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Beetle horns are regulated by the *Hox* gene, *Sex combs reduced*, in a species- and sex-specific manner

Bethany R. Wasik,* Debra J. Rose, and Armin P. Moczek

Department of Biology, Indiana University, 915 E. Third Street, Myers Hall 150, Bloomington, IN 47405-7107, USA

*Author for correspondence (email: bkesselr@indiana.edu)

SUMMARY Discovering the mechanisms that underlie the origin of novel features represents a major frontier in developmental and evolutionary biology. Here we begin to characterize the role of the *Hox* gene *Sex combs reduced* (*Scr*) during the development and evolution of a morphologically novel trait: beetle horns. Beetle horns develop as epidermal outgrowths from the prothorax and/or head, and size and location vary dramatically across species and between sexes. Using both comparative gene expression and larval RNA interference in two species of the horned beetle genus *Onthophagus*, we show that *Scr* functions in patterning adult labial mouthpart identity and suppressing wing development in the prothorax. At the same time, however, our results illustrate that *Scr* has acquired, within its ancestral domain of expression, additional new functions including the regulation of prepupal growth and pupal remodeling of pronotal horn primordia. Furthermore, comparative analyses of our results across both *Onthophagus* species, which differ in location of horn development (thoracic horns vs. thoracic and head horns) as well as patterns of sexual dimorphism (traditional vs. reversed sexual dimorphism), reveal surprising differences in exactly when, where, and to what degree *Scr* regulates horn formation in different sexes. These observations suggest that the interactions between *Scr* and its targets in the regulation of horn development can diversify quickly over remarkably short phylogenetic distances. More generally, our results suggest that the *Hox* complex can play an integral role in the development and evolution of novel complex traits while maintaining traditional patterning responsibilities.

INTRODUCTION

Discovering the mechanisms that underlie the origin of novel features in animal body plans represents a major frontier in developmental and evolutionary biology. Morphological novelties can result from the activation of developmental gene networks in nontraditional tissues, also known as gene co-option (reviewed in True and Carroll 2002). There are several examples of co-option in the development of novel morphologies that span metazoan phylogeny. For instance, appendage patterning genes such as *hedgehog* (*hh*) and *Distal-less* (*Dll*) have become co-opted into butterfly wing tissue during development, contributing to eyespot development and diversity (reviewed in True and Carroll 2002). Similarly, co-option of the *Hox* complex has contributed to the evolution of cephalopod-specific structures such as the brachial crown, funnel tube, and light organ (Lee et al. 2003). Thus, existing patterning gene networks can accommodate significant morphological change to produce novel and diverse structures during development.

The *Hox* complex, in particular, is pivotal in regulating body plan organization. It is composed of 8–10 genes that are conserved as a complex across metazoan phyla and, in the case of arthropods, specify segment identity during development (reviewed in Akam 1989; Carroll 1995). Each *Hox* gene encodes a transcription factor with a highly conserved 60 amino acid homeodomain, and mutations in these genes alter segment identity and cause homeotic transformations of both segments and their appendages (Laughon and Scott 1984; McGinnis et al. 1984a, b). Interestingly, several *Hox* genes have been implicated in the evolutionary diversification of morphological structures.

For example, *Ultrabithorax* (*Ubx*) regulates the membranous wing placement in *Drosophila melanogaster* and *Tribolium castaneum* (the red flour beetle) by promoting haltere identity in the second thoracic segment (mesothorax) in the former and hindwing identity in the third thoracic segment (metathorax) in the latter (Tomoyasu et al. 2005). *Ubx* has evolved a size-specific function as well, interacting with other developmental networks to control and limit the size of the haltere during *Drosophila* development and promoting enlargement of the hind legs of *Acheta domesticus*, the house cricket (Mahfooz et al. 2007; Crickmore and Mann 2008).

Another *Hox* gene, *Sex combs reduced* (*Scr*), regulates segment identity of the labial segment including the posterior mouthparts and prothorax in many insect groups (Struhl

1982; Mahaffey and Kaufman 1987; Martinez-Arias et al. 1987; Pattatucci and Kaufman 1991; Hughes and Kaufman 2000; Curtis et al. 2001). We investigated *Scr* expression and function during the development of beetle horns, a class of novel and highly diverse secondary sexual traits. Beetle horns develop on the dorsal head and/or dorso-lateral prothorax (or pronotum) and thus are likely to share an expression domain with *Scr* during development. At the same time, beetle horns develop in body regions in which insects normally do not produce appendages or other outgrowths. Thus, beetle horns lack obvious homology to other insect structures (Moczek and Rose 2009). Several thousand beetle species spanning multiple beetle families develop such horns, and horn size, shape, and exact location on the head or prothorax can vary dramatically among species, across sexes, and even within sexes (reviewed in Snell-Rood and Moczek in press). Combined, these unique characteristics of beetle horns and horn diversity provide an excellent framework for comparative studies into the development and diversification of novel traits. Here we investigate the role of *Scr* in the development of horns and horn dimorphisms in the genus *Onthophagus*, an exceptionally species-rich and morphologically diverse genus of horned beetles.

In well-studied insect groups, loss-of-function (LOF) *Scr* mutations manifest in several major morphological phenotypes in the posterior mouthparts (the labial segment) and prothorax, resulting primarily from changes in the expression domains of adjacent *Hox* genes. In *Tribolium*, labial mouthparts transform completely into antennae following *Cx* RNAi (*Cephalothorax*, *Scr* ortholog), and both larval RNAi and isolated mutant *Cx* alleles result in ectopic elytra on the lateral prothorax (Beeman et al. 1989; Curtis et al. 2001; Tomoyasu et al. 2005). *Drosophila Scr* mutants show a transformation of labial mouthparts to maxillary identity, ectopic wing growth on the prothorax, a transformation of prothoracic legs to mesothoracic identity, and abnormal male sex comb development (Struhl 1982; Pattatucci and Kaufman 1991; Rogers et al. 1997; Barmina and Kopp 2007). The milkweed bug, *Oncopeltus fasciatus*, displays abnormal prothoracic shape and abnormal male prothoracic sex comb development following nymphal and embryonic *Scr* RNAi, respectively, and a mixed/leg identity in the labial mouthparts (Hughes and Kaufman 2000; Chesebro et al. 2009). The role of *Scr* in the development of sex combs in both *Oncopeltus* and *Drosophila* suggests that *Scr* interacts with sex determination processes.

