

**A METHOD FOR SEXING FINAL INSTAR LARVAE OF THE GENUS  
*ONTHOPHAGUS* LATREILLE (COLEOPTERA: SCARABAEIDAE)**

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**Abstract**

Dung beetles of the genus *Onthophagus* Latreille are used as model systems in behavioral ecology, evolutionary biology, and insect development. In the past, studies on the developmental control of larval and adult form, and studies that seek to integrate development, evolution, and ecology, have been handicapped by experimenters' inability to determine the sex of developing larvae. Here we present a straightforward, reliable, and non-invasive method that permits sexing of *Onthophagus* Latreille larvae during their early to mid third instar.

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Dung beetles of the genus *Onthophagus* Latreille are receiving growing attention as model systems in behavioral ecology and evolutionary and developmental biology (Emlen and Nijhout 2000; Hunt and Simmons 2000). For example, adult provisioning behavior has been used to study how adult morphology, resource availability, and sex of offspring influence parental investment strategies (Lee and Peng 1982; Cook 1988, 1990; Emlen 1994; Sowig 1996*a, b*; Hunt and Simmons 1998, 2000; Moczek 1998, 1999; Herzner 2000). The presence of elaborate secondary sexual traits, such as horns produced by a subset of males in response to larval nutrition, has led to extensive research into the physiology and evolution of alternative male phenotypes (so called *horn polyphenism*; Emlen 1994, 1996, 1997*a*; Moczek and Emlen 1999; Moczek *et al.* in review). The finding that alternative male phenotypes utilize strikingly different reproductive tactics has made onthophagine beetles ideal study systems to investigate the behavioral ecology and sociobiology of complex mating systems composed of multiple, co-adapted tactics (Cook 1988, 1990; Martin-Piera and Lobo 1993; Emlen 1997*b*; Hunt *et al.* 1999; Hunt and Simmons 2000; Moczek and Emlen 2000).

The recent discovery of rich inter- and intraspecific variation in patterns of morph expression in the genus *Onthophagus* has begun to offer promising opportunities for comparative studies into the evolution of horn polyphenisms (Emlen 1996, 2000). Most importantly, recent advances in our understanding of the developmental and endocrine mechanisms that specify adult form during the larval stage have led to the formulation of testable hypotheses about the role of developmental processes in the evolution of horn polyphenisms and the origin of the enormous morphological and behavioral diversity represented by horned beetles (Hunt and Simmons 1998; Stern and Emlen 1999; Emlen and Nijhout 1999, 2000; Emlen 2000; Shafiei *et al.* 2001). However, studies that have tried to understand the origin of morphological variation in adults by investigating the developmental and endocrine events that take place during the larval stage have been severely handicapped by experimenters' inability to

determine the sex of developing larvae (Hunt and Simmons 1997; Emlen and Nijhout 1999; Emlen 2000; Herzner 2000). Many developmental events are sex specific, and some occur only in a subset of individuals within a sex (Emlen 2000). For example, in *O. taurus* Schreber the developmental decision whether to express a horned or hornless morph takes place only in male larvae and only during the last days of the third and final larval instar (Emlen and Nijhout 1999; Shafiei *et al.* 2001). Therefore, standard experimental approaches such as hormone manipulations, hormone titer quantifications, or surgical manipulations aimed at identifying the developmental control of morph determination, have to be conducted on a large number of larvae without knowledge of their sex. Larvae then have to survive to at least the pupal stage before they could be sexed (Emlen and Nijhout 1999). As a consequence, experimenters have had to limit themselves to procedures that larvae were able to survive through pupation. Furthermore, measurements on approximately half of all experimental animals have had to be discarded because larvae developed into females, and hence were uninformative in respect to questions concerning morph determination. Other studies were handicapped by similar problems (*e.g.*, tradeoffs during larval development: Emlen 2000; larval mortality risks as a function of sex and morph: Hunt and Simmons 1997; parental investment into male and female offspring: Herzner 2000). Here we present a straightforward, reliable, and non-invasive method that allows experimenters to sex *Onthophagus* larvae during their early to mid third instar, thus minimizing the number of uninformative experimental animals in future studies.

