



## Original research article

# 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.

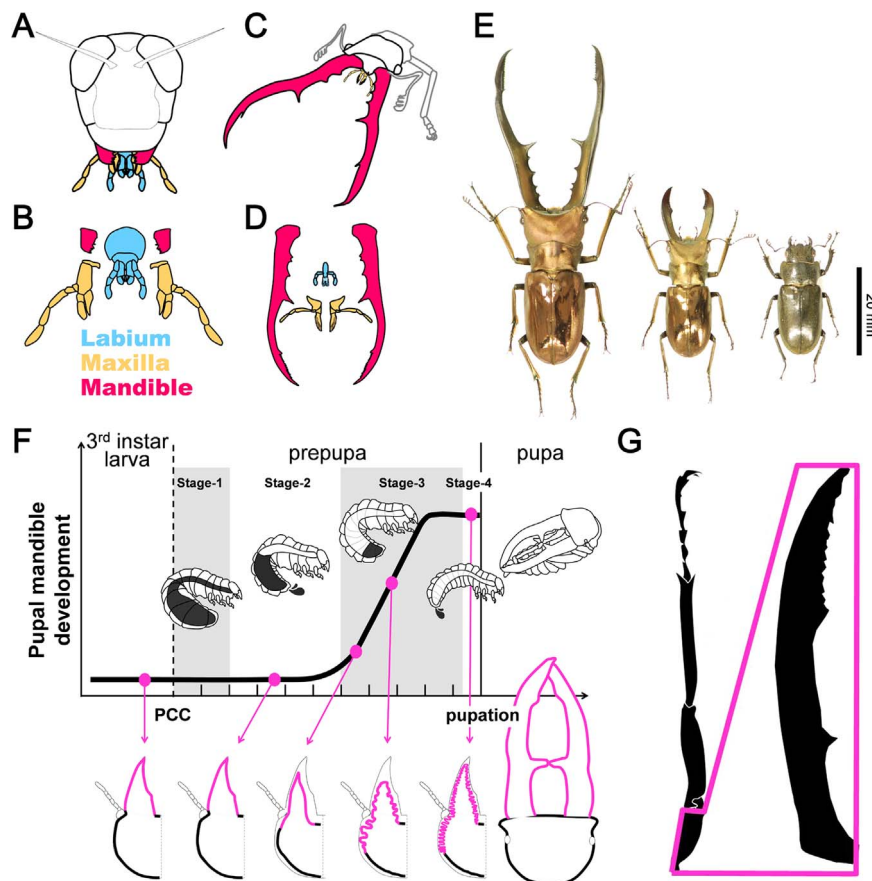
## 1. Introduction

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 and Engel, 2005; Simonnet and Moczek, 2011). The insect mouthpart ground plan is composed of three paired post-oral appendages known as the labia, maxillae, and mandibles (Fig. 1A and B, Snodgrass, 1935; Labandeira, 1997; Jockusch et al., 2004; Grimaldi

and Engel, 2005; Angelini et al. 2012a). 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 and Engel, 2005).

One of the most extreme examples of mouthpart modification can be found in the enlargement of mandibles (Fig. 1C and D). Mandible enlargement has evolved multiple times in insects; for example, in the sickle-like mandibles of many Neuroptera, (Contreras-Ramos, 2011),

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**Fig. 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 and 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.

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 and 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 and Moczek, 2011; Angelini et al., 2012a).

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 disproportionately 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.

### 1.1. 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 and Kaufman, 2005). The developmental patterning of this proximal-distal axis in insect appendages is highly conserved and regulated by many genes (Kojima, 2004; Angelini and Kaufman, 2005). Based on previous studies of appendage morphogenesis in *Drosophila* and *Tribolium* (Lecuit and Cohen, 1997; Kojima, 2004; Angelini et al., 2009; Angelini et al., 2012a, 2012b), we focused on seven of these appendage-patterning genes, *Distal-less* (*Dll*), *aristaless* (*al*), *Epidermal growth factor receptor* (*Egfr*), *Keren* (*Krn*), *dachshund* (*dac*), *homothorax*

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