Here we detail our characterization of *Scr* in beetle horns of two species in the genus *Onthophagus*: *O. nigriventris* and *O. sagittarius*. Both species differ in adult horn morphologies, nature, degree of adult sexual dimorphism, and developmental mechanisms used to generate differences in horn formation (summarized in Fig. 1). We find that *Scr* functions in patterning adult labial mouthpart identity and suppressing wing development in the prothorax. At the same time, our results show that *Scr* has acquired additional new functions within its ancestral domain of expression, including the regulation of prepupal growth and pupal remodeling of horn primordia and executes these functions in a body region-, sex-, and species-specific manner.

MATERIALS AND METHODS

Rearing conditions

O. nigriventris was reared as described in Moczek et al. (2006). Laboratory colonies of *O. sagittarius* were reared from animals collected from cow pastures in Oahu, Hawaii. The colony was kept in growth chambers at Indiana University at 27°C under a 16:8 light:dark cycle. Colony maintenance, breeding and sexing are described in Moczek and Nagy (2005). First day prepupal animals were used for cryosectioning and immunohistochemistry analyses as described in Moczek et al. (2006).

Scr cloning

O. nigriventris Scr was cloned through PCR from *O. nigriventris* genomic DNA with degenerate primers designed to nucleotides encoding amino acids 1–8 and 173–179 in the *T. castaneum Scr* ortholog, *Cephalothorax* (*TcCx*, GenBank Accession Number: AF227628). *O. sagittarius Scr* was cloned through PCR from cDNA with degenerate, nested forward primers designed to the *TcCx* nucleotide sequence encoding amino acids 1–9 and 12–18 and degenerate, nested reverse primers designed to amino acids 305–312 and 283–291 encoded by *TcCx*. For *O. sagittarius* dsRNA, the same forward nested primers were used while reverse nested primers were designed to amino acids 305–312 and 173–179 encoded by *TcCx*. Primer sequences are highlighted in Figure S1. *OnScr* and *OsScr* PCR products were cloned into a pCRII-TOPO vector with TOPO TA Cloning kit (Invitrogen, Carlsbad, CA, USA) or the pSC-A vector with a Strataclone PCR Cloning kit (Stratagene/Agilent, Santa Clara, CA, USA), respectively (also in Moczek and Rose 2009). All *Scr* constructs were sequenced as described in Moczek and Rose (2009) and submitted to GenBank (*OnScr*: FJ890927, *OsScr*: FJ898369).

In situ hybridization and immunohistochemistry

In situ hybridization and immunohistochemistry were performed as described in Moczek et al. (2006). RNA probes were constructed from a 592 bp *OnScr* nucleotide sequence encoding the region between the octapeptide motif and the YPWM motif directly preceding the homeobox (Fig. S1; Mavilio et al. 1986; LeMotte et al. 1989; Curtis et al. 2001) and used on sagittal, larval cryo-sections of both *Onthophagus* species. Immunohistochemistry was also performed on sagittal, larval cryosections with a cross-reacting polyclonal *Drosophila* Scr antibody (courtesy of T. C. Kaufman), incubated with CY3-conjugated secondary antibody (Jackson Labs), and counterstained with DAPI (Sigma-Aldrich; St. Louis, MO, USA) for nuclei visualization.

dsRNA construction and injection

dsRNA constructs for *OnScr* (592 bp) and *OsScr* (559 bp) were created from the same nonhomeodomain nucleotide regions as the

