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Appendage-patterning genes regulate male and female copulatory structures in horned beetles

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SUMMARY Explaining the extraordinarily rapid diversification of insect copulatory structures has been a longstanding objective in evolutionary biology. However, remarkably little is known about the developmental genetic underpinnings of their formation. Furthermore, recent work has questioned whether male genitalic structures in beetles are serially homologous to appendages, or even homologous to the genitalia of other orders. Using RNA interference, we demonstrate that several cardinal appendage-patterning genes regulate the formation of copulatory structures in *Onthophagus* beetles of both sexes. These results are in strong disagreement with previous findings in the model beetle species *Tribolium castaneum*, but congruent with earlier studies in true bugs and flies. Our results support the hypotheses that genitalic development is largely conserved across insect orders, and that genitalia constitute serial appendage homologues. Moreover, we identify two patterning genes with striking phenotypic effects in both sexes. In these cases, the affected structures are known to interact functionally during copulation, but are not homologous to each other. This suggests that shared developmental regulation of male and female copulatory structures may extend beyond components related by descent to those related by function.

INTRODUCTION

Explaining the extreme diversity of insect genital morphology has been a longstanding objective in evolutionary biology (Eberhard 1985; Hosken and Stockley 2004; Eberhard 2010). Copulatory structures evolve far more rapidly than most external morphological traits, and are typically the first - and often only - morphological structure to diverge noticeably among closely related species. Evolutionary modifications of copulatory organs are thus believed to play critical roles in the evolution of reproductive isolation and speciation (Eberhard 1985; Hosken and Stockley 2004; Eberhard 2010). Rapid genitalic divergence has generally been attributed to shape, rather than size, variation (Arnqvist and Thornhill 1998; Soto et al., 2007; Eberhard et al., 2009; Macagno et al., 2011), and recent work has demonstrated that structures that interact tightly during the copula coevolve in males and females (McPeek et al., 2009; McPeek et al., 2011; Macagno et al., 2011; Simmons and Garcia-Gonzalez 2011). However, apart from work on *Drosophila* (Keisman and Baker 2001; Sánchez and Guerrero 2001; Gorfinkiel et al., 2003; Foronda et al., 2006; Chatterjee et al., 2011), little is known about the developmental genetic mechanisms that pattern copulatory structures across insects, and virtually nothing is known about the degree to which shape and size might be under separate control, or the degree of sex-shared developmental regulation of copulatory structures. What little comparative functional genetic information exists comes from a single study (Aspiras et al., 2011), which investigated the roles and regulation of appendage-patterning genes during genitalia development in the milkweed bug *Oncopeltus fasciatus*, and in the red flour beetle *Tribolium castaneum*, two well-established model systems in the Hemi- and Holometabola, respectively. This study found that a large number of appendage-patterning genes are indeed involved in the formation of male and female genitalia of *O. fasciatus*, in ways reminiscent of earlier findings in *Drosophila*. In contrast, in *T. castaneum*, the orthologous genes played no role in the development of male genitalia, and only a marginal role in females. This study raised the intriguing possibility that insect orders may have diverged dramatically in the basic developmental genetic regulation of copulatory structures, and/or that beetle copulatory structures are not homologous to those of true bugs (Hemiptera) or flies (Diptera).

Here, we address these and additional issues by investigating the role of appendage-patterning genes in the development of genital form in two beetle species in the genus *Onthophagus* (Scarabaeidae). We targeted four patterning genes whose effects on appendage formation had previously been documented in *Onthophagus* (Moczek and Rose 2009; Wasik and Moczek 2011) and many other insects (Kojima 2004), yet none of which were found to affect genital development in male *T. castaneum* (Aspiras et al. 2011). Specifically, we examined the function of:

