

## Research Article

# Improved Visualization of *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae)—Part I: Morphological Features for Sex Determination of Multiple Stadia

J. F. Esquivel,<sup>1</sup> T. L. Crippen,<sup>2</sup> and L. A. Ward<sup>3</sup>

<sup>1</sup>Areawide Pest Management Research Unit, Southern Plains Agricultural Research Center, Agricultural Research Service, United States Department of Agriculture, 2771 F&B Road, College Station, TX 77845, USA

<sup>2</sup>Food and Feed Safety Research Unit, Southern Plains Agricultural Research Center, Agricultural Research Service, United States Department of Agriculture, 2881 F&B Road, College Station, TX 77845, USA

<sup>3</sup>Department of Entomology, Texas A&M University, College Station, TX 77843, USA

Correspondence should be addressed to J. F. Esquivel, [jesus.esquivel@ars.usda.gov](mailto:jesus.esquivel@ars.usda.gov)

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The lesser mealworm, *Alphitobius diaperinus* (Panzer), is a perennial pest of poultry facilities and known to transmit pathogens of poultry and humans. Ongoing research examining reservoir potential of *A. diaperinus* revealed the need for a comprehensive, user-friendly guide for determining sex of *A. diaperinus* at different stadia. This paper is unique in providing a comprehensive illustrated guide of characters used for differentiation of sexes in *A. diaperinus*.

## 1. Introduction

The lesser mealworm, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae), is a perennial pest of poultry facilities. The species is of tropical origin from sub-Saharan Africa and has adapted to the moist, temperature controlled environments of poultry production facilities [1]. As a nocturnal species, the low-light conditions within broiler grow-out houses are conducive for populations to thrive [2]. Peak population densities occur during the warmer summer season but the pest is present throughout the year [3]. Omnivorous feeding habits allow *A. diaperinus* to feed on manure, feed, chicken carcasses, detritus, and other larvae and beetles [4–7].

Generally, 5 to 8 wks are required for *A. diaperinus* to complete a life cycle within a broiler grow-out house; ambient temperature and humidity will affect the life span [8]. Female beetles oviposit in sheltered areas of the house, depositing 1000 to 1800 fertile eggs over their life span [2, 9–11]. Eggs hatch in 4–7 days and larvae live in the moist litter and along the building walls [12]. The larvae progress through 6 to 11 stadia over a span of 30 to 100 days before

reaching adulthood [9, 13, 14]. Late instars nearing pupation seek a drier environment by tunneling into compacted earthen floors beneath the litter or into the insulation and crevices of the building walls [15]. Adults emerge after 7 to 8 days. Under laboratory conditions, life span of an adult can exceed a year; however, in field setting, longevity of adults and larvae is influenced by predation from poultry and abiotic conditions.

In addition to causing structural damage to poultry houses, *A. diaperinus* has been associated with the transmission of pathogens (e.g., *Salmonella*) which can be potentially fatal to poultry and, more importantly, humans [16]. Consequently, studies are ongoing to further determine the reservoir capacity of lesser mealworm. Factors that may affect reservoir capacity and transmission potential are sex of beetles and larvae and size of the respective alimentary canals. Size of the *A. diaperinus* alimentary canal and its respective sections are presented in a companion paper in this issue [17]. Rapid determination of sex in the field settings of the poultry facilities would enable more timely and proactive approaches to beetle management. However, during the course of examining, the reservoir potential and alimentary

canal capacity of *A. diaperinus*, it was determined that a comprehensive assessment of characters for determining sex of *A. diaperinus* was lacking. Schematics of genitalia for determining sex of pupae and beetles are available [18–20], but these schematics fail to clearly reflect the orientation and morphology of said features. Further, secondary sexual characteristics, such as orientation of tibial spurs within *A. diaperinus* adults differ and reports vary in the description and use of either mesothoracic or metathoracic tibial spurs to determine sex [21, 22]. The objective of this paper is to improve upon extant schematics and establish a definitive illustrated guide of morphological characters for determining sex of *A. diaperinus*.