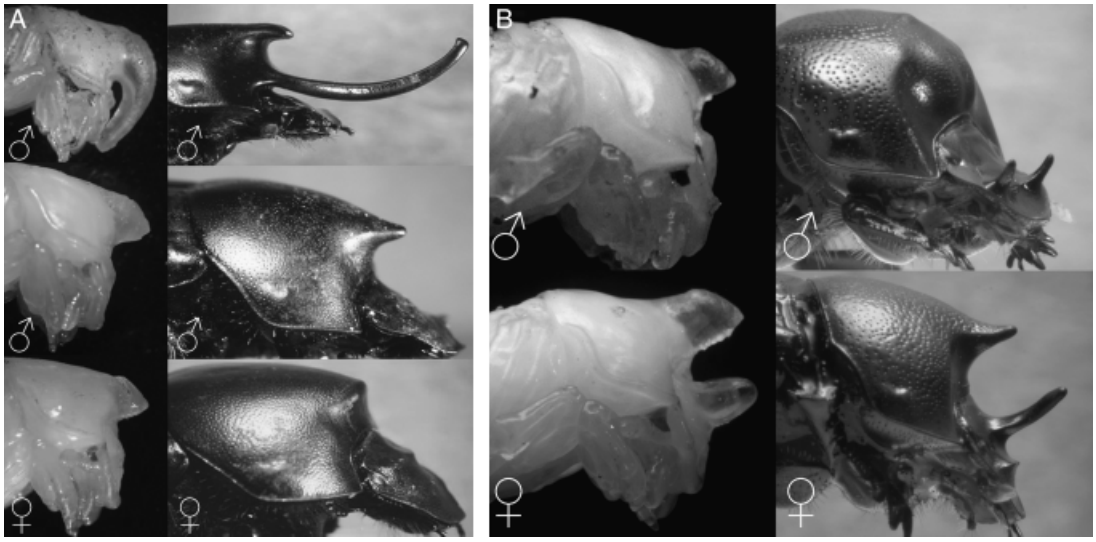


Fig. 1. Pupal and adult morphologies of *Onthophagus nigriventris* and *Onthophagus sagittarius*. Pupal (left) and adult (right) morphologies are shown for (A) *O. nigriventris* and (B) *O. sagittarius*. (A) In *O. nigriventris*, differential growth of pronotal horn primordia during the prepupal stage results in a pupal sexual dimorphism over most of the body size range, causing male pupae to develop slightly larger pronotal horns than female pupae (left panel). Sexually dimorphic prepupal growth is followed by sexually dimorphic resorption of pupal pronotal horn tissue in females only, generating a substantial sexual dimorphism in adult horns (right panel). Prepupal horn growth is dimorphic among males, resulting in large curved pronotal horns in the very largest males only and small pointed horns in males of smaller body sizes. Males in this study rarely reached this largest size class, and our analyses are limited to the remainder of the *O. nigriventris* body size range over which all treatment groups were well-represented. (B) *O. sagittarius* exhibits nearly identical prepupal pronotal horn growth in both sexes, resulting in sexually monomorphic pupae regarding pronotal horn length (left panel). Prepupal growth is followed by sexually dimorphic resorption of pupal pronotal horn tissue. Pupal pronotal horns in males undergo extensive resorption resulting in the formation of adult pronotal ridges, whereas females convert most of their pupal pronotal horn tissue into an adult horn. Altogether, this results in a rare sex-reversed adult sexual dimorphism (right panel). Furthermore, both sexes of *O. sagittarius* develop head horns. Females have a large head horn extending from the center of the head, and males have a pair of small head horns near the anterior edge of the head. Sexual dimorphism in adult head horns is the result of differential prepupal growth, and very little head horn tissue resorption occurs during the pupal stage.

in situ RNA probe (Fig. S1B). In vitro transcription and injection of *OsScr* and *OnScr* dsRNA constructs were done as described in Moczek and Rose (2009) except for the use of 33 G 1 in. needles in *O. sagittarius*. Control animals were generated as described in Moczek and Rose (2009). *Scr* RNAi and control adults in both species were weighed on the second day of adulthood, preserved in 70% EtOH, and stored at -20°C.

Allometric measurements

Control and RNAi pupae and adults were measured as described in Moczek and Rose (2009). Body mass was used as an indicator of both pupal and adult size, and pronotal and head horns were measured as described previously and recorded to the nearest 0.001 mm (Moczek 2006, 2007; Moczek and Rose 2009). Left and right head horn measurements in *O. sagittarius* pupal and adult males were averaged per individual.

Statistical analyses

All analyses were conducted using JMP (v. 7.0, SAS Institute Inc., Cary, NC, USA, 1989–2007) with a fixed-factor analyses of variance model. Separate analyses were conducted for males and females of both species, as well as pupal and adult stages. Horn length was designated as the response variable and body weight, treatment (wild-type, control and RNAi), and *weight* × *treatment* interactions were designated as model effects. When treatment effects were detected, we replicated the analysis adding a *sex* × *treatment* interaction term to the model to test for possible sex-specific differences in treatment effects. Unless otherwise noted, we reported the results of the simple model (each sex analyzed separately). Where *sex* × *treatment* interactions are reported, the use of the complex model did not result in qualitative changes in the results of the remaining model effects. We repeated the analysis (adults only) by further subdividing RNAi animals into groups of "mild" or "severe" *Scr* RNAi phenotypes to examine possible correlations between the severity of *Scr* RNAi on horn formation. We used the same approach to examine possible treatment effects on fore tibia length (adults only) and head horn length (*O. sagittarius* only).

RESULTS

Scr cloning and sequence analysis

We isolated *Scr* fragments from *O. nigriventris* encoding amino acids 1–197 (592 bp) and from *O. sagittarius* encoding amino acids 12–309 (894 bp). An octapeptide motif unique to *Scr* in insects and mammals (MSSYQFVN) was conserved in *O. nigriventris* (Fig. S1A; Mavilio et al. 1986; LeMotte et al. 1989; Curtis et al. 2001). The cloned *O. sagittarius Scr* nucleotide sequence starts immediately downstream of the region

encoding the octapeptide motif at amino acid 12 and includes a portion of the homeobox (Fig. S1A). Additionally, the proteins encoded by *OnScr* and *OsScr* contain a conserved PEST motif between amino acids 110-134 (Fig. S1A; Rogers et al. 1986, 1997; Andrew 1995; Rechsteiner and Rogers 1996; Curtis et al. 2001). Proteins encoded by *OnScr* and *OsScr* fragments share 97.8% amino acid identity to one another and 80.4% and 85.7% amino acid similarity to the corresponding protein region encoded by the *Tribolium* ortholog, *TcCx* (GenBank Accession Number: AF227628), respectively. In contrast to *TcCx*, proteins encoded by both *OnScr* and *OsScr* contain several small amino acid insertions (Fig. S1A).

Scr expression

Scr mRNA expression was observed throughout the larval prothorax including horn tissue and anterior prothoracic legs, and also in the labium in both species (data not shown). A cross-reacting polyclonal *Drosophila* Scr antibody detected *Onthophagus* Scr protein expression in the same locations as mRNA expression in both species (Fig. 2). Both *Scr* mRNA and protein expression patterns were consistent with expression patterns seen in other insects (Mahaffey and Kaufman 1987; Martinez-Arias et al. 1987; Rogers et al. 1997; Curtis et al. 2001).