### Material and Methods

**Species.** We used three species for our study: *Onthophagus taurus* Schreber, *O. hecate* Panzer, and *O. nigriventris* d'Orbigny. All species were kept as at least second generation laboratory colonies in an insectary at Duke University at 26°C, 60% relative humidity (RH), and a 16:8 light:dark (L:D) photoperiod. *Onthophagus taurus* and *O. hecate* were originally collected from pastures near Durham, NC, whereas *O. nigriventris* was collected from pastures near Beechmont, Queensland, Australia. Most of the work presented here was done on *O. taurus*, whereas the latter two species were used to test whether our results can be extended to other species.

**Breeding.** Five pairs of adult beetles were placed in plastic containers 35 cm in height and 20 cm in diameter, 3/4 filled with a moist 2:1 sand:soil mixture and provided with *ca.* 1 liter of fresh cow dung, and allowed to breed. Adults and brood balls were collected after one week. Brood balls were stored for 1–2 days in containers filled with moist sand:soil mixture. Brood balls were then opened and larvae transferred into artificial brood chambers.

**Artificial Brood Chambers.** Larvae normally complete their entire development within the confines of a brood ball, which provides both food and shelter to growing larvae (Halffter and Edmonds 1982). Our experiment required daily observation and frequent manipulation of larvae. We therefore developed artificial brood chambers that allowed us to examine larvae when needed while minimizing disturbance and ensuring continued food supply. We used sterilized 12-well tissue culture plates (Costar<sup>®</sup>) to design artificial brood chambers. Each well was filled with cow manure that had previously been squeezed through a cheese cloth to remove excess moisture and to obtain a consistency similar to that of brood balls naturally produced by adult beetles. Dung was pressed tightly into each well and a small chamber was carved into

the center to provide room for a larva. Each well contained a single larva. The amount of dung in each well was in excess of the amount typically provisioned by adult beetles. Larvae usually started feeding on the provided manure within 1 h and regularly completed larval development, pupated, and eclosed without any indication that growth conditions were suboptimal. Both male morphs were obtained from rearing beetles in this fashion. Except for a brief examination (30 min per day) artificial brood chambers were kept in the dark at 26°C, 60% RH.

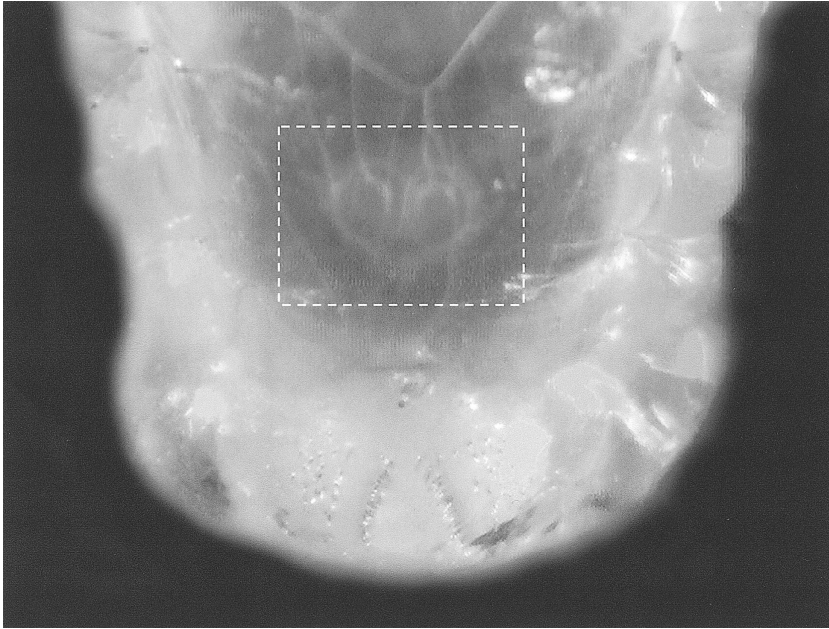
**Observations.** One group of larvae was observed every other day starting with the first instar and ending with the pupal stage. A second group was used to examine the third instar in detail. Larvae in this group were transferred to artificial brood chambers as late second instars and observed daily after the molt to the third instar until pupation. Observations consisted of careful, whole-body examination (approximately 3 min/larva) using a Wild<sup>®</sup> dissecting scope. We gave particular attention to the abdominal segments to detect possible morphological changes associated with the early development of future primary sexual traits. Observations were recorded and later correlated with the actual sex expressed by the individual. Actual or final sex was determined in pupae (clearly evaginated aedeagus in males only) or adults (horns in males only, pygidium morphology (Balthasar 1963), presence/absence of aedeagus). Larvae that died before reaching the pupal stage were excluded from the analysis.

