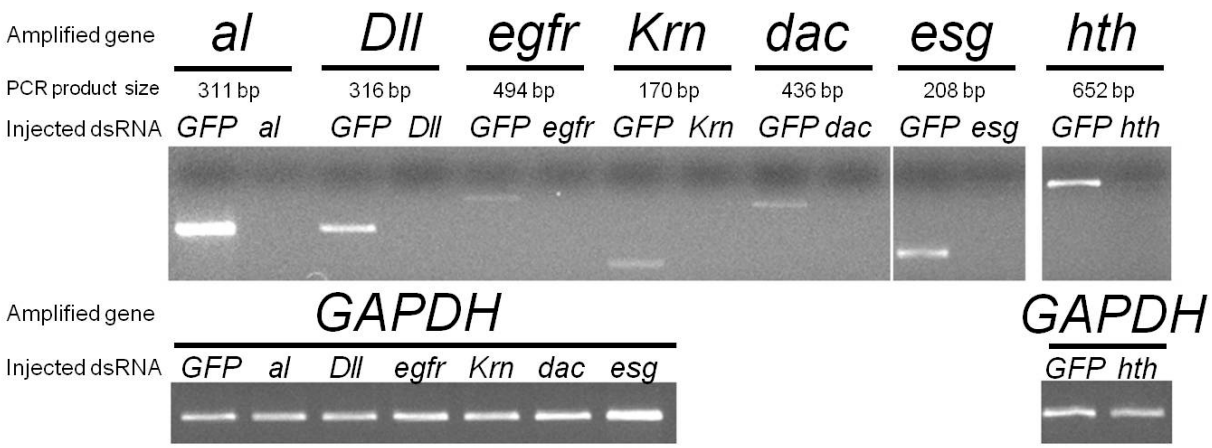

Figure S2. Validation of RNAi knockdown effect via RT-PCR.

RNAi efficiency was validated with RT-PCR. Each target gene was amplified from cDNA template derived from target gene dsRNA injected larvae or *GFP* dsRNA injected larvae. Only for *hth*, 100 ng of total RNA was used for cDNA synthesis while 600 ng was used for other six genes. In all seven genes analyzed, dsRNA injection sufficiently decreased the target gene expression level.

Supplementary Tables

Table S1: List of degenerate primer sequences for gene cloning

Gene	Forward primer 5' - 3'	Reverse primer 5' - 3'
Distal-less (Dll)	CGCCTTCRTVGAGYTRCAGCARC	GSGCSGCCTTCATCATCTTRTTG
aritaless (al)	GACGAGTTYGCSCCCAAGAGGAARCAG	GGYCTYTGVGCYGCCGAAATGTT
Epidermal growth factor receptor (Egfr)	ACATCCGYGARGTRACSGGNTACGT	ACRCACGTBGCRCCRTAMGCRTACTT
Keren (Krn)	TAYGCSGMSTGGTACTGCCTYAACG	CGTSRCBCCVCCSGCDATACTSGCC
dachshund (dac)	TACAACAGCCCRCCGCCGAT	GACACRTTCARCACGGGRCTGTG
homothorax (hth)	TTYTCCAAACAGATMCGWCAAGAGAARCC	TGRTCGATCATAGGTTGRACTATTCTCCG
escargot (esg)	CCGGSCTNTCCAAGCAYCRDCAGTT	CGARTACTTCTTSACGTCGSWGTGYGTCTG

Table S2: List of primer sequences for knockdown validation via RT-PCR

Gene	Forward primer 5' - 3'	Reverse primer 5' - 3'
Distal-less (Dll)	CACCACTTTAATTCGCCGGCGGGAAATGC	GGGCCAAGTATTGCGTCCTCTG
aristaless (al)	CTCCAGGACACACTATCCGGACG	GGGGTGGCGTCGGGAGGTACCCCGG
Epidermal growth factor receptor (Egfr)	GCAGATCATCCGTGGAAGGACGTTGTTC	CCTGCATACTAGGGCAGTCTGCTGTAC
Keren (Krn)	CACGTATGAATGCCCTCCAGCATATGCTG	GTTGAGACGGCAAATATGATCTGTC
dachshund (dac)	CCGGGTAGACCCCCGAAAAGAGCACCCG	CTAACGGTATCGGCCTGTCGGGGTCACCC
homothorax (hth)	GCAGACCCAGAAGTAGATTCGTTAATGG	GATGTTGAAAGAGCCACGCTCTGAG
escargot (esg)	CTGCGACAAAGTCTACGTCTCCC	GAGGTGCGCCCTCAAGTTCGAGC
GAPDH	GGGCGCCAAAAGGGTTATT	GCCTTGGCAGCACCGGTTGATGCGGG

Table S3: Results from larval RNA interference

Gene	Size (bp)	Injected amount (µg)	Males				Females			
			Injected	Successfully pupated	Survive to eclose	Phenotype penetrance (%)	Injected	Successfully pupated	Survive to eclose	Phenotype penetrance (%)
*GFP**	720	1	23	20	20	0	13	13	13	0
Distal-less (Dll)	452	20	2	1	1	100	-	-	-	-
		5	5	3	2	100	-	-	-	-
		1	20	11	9	100	11	9	9	100
aristaless (al)	451	1	8	0	0	-	3	0	0	-
		0.5	15	5	5	80	10	3	3	100
Epidermal growth factor receptor (Egfr)	434	1	5	0	0	-	3	0	0	-
Keren (Krn)	142	1	6	1	1	100	5	1	1	100
		0.5	11	7	7	100	3	2	2	100
dachshund (dac)	487	1	31	25	23	100	10	8	7	100
homothorax (hth)	760	1	10	0	0	-	3	0	0	-
		0.1	17	3	1	100	6	1	0	-
escargot (esg)	256	1	3	3	3	0	2	2	2	0

* Include samples from Gotoh et al. 2014