Scr RNAi effects on non horn structures

Scr RNAi in both *Onthophagus* species resulted in adults with homeotic transformations of the labium (composed of labial palps and hypopharyngeal sclerites) and induction of ectopic wing tissue on the lateral prothorax, with bristle patterns indicative of mesothoracic elytral identity (Fig. 3, D–E). RNAi phenotypes were scored "mild" or "severe" based on the severity of mouthpart transformation and presence of ectopic wing tissue. In mildly affected animals of both species, the distal-most segment of the labial palps adopted an incomplete maxillary fate exhibited by bristle loss and elongation (Fig. 3B). In severely affected animals, both the labial palps and hypopharyngeal sclerites transformed into near complete maxillary structures (Fig. 3C). Prothoracic legs were affected in both species solely in size but not identity. Fore tibia length of males and females of both species was significantly reduced following *Scr* RNAi, compared with fore tibia length in control animals (Fig. S2, Table S3).

Scr RNAi effects on prepupal pronotal horn growth

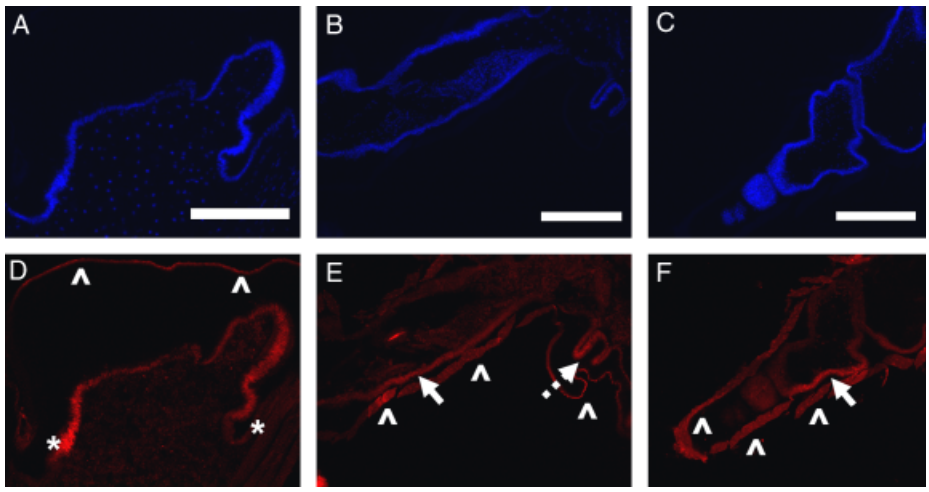
We used morphometric measurements and a fixed-factor analyses of variance model to quantify *Scr* RNAi-induced changes in size and shape of horns, compared with control and untreated wild-type animals (Tables S2, S4). Pupal pronotal horn length was moderately but significantly reduced following *Scr* RNAi in male (Fig. 4; $P<0.0001$) but not female ($P=0.53$) *O. nigriventris* pupae. However, the *sex* × *treatment* interaction term was nonsignificant. In contrast, pupal pronotal horns in *O. sagittarius* were strongly reduced in both sexes ($P<0.0001$; *sex* × *treatment* interaction = NS). These data suggest that *Scr* regulates prepupal pronotal horn growth but does so differently in different species and sexes.

Fig. 2. *On*Scr immunohistochemistry. All tissue cryo-sections were generated from prepupal *Onthophagus nigriventris* larvae, sectioned sagittally with dorsal up, anterior to the right, and posterior to the left. (A–C) DAPI nuclear expression (blue) in the (A) pronotal horn primordium, (B) prothoracic leg and labium, and (C) distal prothoracic leg. (D–F) *Dm* anti-Scr (red) expression in the (D) pronotal horn primordium (white asterisks mark extent of expression domain), (E) anterior prothoracic leg (solid white arrow) and labium (dashed white arrow), and (F) distal anterior prothoracic leg (solid white arrow). In (D–F), white carets (^) mark artifactual staining of the larval cuticle. Immunohistochemistry expression patterns did not differ in *Onthophagus sagittarius* (not shown). Scale bars indicate 200 μm. Note: carets in (D–F) are below but not overlaying cuticle.

Scr RNAi effects on adult pronotal horn development

O. nigriventris male adult horns were dramatically reduced in length ($P<0.0001$), while female adult pronotal horn length was only moderately reduced ($P<0.0254$). Unlike in the pupal stage, sex-specific differences in treatment response were also evident in a highly significant *sex* × *treatment* interaction term ($F_{2.161}=13.75$; SS = 2.41; $P<0.0001$). In contrast, adult *O. sagittarius* exhibited a significant and dramatic response to *Scr* RNAi only in female adults. Male *Scr* RNAi adults did not differ significantly from control-injected animals, even

Fig. 3. *Scr* RNAi effects on nonhorn structures. (A–C) Mouthpart structures in *Onthophagus nigriventris* wild-type and *Scr* RNAi adults and (D–E) lateral prothorax in *Onthophagus sagittarius* wild-type and *Scr* RNAi adults. (A) Wild-type mouthparts most relevant in the present study include maxillary palps (characterized by elongated segments with smooth surfaces without bristles – red dashed and solid boxes), labial palps (characterized by stout segments with rough cuticular surfaces and distinct bristle patterns – blue dashed and solid boxes), and the hypopharynx (characterized by paired sclerites with dense, long bristles – green arrows and solid boxes). (B) A mild *Scr* RNAi phenotype with wild-type maxillary palps, distally transformed labial palps with bristle loss and elongation, and paired moderately transformed hypopharynx. (C) A severe *Scr* RNAi phenotype with wild-type maxillary palps, labial palps with smooth texture and few bristles, and a fused hypopharynx with color and bristle pattern similar to the maxillary galea (mg). (D) Wild-type lateral prothorax in an *O. sagittarius* female and (E) a female *Scr* RNAi adult with ectopic prothoracic tissue bulging laterally with a textured pattern matching those normally observed on the elytra (indicated by black arrow).