a) *Distal-less* (*Dll*), which is required for distal appendage formation across diverse arthropods including beetles; b) *homothorax* (*hth*), which specifies where developing appendages insert proximally into the body wall; c) *dachshund* (*dac*), which specifies medial appendage identity; and d) *decapentaplegic* (*dpp*), which plays diverse roles in appendage growth, including proper establishment of the proximo/distal and dorso/ventral axes (Kojima 2004). Furthermore, we assessed the degree to which these appendage-patterning genes are involved in the development of copulatory structures that are related by function, though not by descent, in *Onthophagus* beetles (Fig. 1). Specifically, in males, we analyzed the aedeagus, whose serial homology with appendages has been questioned in beetles (Aspiras et al., 2011), but is generally supported by classical comparative anatomy (Minelli 2002; Rosa-Molinar and Burke 2002; Boxshall 2004) and developmental data from flies and true bugs (Sánchez and Guerrero 2001; Aspiras et al., 2011). At the same time, we examined the pygidial flap – the female copulatory structure that provides the mechanical coupling site for the aedeagus during copulation. The pygidial flap originates as part of the body wall, and while it interacts functionally with the aedeagus during copulation, both structures are not homologous to each other. However, previous work has shown that these structures coevolve (Simmons and Garcia-Gonzalez 2011) and diverge in concert between the sexes across closely related populations and species (Macagno et al., 2011).

Our study aimed to answer the following questions: (1) Are appendage-patterning genes involved in the development of genital form in *Onthophagus*? (2) If so, do appendage-patterning genes primarily regulate the relative size of genitalic parts or their shape? (3) Which, if any, appendage-patterning genes instruct the development of copulatory structures in both sexes, and might therefore constitute especially interesting candidates for parallel evolution?

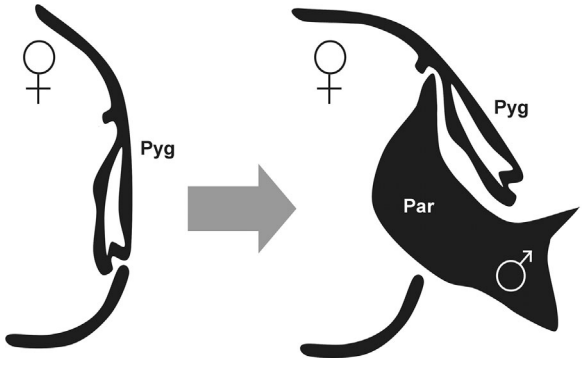

Figure 1. Interaction of parameres (Par) and pygidial flap (Pyg) during copulation in *Onthophagus* [cross-section of the distal portion of female abdomen, modified after Macagno et al. (2011)].

MATERIALS AND METHODS

Animals used here derive from two previous studies focused on the role of appendage-patterning genes in beetle horn development (*Distal-less*,*homothorax*, *dachshund*: Moczek and Rose 2009; *decapentaplegic*: Wasik and Moczek 2011; Table 1). Details on husbandry, rearing, staging, cloning, sequence analysis, and RNAi approaches can be found in these earlier studies. Below we summarize the methods most critical to the present analysis.

Cloning, sequence analysis, dsRNA construction

Fragments of all four candidate genes were cloned into pCRII-TOPO vector (Invitrogen) and analyzed as described in Moczek and Rose (2009) and Wasik and Moczek (2011). dsRNA constructs were generated via *in vitro* transcription (MEGA-script kit, Ambion) to produce both sense and antisense RNA strands for each fragment. DNA sequences are deposited in GenBank [accession numbers: DQ452570 (*Otdac*), EU732589 (*Othth*), EU779933 (*OtDll*), EU779932 (*ObDll*), HM632025 (*Obdpp*)].

dsRNA injection

Larvae were injected during the third (=final) instar, at least 5–10 days prior to entering the prepupal stage, with 3 μl of a solution containing 0.5–5 μg of dsRNA in injection buffer (5 mM KCl, 1 mM KPO4 ph6.9). Two control groups were used: (i) Control-injected animals reared under the same conditions, but injected instead with dsRNA from a 167bp PCR product derived from a pBluescript SK vector in injection buffer. (ii) Untreated (wild-type) animals, reared under the same conditions but not injected.

Table 1. Number of individuals inspected and source study for each target gene

	Onthophagus taurus		*Onthophagus binodis*	
Gene knockdown	Males	Females	Males	Females
Distal-less	7 (a)	6 (a)	8 (a)	8 (a)
homothorax	5 (a)	5 (a)		
dachshund	7 (a)	7 (a)		
decapentaplegic			12 (b)	8 (b)
Ctrl-inj	7 (a)	7 (a)	10 (b)	10 (b)
Ctrl-WT	20	20	20	20

[2009](a) Moczek and Rose (2009); (b) Wasik and Moczek (2011). Ctrl-inj, control-injected; Ctrl-WT, wild-type individuals.