## 2. Materials and Methods

**2.1. Insect Rearing.** The Southern Plains Agricultural Research Center (SPARC) starter colony of *A. diaperinus* was obtained from a colony originally isolated from a poultry farm in Wake County, NC, maintained by Dr. D. W. Watson (North Carolina State University, Raleigh, NC). The SPARC colony has remained in production since 2004. The colony was reared in plastic containers (15 × 15 × 30 cm) with screened tops; containers held 1000 mL wheat bran (Morrison Milling Co., Denton, TX) on the floor surface. A 6 cm<sup>2</sup> sponge moistened with deionized water and a slice (0.5 cm thick) of apple provided a water and food source, respectively. Both were replenished twice per week. The moistened sponge was placed on a piece of aluminum foil to prevent contact with the bran. Also, 30 mL of fishmeal (Omega Protein, Inc., Hammond, LA) were added to the wheat bran once per week; new wheat bran was added as it was depleted by dropping through the screened bottom of the cage. Layered black construction paper (6 × 6 cm) was provided as an oviposition substrate to allow collection of eggs to sustain the colony. Eggs were transferred to a separate container and resulting larvae, pupae, and adults were maintained as described above. The colony was maintained at 30°C in an 8:16 hr (light:dark) photoperiod.

**2.2. Validity of Characters for Determining Sex.** Late instars reportedly retain pygopods upon pupation and resulting pygopods on pupae reflect female pupae [18]. Three cohorts of 25, 85, and 96 larvae were sorted based on presence of pygopods and development was monitored from larvae to adulthood. Resulting pupae were sorted based on previously reported characters [18, 23] and eclosing adults were sexed to confirm relationship with pygopods on pupae. Paired pygopods in the larval stage, present ventrally on the 10th abdominal segment, aid in locomotion [24].

**2.3. Preparation of Specimens.** Late-instar larvae, pupae, and adults were collected from rearing containers and placed in respective 25 dram vials. Vials containing larvae and pupae were placed in a freezer to kill the insects; adults were killed by adding 80% ethyl alcohol (EtOH) to vials. Adults in EtOH-filled vials were briefly agitated by hand to remove adhering diet and frass. Larvae and pupae were cleaned by

brushing with a fine-tipped paintbrush dipped in EtOH, since full immersion increased transparency of their more weakly sclerotized, lighter pigmented cuticle.

Specimens were allowed to air dry on a clean Kimwipe towel. Larvae and pupae were pinned through the thorax to prepare them for imaging. For adults, slight pressure was applied to the abdomens using fine-tip forceps to evert genitalia before pinning. Additional adults were processed to document differences and orientation of tibial spurs between sexes. Tibiae of the mesothoracic legs were excised and individually point mounted by their proximal ends for imaging apical spurs.

**2.4. Imaging.** A specially constructed viewing arena consisted of an 80 LED ring light (Model KD-200; Gain Express Holdings Ltd, Hong Kong) with a modified pinning stage located within the center of the ring. A small (7.62 × 2.54 × 1.27 cm) Styrofoam bar attached to the internal face of the ring light provided an additional, vertical pinning surface to facilitate orientation of specimens. A removable hemisphere-shaped dome with a 2.7 mm viewing aperture fit over the outer edge of the ring to uniformly distribute light within the arena.

Point mounted or direct-pinned specimens of all stadia and excised tibiae were placed in the viewing arena and manipulated for best orientation under a Leica MZ16 microscope equipped with APO lens (Leica Microsystems, Wetzlar, Germany). A ProgRes 3008 digital camera mounted on the microscope interfaced with a Windows-driven operating system. Sequentially focused images of each specimen were captured using PictureFrame 2.3 software (Optronics, Goleta, CA). Each image series was subsequently processed using Auto-Montage Pro software 5.01.0005 (Syncroscopy USA, Frederick, MD) to construct one composite image with enhanced depth of field. Adobe Photoshop CS5 (Adobe Systems, Inc., San Jose, CA) was used to improve clarity of composite images.

## 3. Results

**3.1. Larvae.** All late-instars in two cohorts ( $n = 110$  total) possessed a pair of prominent fleshy pygopods ventral to the pygidium (Figures 1(a) and 1(b)). In the third cohort, a small percentage of larvae (12.5%;  $n = 96$ ) did not exhibit prominent pygopods; instead the pygopods were unapparent, or much reduced (Figures 1(c) and 1(d)). A previous report suggested that the pygopods [24] were retained to the female pupal stage (discussed below). However, sex ratios of resulting pupae from sorted larval cohorts indicate that presence of pygopods does not exclusively reflect females. Of the larvae with prominent pygopods surviving to adulthood, 56.9% ( $n = 86$ ) of adults were males. Similarly, adult males comprised 58.3% ( $n = 12$ ) of the larvae with unapparent pygopods. Both sexes possess a urogomphus (Figure 1).