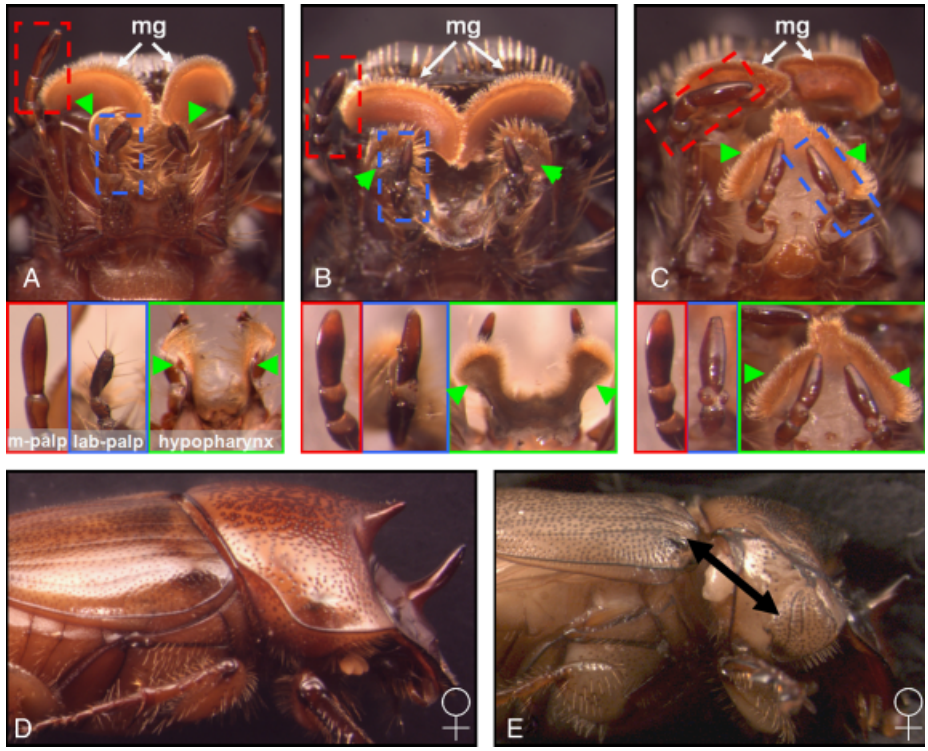


though they had exhibited major differences in the preceding pupal stage. Unlike in the pupal stage, but similar to *O. nigriventris*, sex-specific differences in treatment response were now also evident in a highly significant *sex* × *treatment* term ($F_{2,16} = 47.03$; SS = 1.26; $P < 0.0001$).

Additional species-specific differences in *Scr* RNAi response emerged when adults were subdivided into categories of "mild" and "severe" mouthpart transformation. In *O. nigriventris*, only males with "severe" phenotypes exhibited significant reductions in adult pronotal horn length ($P < 0.05$) (Fig. 4), whereas males with "mild" phenotypes had pronotal horn lengths indistinguishable from control or wild-type animals. In contrast, *O. sagittarius* females with "mild" and "severe" phenotypes exhibited similarly significant reductions in pronotal horn length, compared with control-injected and wild-type animals ($P < 0.05$) (Fig. 4).

Scr RNAi effects on head horns

In *O. sagittarius*, head horn length of males and females was unaffected by *Scr* RNAi at both pupal and adult stages (Fig. S2). The only exception was a small but significant effect