Knockdown validation

Western blot (*dac*, *hth*), Northern blot (*Dll*), and qRT-PCR were used to evaluate the depletion of protein and mRNA levels after RNAi-mediated knockdown (Moczek and Rose 2009; Wasik and Moczek 2011). All three methods documented substantial reductions in gene product for all four genes, and across all tissues tested, compared to wild-type.

Dissection and imaging

We dissected the copulatory structures of beetles that exhibited obvious external RNAi phenotypes for each gene of interest (Moczek and Rose 2009; Wasik and Moczek 2011), which were then compared to wild-type and control-injected individuals. To increase sample size, we also included in the analysis some *O. taurus* that did not show obvious external knockdown phenotype: 2 *hth*-RNAi males, 2 *hth*-RNAi females, 3 *dac*-RNAi males, and 1 *dac*-RNAi female. Images were collected using a 2D image analysis setup including a stereoscope (Leica MZ-16, Bannockburn, IL, USA) and a digital camera (Scion, Frederick, MD, USA).

RESULTS

Wild-type copulatory structures

In wild-type *Onthophagus*, the male copulatory structure (aedeagus) is retracted inside the abdomen when not used in copulation. It consists of two heavily sclerotized regions: a proximal, tubular phallobase, and two distal, hook-like, paired parameres (Fig. 2A and 3A). The highly membranous endophallus is contained within the aedeagus. During copulation (Fig. 1), the aedeagus is extruded from underneath the pygidium (the dorsal side of the last abdominal segment). The tips of the parameres are then inserted into pits located within the female pygidial flap (Fig. 2E and 3C). Having achieved a stable copulation position, the endophallus is inflated inside the female genital tract (Werner and Simmons 2008).

RNAi phenotypes

The copulatory structures of control-injected beetles were virtually indistinguishable from wild-type. In contrast, *Dll*-RNAi males had most of the distal portion of the parameres deleted in both *O. taurus* (Fig. 2B, n = 6/7) and *O. binodis* (Fig. S1, n = 7/8), whereas the proximal phallobase appeared unaffected. *dac*-RNAi (Fig. 2C, *O. taurus*), on the other hand, resulted in both a deformation of the parameres (n = 2/7) and a failure of the phallobase to sclerotize properly (7/7). However, neither *Dll*-RNAi nor *dac*-RNAi affected the female pygydium in any obvious manner, in marked contrast to *hth*-RNAi (*O. taurus*): here, 2/5 females showed a dramatic deformation of the pygidial flap, especially in the area of the pygidial pits (Fig. 2F). Additionally, 4/5 males exhibited aedeagal phenotypes ranging from partial (Fig. 2D) to near complete obliteration (Fig. S2). More specifically, in weak male phenotypes, paramere size appeared disproportionally larger relative to the size of the phallobase (Fig. 2D), whereas in more severe cases, *hth* knockdown resulted in major changes in both size and shape (Fig. S2).

Copulatory structures of both sexes were also affected by the knockdown of *dpp*. Specifically, in *O. binodis*, 6/12 males failed to properly sclerotize the distal portion of the phallobase, 3/12 males failed to properly develop their distal parameres (Fig. 3B), and all females examined (8/8) had a substantially reduced pygidial flap (Fig. 3D).

DISCUSSION

We investigated the role of appendage patterning genes in genitalic development of *Onthophagus* beetles. In these beetles, the formation of adult outgrowths, including appendages, does not rely on "imaginal discs" as seen e.g. during the highly derived development of *Drosophila*, but instead involves epidermal outbuddings generally initiated late in larval development, similar to appendage formation across a wide range of holometabolous insects (see Švácha 1992 for examples and discussion of the imaginal disc concept). In *Onthophagus*, this process utilizes epidermis belonging to either structures already in existence in the larval stage (e.g., the larval leg, antenna, or mouthparts), or previously undifferentiated epidermal regions (e.g., wings, horns, aedeagus). However, male genitalia stand out because their growth precedes that of any other adult structure and is visible through the translucent abdominal cuticle already during the first third of the last instar, whereas legs, antennae, mouthparts, and wings initiate their growth not until the final days of the larval stage (Moczek and Nijhout 2002; Moczek 2006).