**Cauterization.** Observations indicated a pair of abdominal imaginal disc-like structures (henceforth also referred to as *discs*) to be associated with males only (see *Results*). To identify the adult trait produced by these discs we cauterized the disc region of ten *O. taurus* between day 8 and 12 of the third instar. We recorded any morphological changes in pupae (by observation) and adults (dissection of abdomen).

**Applicability to Other Species.** To test whether our results can be extended to other species, we reared *O. hecate* and *O. nigriventris* larvae as described above. Third-instar larvae were checked every other day for the appearance of discs. Presence or absence of discs was then correlated with the sex of the adult. Unless otherwise noted, all results are presented as means  $\pm$  SD.

## Results and Discussion

We were unable to detect any differences between male and female larvae during the first and second instar. The only noticeable and recurring difference between larvae occurred in the third instar and consisted of a pair of disc-like structures that developed in 69 (= 54%;  $n = 128$ ) of these larvae (Fig. 1). Disc-like structures became visible on day  $7 \pm 1$  ( $n = 29$ ) of the third instar, which on average lasted  $16 \pm 1.7$  days under laboratory conditions ( $n = 41$ ). Discs appeared near the second-last abdominal segment and were visible just below the ventral abdominal cuticle but above the abdominal muscles and the gut system. Discs started out as translucent structures and became increasingly opaque in the second half of the third instar. Discs grew to a final size of approximately 1mm in diameter (both discs combined) by the time larvae purged their gut. By that time both discs were no longer discernible as two separate structures but appeared as one nearly perfectly round structure. All larvae in which disc-like structures were observed developed into males ( $n = 69$ ), whereas the remainder, where no such discs were observed, developed



**Fig. 1.** Ventral abdominal segments of a male *Onthophagus taurus* larva on day 10 of the third instar. Note pair of translucent, disc-like structures in the center of the image (surrounded by dashed line). Discs are the larval precursors of the adult male aedeagus. Cautery of larval discs results in absence of the aedeagus in adult males. Females do not express discs-like structures during the larval stage.

into females ( $n = 59$ ). This finding indicates that discs are expressed in a sex-specific manner.

We cauterized the region of the abdominal discs in ten presumptive male *O. taurus* larvae. Nine larvae pupated and eclosed to adulthood. In eight out of nine pupae the otherwise clearly evaginated pupal aedeagus was missing. The ninth individual expressed a vestigial pupal aedeagus. Dissection of adults revealed that the entire aedeagus was lacking ( $n = 8$ ) or only partially developed ( $\sim 2/5$  of normal length;  $n = 1$ ) in otherwise fully intact animals.

Disc-like structures were also observed in six out of nine *Onthophagus hecate*, and in two out of six *O. nigriventris* larvae. In all cases, only larvae that expressed abdominal disc-like structures developed into males, whereas the remainder developed into females.

Combined, these results suggest strongly that the disc-like structures described in this study are responsible for the development of the adult male copulatory organ. Consequently, appearance of these discs should be sex-specific and should enable experimenters to reliably identify sex of developing larvae in a non-invasive fashion. Our results also suggest that this may be true for *Onthophagus* species in general. *Onthophagus* beetles promise great potential in the current quest for an integration of developmental and evolutionary biology (Emlen 2000; Schlichting and Pigliucci 1999) and we hope that

our results will help to ease and optimize the design of future experiments on this group of beetles.

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