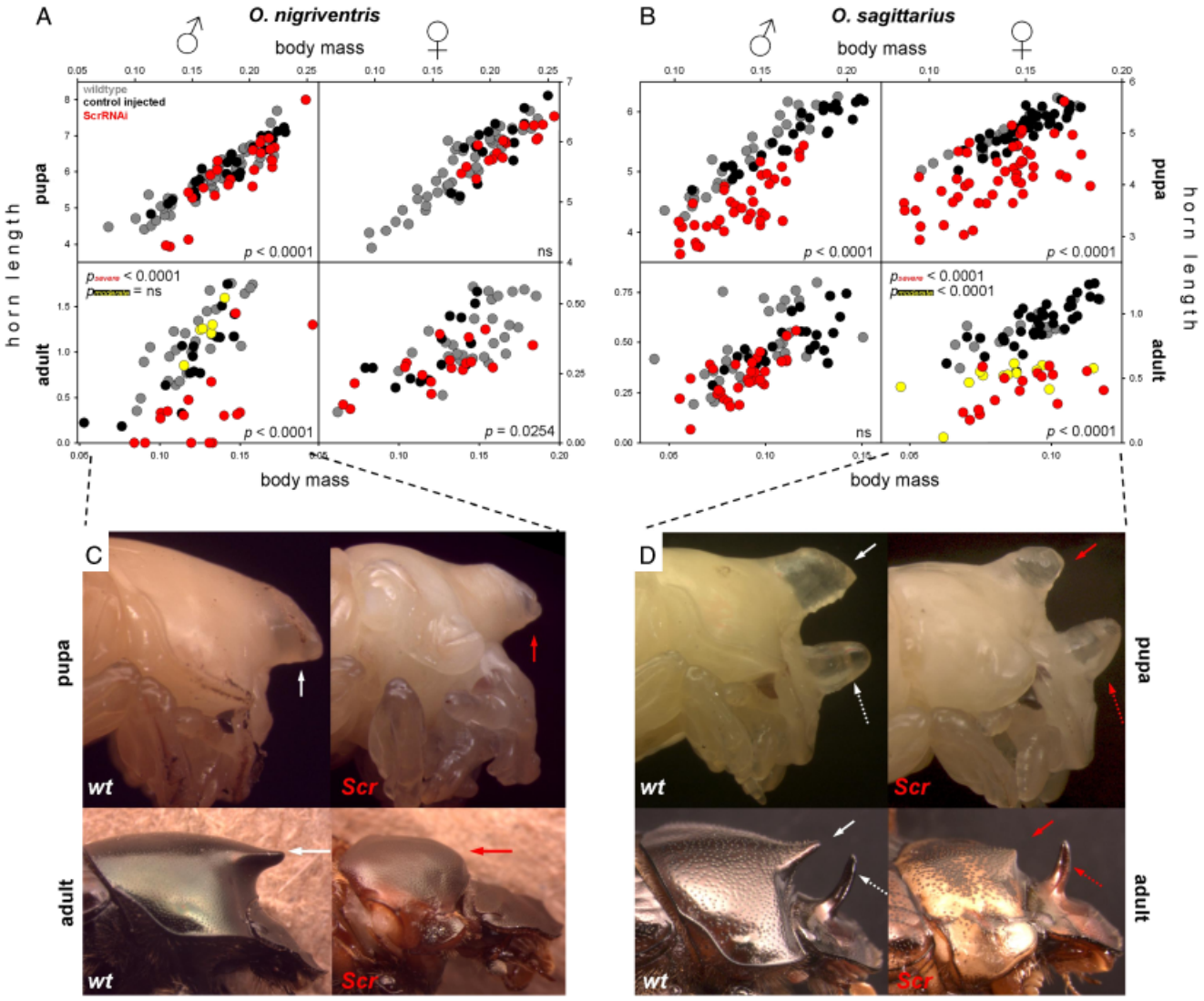


Fig. 4. *Scr* RNAi effects on pronotal horn development and representative phenotypes in two *Onthophagus* species. (A, B) Body mass (g) versus pronotal horn length (mm) allometries in (A) *Onthophagus nigriventris* and (B) *Onthophagus sagittarius* pupae (top) and adults (bottom). Males are left and females are right for both species. Shown are measurements obtained from untreated wild-type (gray), control-injected (black), and *Scr* RNAi (red) animals. *P*-values indicate significance of treatment effect in the ANOVA. A significant effect of *Scr* RNAi injections was detected in each category except female *O. nigriventris* pupae. A significant treatment effect was also detected in adult male *O. sagittarius*, but pairwise comparisons revealed that this was due to significant differences only between wild-type and *Scr* RNAi, but not control-injected and *Scr* RNAi animals. No significant differences between wild-type and control-injected animals were detected in any of the categories, including adult male *O. sagittarius*. The strongest effects of *Scr* RNAi on pronotal horn length were detected in male *O. nigriventris* and female *O. sagittarius*. Subdividing these *Scr* RNAi animals into mild and severe categories based on degree of mouthpart transformation (see Results) showed that only severely affected *O. nigriventris* males also displayed a significant reduction of pronotal horn length as adults, whereas pronotal horn length in *O. sagittarius* was similarly reduced in both mildly and severely affected individuals. (C, D) Shown are four individuals first as pupae (top) and then again as adults (bottom), size-matched within species by mass. In each panel, left corresponds to wild-type and right to *Scr* RNAi treatment. Note the presence of a substantial pronotal horn in wild-type and *Scr* RNAi *O. nigriventris* pupae (solid arrows). The corresponding adult pronotal horn remains visible only in wild-type but undergoes complete resorption in the *Scr* RNAi animal. In contrast, *O. sagittarius* displays a substantial reduction in pronotal horn length in the pupa, followed by an even greater reduction in the adult. Also note absence of obvious effects on head horn development in female *O. sagittarius* (dotted arrows).

in male adult head horn length ($F_{2.87} = 0.057$; SS = 3.74; $P = 0.028$), but pairwise comparisons revealed that this was due to significant differences only between head horn lengths in wild-type and *Scr* RNAi animals, not in control-injected and *Scr* RNAi animals. No significant differences between head horn lengths in wild-type and control-injected animals were detected in any of the categories. Thus, horns that develop in two different segments in the same individual were differentially sensitive to *Scr* RNAi.

RNAi effects on Scr protein depletion

Western analyses with both anti-Scr and anti-α-tubulin (DM1α) antibodies verified Scr protein depletion. *Scr* RNAi reduced Scr protein abundance in the prothorax in *O. sagittarius* tissue samples from first day female and male pupae with severe phenotypes (Fig. S4). We also observed significant Scr protein depletion in both female and male prothoracic legs. As expected, there was little Scr expression in abdominal tissue and anti-α-tubulin (DM1α) was uniformly expressed among samples (Fig. S4).

RNAi effects on penetrance and survival

Survival rates and penetrance are described in Table S1. Location of ds*Scr* injection did not affect the resulting knockdown phenotypes in either species (data not shown). We observed no obvious effect of *Scr* dsRNA concentrations (ranging from 0.5 to 5 μg/ml) on phenotype severity. Specifically, higher concentrations of *OnScr* or *OsScr* dsRNA did not result in a higher frequency of animals with "severe" phenotypes.

DISCUSSION

Here we show that *Onthophagus Scr* functions in the developmental regulation of adult mouthpart and prothoracic identity, but has also acquired important new roles in the development and diversification of pronotal horns in *Onthophagus* beetles. Specifically, we illustrate *Onthophagus Scr* functions in pronotal, but not head horn, development in two closely related *Onthophagus* species and executes its function in a species- and sex-specific manner. Our results suggest that evolutionary changes in *Scr* function, and perhaps the functions of *Hox* genes generally, may mediate the evolution of novel structures and can do so without compromising traditional expression and function. Below we briefly discuss important implications of our results.