We found that experimental down-regulation of all four appendage-patterning genes analyzed substantially affected the formation of male copulatory structures in *Onthophagus* beetles. These results are in marked disagreement with previous findings in the model beetle species *Tribolium castaneum* (Aspiras et al. 2011), yet congruent with earlier results in flies and true bugs (Sánchez and Guerrero 2001; Aspiras et al. 2011). Specifically, knockdown phenotypes of copulatory organs paralleled those described previously for other *Onthophagus* appendages (Moczek and Rose 2009), and arthropod appendages in general (Kojima 2004), to a significant degree. *Dll*-RNAi affected the distal region of the aedeagus, similar to what has been observed previously for legs and antennae in *Onthophagus* and diverse arthropods. Similarly, *dac*-RNAi affected the medial regions of the aedeagus, mirroring its effects on a range of other arthropod appendages, including *Onthophagus*. Lastly, *hth*-RNAi resulted in substantial modifications of the architecture of the entire aedeagus, again paralleling

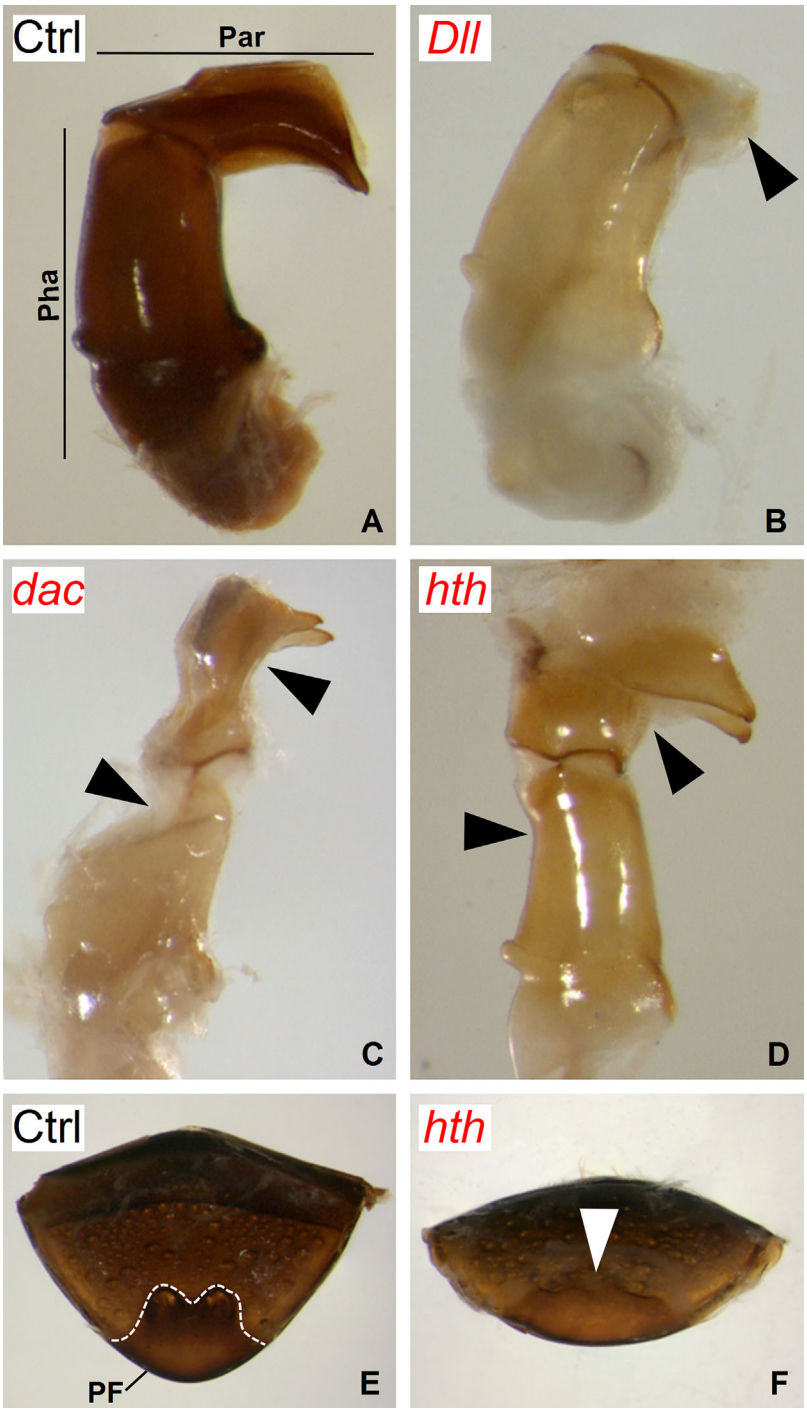

Figure 2. *Distal-less* (*Dll*)-, *homothorax* (*hth*)-, and *dachshund* (*dac*)- knockdown phenotypes in male (A-D) and female (E-F) copulatory structures of *O. taurus*. Arrows highlight anatomical areas where RNAi phenotypes are most evident. Note that tanning (darkening) increases gradually after adult molt; most RNAi individuals died or were fixed prior to achieving the level of tanning typically observed in older wild-type individuals. (A) Male aedeagus, wild-type, left side—parameres (Par) and phallobase (Pha) highlighted. (E) Female pygidium, wild-type, pygidial flap (PF) highlighted. All images are taken at the same magnification.