Scr regulates non horn structures

Scr RNAi resulted in a homeotic transformation of the labium and an induction of ectopic wing tissue on the lateral prothorax, similar to some phenotypes observed in previous studies (Struhl 1982; Mahaffey and Kaufman 1987; Martinez-Arias et al. 1987; Pattatucci and Kaufman 1991; Hughes and Kaufman 2000; Curtis et al. 2001; Tomoyasu et al. 2005; Chesebro et al. 2009). Specifically, the *Onthophagus* labial mouthparts showed a transformation to maxillary identity. The duplication of maxillary mouthparts observed in animals with "severe" phenotypes suggests labial identity is regulated by *Scr* during the third larval instar of *Onthophagus* development.

Interestingly, in the only other beetle species for which *Scr* RNAi phenotypes are known, *Tribolium*, embryonic *Scr* RNAi results in a transformation of labial mouthparts to antennae (Curtis et al. 2001). This difference in the outcome of labial transformation between *Onthophagus* and *Tribolium* may be a result of diverged *Scr* function in labial development among beetle species, or alternatively reflect ontogenetic changes in the patterning function of *Scr* from embryonic to late larval development.

Scr RNAi only affected prothoracic leg length but not identity in both species. In contrast, *Drosophila Scr* LOF mutants exhibited a transformation of prothoracic to mesothoracic leg identity, as well as an alteration of sex comb identity (Struhl 1982; Barmina and Kopp 2007). *Oncopeltus* males also exhibited abnormal prothoracic sex comb development after *Scr* embryonic (but not postembryonic) RNAi (Hughes and Kaufman 2000; Chesebro et al. 2009). Our results indicate the possibility that *Onthophagus Scr* regulates the development, but not identity, of legs throughout the larval stage. However, both Western blotting data and incomplete transformations (e.g., elytra) show we were unable to completely repress *Scr* through larval RNAi, so it is conceivable that some of the phenotypes detected may be a result of the hypomorphic nature of larval RNAi in *Onthophagus* beetles.

Scr regulates prepupal pronotal horn growth

Our results suggest *Scr* regulates prepupal pronotal horn growth but does so differently in different species and sexes. Following larval *Scr* RNAi, pupal pronotal horn length in *O. nigriventris* males and both *O. sagittarius* sexes was significantly reduced compared with pupal pronotal horn length in control animals, indicating an effect of *Scr* on prepupal horn growth. Since prepupal horn growth occurs during a critical period immediately before pupation in the late third instar, significant reductions in horn growth imply that *Scr* is still regulating the identity of the prothorax well into late larval stages. Significant reductions in growth may result from disrupted *Scr* interactions with other developmental genes expressed simultaneously throughout the prothorax, including pronotal horn formation. The role of *Scr* in pronotal horn development may be similar to observations on the role of *Ubx* in the enlargement of metathoracic legs in *A. domesticus* (the house cricket) which exhibit distinct *Ubx* expression dur-

ing development, but are reduced dramatically following *Ubx* RNAi (Mahfooz et al. 2007). Similar mechanisms may be occurring in the mouthparts or the prothorax following *Scr* RNAi in *Onthophagus* beetles. For example, reductions in *Scr* expression may be negatively affecting appendage development genes, such as *dpp*, in the mouthparts and prothorax, reducing final appendage size. *dpp* has independently been found to have a crucial role in pronotal horn growth and mouthpart development in *Onthophagus* pupae and adults (B. R. Wasik, unpublished data), though it is unclear whether *dpp* specifically interacts with *Scr* during pronotal horn growth.

Scr RNAi regulates adult pronotal horn development

Adult pronotal horn length was also affected in a species- and sex-specific fashion and suggests the effects of *Scr* on horn development extend beyond the initial prepupal growth phase into the pupal horn remodeling phase of development. *O. nigriventris* pupae of both sexes exhibited only modest, if any, reductions in pupal pronotal horn length, while the corresponding adult horns exhibited dramatic reductions in males. Similarly, sex-specific differences in *O. nigriventris* pronotal horn length were significant only in the adult stage. Similar patterns were observed for *O. sagittarius* pronotal horn development. Here, pupal pronotal horns were similarly reduced in both sexes (*sex* × *treatment* interaction = NS), while pronotal horn length in the resulting adults revealed a highly significant *sex* × *treatment* interaction term due to far stronger effects of *Scr* RNAi in females than males.

Additional and unexpected species- and sex-specific differences in adult *Scr* RNAi responses emerged when individuals were subdivided according to "mild" and "severe" mouthpart transformation. Only *O. nigriventris* males with severe mouthpart transformations exhibited significant reductions in adult pronotal horn length, whereas *O. sagittarius* females exhibited similarly significant reductions in pronotal horn length regardless of the degree of mouthpart transformation. These observations suggest that posterior mouthparts and pronotal horns in the same individual are differentially sensitive to *Scr* RNAi, and that the magnitude of these differences can diverge between species.

Scr may be regulating sex-specific pronotal horn development similarly to its role in the sex-specific regulation of male sex comb development in *Drosophila* (Barmina and Kopp 2007). Specifically, sex-specific pupal horn remodeling via *Scr* may be mediated through mechanisms such as programmed cell death (PCD), which has been documented as a major contributor to morphogenetic processes during development (reviewed in Adachi-Yamada and O'Connor 2004; Domingos and Steller 2007). Preliminary data suggest PCD has a significant role in the remodeling of *Onthophagus* beetle horns during the pupal stage (Moczek 2006; T. Kijimoto, unpublished data). However, preliminary examination of cryo-sectioned pupal tissue stained with a PCD indicator (TUNEL) has thus far failed to produce a consistent connection between *Scr* RNAi and changes in levels of PCD in the pronotal horn (B. R. Wasik, unpublished data). Pupal horn resorption occurs within 48 h following pupation, and while subtle changes in PCD during this period may be sufficient to yield substantial changes in the amount of horn resorption, they may not be readily detectable with single time point measurements.

CONCLUSIONS

This study illustrates that co-option of a member of the *Hox* complex contributes to *Onthophagus* pronotal horn development and diversity. Specifically, we have shown that *Hox* genes can play an integral role in regulating a novel and complex trait, and can do so while maintaining traditional patterning responsibilities. Our results therefore suggest that evolutionary changes in the functions of *Scr* (and perhaps those of *Hox* genes generally) have the capacity to mediate the evolution of novel structures, without compromising traditional expression and function. Further, these regulatory roles extend far into postembryonic stages and provide insight into the dynamic spatial and temporal expression of patterning networks over multiple developmental stages. Recent work in *Tribolium* and *Oncopeltus* document such dynamic behavior not only for the *Hox* complex but also for appendage patterning networks (Tomoyasu et al. 2005; Angelini et al. 2009; Chesebro et al. 2009). However, we know little about spatial and temporal diversity in patterning of late developmental stages in nonmodel insects, especially adults. Thus, it is conceivable that members of the *Hox* complex may be involved in regulating many other novel complex traits in adult insects, such as abdominal claspers in sepsid flies or photic organs in fireflies (Bowsher and Nijhout 2007). Taking advantage of the tremendous diversity of novel structures found among adult insects has the power to elucidate how existing patterning gene networks may promote novel and diverse morphological changes during development.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. *Onthophagus* Scr Amino Acid Sequence Alignment. (A) This alignment was constructed from Scr sequences from several insects, *O. nigriventris*, and *O. sagittarius* using Clustal X (Thompson et al. 1997). Conserved residues and motifs are highlighted in bold, the octapeptide motif has a double-underline, PEST motifs have a dashed underline (configured with http://www.ngbw.org; Rifaieh et al. 2007), and the homeodomain has a single-underline. Small insertions in both *On*Scr and *Os*Scr are present at positions 20-21, 38-39, 92-93, and 178, and larger insertions occur at positions 100 (4 amino acids) and 150 (7 amino acids). *O. sagittarius* (*O.s*: ACR58763), *O. nigriventris* (*O.n*: ACR56892), *Tribolium castaneum* (*T.c*: AAF42868), *Bombyx mori* (*B.m*: BAA76868), *Nasonia vitripennis* (*N.v*: NP_001128396), *Apis mellifera* (*A.m*: XP_623903), *Drosophila melanogaster* (*D.m*: AAA19240), and *Anopheles gambiae* (*A.g*: AAC31944) are compared in this alignment. Asterisks indicate the boundaries of the *Onthophagus* sequences highlighted in (B). (B) This alignment was also constructed using Clustal X (Thompson et al. 1997) and

depicts regions of *OnScr* and *OsScr* used for either RNA probes or dsRNA construction. Conserved residues and motifs are highlighted in bold. Nucleotide regions with a single underline indicate the sequence used for the *O. nigriventris Scr* RNA probe and dsRNA (592 bp) and the *O. sagittarius* dsRNA construction (559 bp). Nucleotides with a double-underline are primer regions. Both presumed *Onthophagus* fragments align well with known insect Scr sequences, consistent with the hypothesis that *On*Scr and *Os*Scr are the *Onthophagus* Scr orthologs.

Fig. S2. Allometric Data for Fore Tibia Length. *Scr* RNAi effects on adult fore tibia length in two *Onthophagus* species. Body mass (g) – adult fore tibia length (mm) allometries in *O. sagittarius* (top) and *O. nigriventris* (bottom). Males are shown on the left, females on the right. Shown are measurements obtained from untreated wild-type (grey; *O. sagittarius* only), control-injected (black) and *Scr* RNAi (red) animals. P values indicate significance of treatment effect in the ANOVA (see Tables S4 and S5). A significant effect of Scr RNAi injections was detected in each category.

Fig. S3. Allometric Data for Head Horn Length. *Scr* RNAi effects on head horn development in *Onthophagus sagittarius*. Body mass (g) – head horn length allometries (mm) in pupal (top) and adult (bottom) individuals. Males are shown on the left, females on the right. Shown are measurements obtained from untreated wild-type (grey), control-injected (black) and *Scr* RNAi (red) animals. No significant effect of *Scr* RNAi injections was detected in any category (see Table S6) except for male adults which exhibited slightly but significantly increased horn lengths in *Scr* RNAi individuals when compared to wild-type, but not when compared to control-injected animals. No significant differences between wild-type and control-injected animals were detected in any of the categories.

Fig. S4. Western Analysis of *Scr* RNAi in *O. sagittarius* pupae. Western blot analyses results are shown from thorax (T), abdomen (A), and prothoracic leg (L) tissue samples from *O. sagittarius* wild-type females and males, and *Scr* RNAi females and males. The top, left panel shows wild-type levels of Scr in thorax and leg tissue of both sexes, and the top, right panel shows reduced Scr levels in thorax and leg tissue of both sexes. Abdominal Scr expression was minimal in all cases. The bottom panel shows identical tissue as the top panel but displaying α-tubulin (DM1α) expression, which remained unchanged in all treatments. Different sets of wild-type and *Scr* RNAi pupal tissue produced similar results.

Table S1. Phenotypic penetrance and survival for control-injected and *Scr* RNAi individuals.

Table S2. Analysis of variance of the effect of *Scr* RNAi on pronotal horn length in *O. nigriventris* (top) and *O. sagittarius* (bottom).

Table S3. Analysis of variance of adult fore tibia length on *O. nigriventris* (only data on control-injected and Scr RNAi animals available) and *O. sagittarius*.

Table S4. Analysis of variance of the effect of *Scr* RNAi on head horn length in *O. sagittarius*.

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