previous findings for *Onthophagus* legs and antennae (Moczek and Rose 2009), and in line with one of *hth*'s basic functions, namely to specify where developing appendages insert proximally into the body wall. *dpp*-RNAi, in turn, affected both parameres and phallobase, in keeping with its diverse roles in overall appendage growth, such as the proper establishment of the proximo/distal and dorso/ventral axes (Kojima 2004; Wasik and Moczek 2011). Our results therefore reject the hypothesis of non-appendicular origin of male copulatory structures in beetles.

When compared to the wild-type, knockdown phenotypes mainly involved changes in shape rather than in the size of genitalic parts relative to each other, recalling the general observation that genitalic shape is more evolutionarily labile than size, and that shape and size might therefore be under separate developmental control (Arnqvist and Thornhill 1998; Soto et al.,

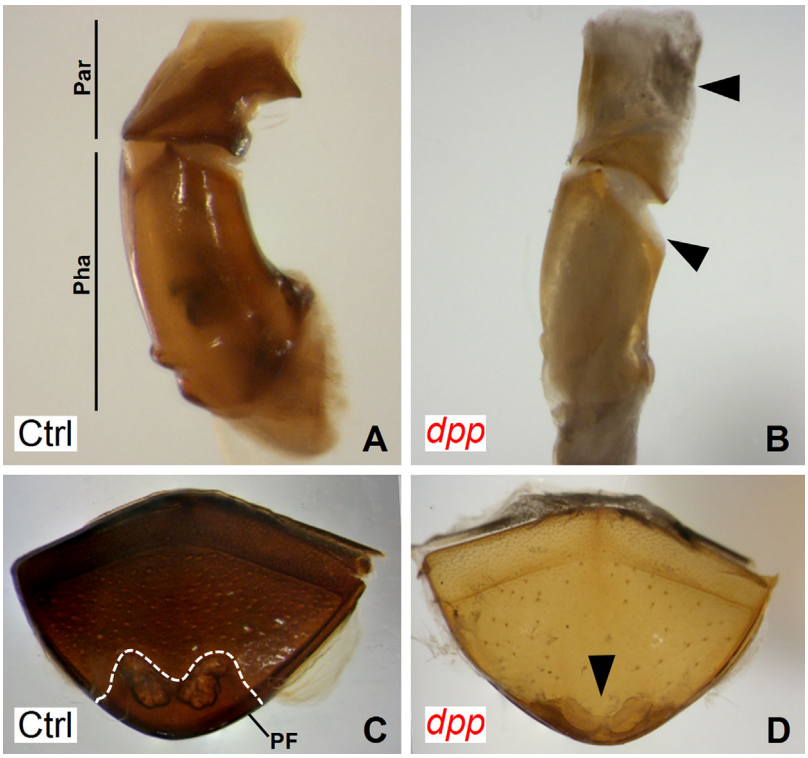

Figure 3. Effects of *decapentaplegic* (*dpp*)-RNAi on the copulatory structures of *O. binodis*. Arrows highlight anatomical areas where effects are most evident. (A-B) Male aedeagus, left side, parameres (Par) and phallobase (Pha) highlighted. (C-D) Female pygidium, pygidial flap (PF) highlighted. All images are taken at the same magnification.

2007; Eberhard et al., 2009; Macagno et al., 2011; Simmons and Garcia-Gonzalez 2011). Future work is clearly needed to further assess this issue. Interestingly, *hth*-RNAi phenotypes constituted an exception to this rule, and involved drastic modifications in both shape and relative size. In weak male phenotypes, paramere size appeared disproportionally larger relative to the size of the phallobase (a modification that resembles some interspecific differences observed in nature, e.g., between *O. taurus* and *O. sagittarius*—Kijimoto et al., 2012 suppl. Fig. S3), whereas in more severe cases, *hth* knockdown resulted in major changes in both size and shape. Notably, *hth*-RNAi also affects the relative sizes of anatomical components of other appendages such as the antenna, causing for example an enlargement of the antennomeres of the scape and funicle, but a reduction of the antennal club (Moczek and Rose 2009).

Collectively, our results firmly support the notion that the male copulatory structures of beetles are homologous to those of other insect orders [Hemiptera (*Oncopeltus fasciatus*: Aspiras et al., 2011), Diptera (*Drosophila*: Sánchez and Guerrero 2001)], and serially homologous to other appendages such as legs and antennae. This is further supported by a recent study on *Photuris* beetles ("fireflies"), where down-regulation of the Hox gene *Abdominal-B* transformed genitalia into legs complete with tarsal claws (Stansbury and Moczek 2014), and raises the question why no such effects were detected in male *Tribolium castaneum* (Aspiras et al. 2011). At least three potential explanations emerge. First, *Tribolium* genitalia may represent a case of massive divergence in the developmental basis of otherwise morphologically homologous structures (developmental systems drift: True and Haag 2001). Alternatively, the negative results of Aspiras et al. (2011) might be explainable based on the observation that the male genitalia of *Tribolium* are partially reduced compared to those of most beetles, including *Onthophagus*. For example, *Tribolium* genitalia do not have obvious parameres (i.e., the structures affected by *Dll*-RNAi in *Onthophagus*; Aspiras et al. 2011). The absence of *Dll*-RNAi phenotypes in male copulatory structures of *Tribolium* may therefore reflect loss/substantial reduction of aedeagal regions normally patterned by *Dll*. However, this does not explain the lack of any effects of *hth* or *dac* down-regulation, which would still be expected to pattern the basal and medial regions of *Tribolium* aedeagi, respectively, raising instead the possibility that RNAi as administered in Aspiras et al. (2011) may simply have been insufficient to detect genitalic phenotypes in *Tribolium*. In their study, qRT-PCR clearly demonstrated the down regulation of target gene transcript abundances, however, timing of dsRNA injections may have occurred too late to affect genitalic growth. In line with other holometabolous insects, *Onthophagus* genitalia start proliferating up to 10 days prior to entering the prepupal stage (Moczek and Nijhout 2004), when other structures such as legs and wings initiate most of their growth. Because in Aspiras et al. (2011) dsRNA was not injected until the prepupal stage, transcript reduction might have occurred too late to affect genital formation in *Tribolium*. More generally, our findings underscore the importance of using emerging model systems, where available, to validate conclusions derived from single model studies.

At the same time, we identified two appendage-patterning genes whose down-regulation also affected *female* copulatory structures. *hth*-RNAi caused a deformation of the entire pygidial flap, while *dpp*-RNAi deleted the pits that male parameres normally hook into during mating. Being part of the body wall, the pygidial flap does not constitute a serial homolog to the male aedeagus, though it is clearly related to it by function, as it interacts tightly with the parameres during copulation (Werner and Simmons 2008). Previous work has shown that the male and female copulatory structures that interact tightly during copula tend to coevolve in *Onthophagus* (Macagno et al., 2011; Simmons and Garcia-Gonzalez 2011) as well as other invertebrates such as Odonata (McPeek et al., 2009; McPeek et al., 2011). Our results suggest that *hth* and *TGFβ signaling* (which *dpp* is part of) may be involved in instructing the concerted development of both aedeagus and pygidial flap, thus representing pathways or pathway components whose targets might facilitate the parallel evolution of male and female copulatory structures, even in cases in which copulatory structures are related only by function but not by descent. Further studies are needed to investigate this intriguing possibility.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site

supporting information Figure S1: Male copulatory structure of *O. binodis*: effect of *Distal-less* (*Dll*)-RNAi. The tips of the parameres are deleted.

supporting information Figure S2:Male copulatory structure of *O. taurus*: strong *homothorax* (*hth*)-RNAi phenotype.