**3.2. Pupae.** The pupae of both sexes of *A. diaperinus* possess a pair of urogomphus dorsally (Figure 2). The larval urogomphus are retained to the pupal stage [18], and,

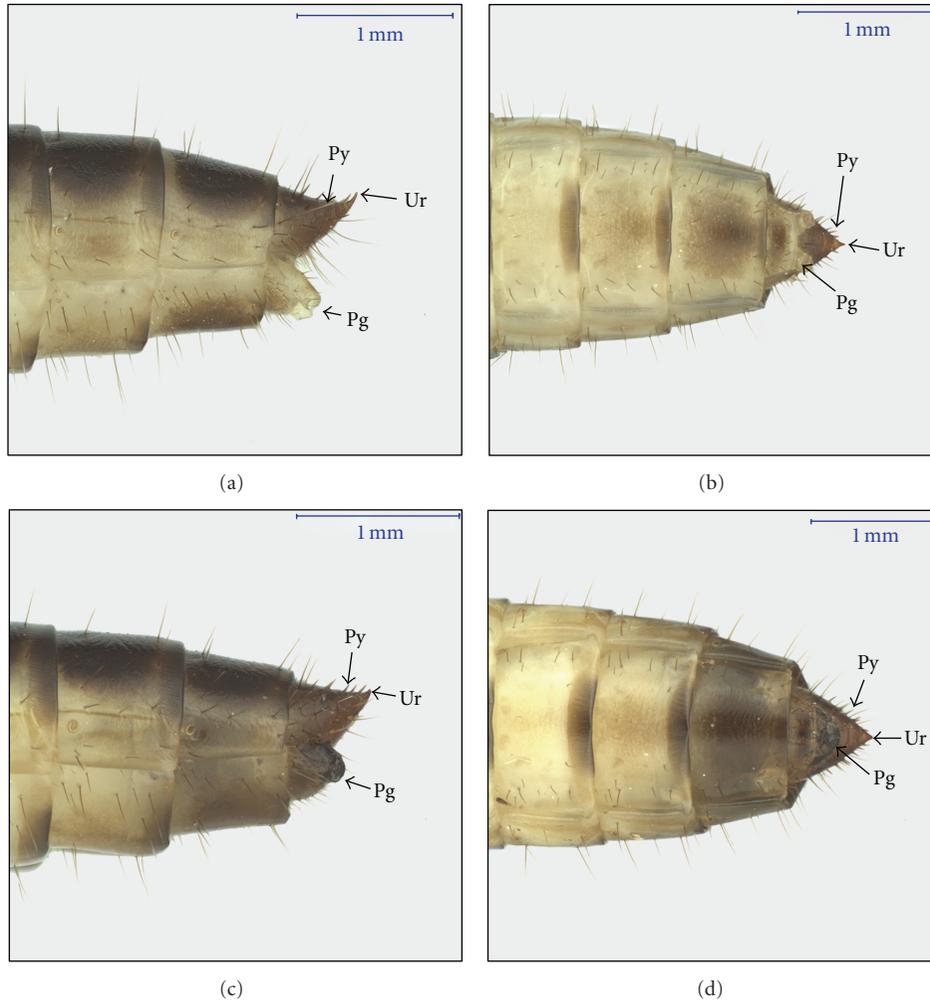


FIGURE 1: External posterior characteristics of late-instar *A. diaperinus*: with prominent pygopods ((a) lateral view and (b) ventral view) and unapparent, or much reduced, pygopods ((c) lateral view and (d) ventral view). Pg, pygopod; Py, pygidium; Ur, urogomphus.

reportedly, the additional pair of prominent processes seen ventrally in female pupae (Figures 2(a) and 2(b)) are the paired pygopods retained from the larval stage (Figure 1(a)) [19]. However, as previously demonstrated, not all larvae with prominent pygopods are females. Conversely, all pupae with prominent pygopods ( $n = 37$ ) yielded female beetles; males resulted from all pupae without pygopods ( $n = 49$ ) (Figures 2(c) and 2(d)). Thus, in the pupal stage, pygopod presence is a reliable indicator of sex. Newly formed pupae are typically a pearly white color [18] but the urogomphus and the pygopods, if present, darken as the pupae age [25].

**3.3. Tibial Spurs of Adults.** Paired tibial spurs are present apically on the tibiae of all legs, but orientation of the spurs on the mesothoracic and metathoracic legs can aid in differentiation of sexes. The spurs arise from the anterior and posterior apical corners of the tibiae (Figure 3). In females, both spurs of the mesothoracic tibiae are parallel to each other and align along the longitudinal axis of the tibia (Figure 3(a)).

Conversely, the mesothoracic tibial spurs on the male are not parallel to each other. In males, the anterior spur curves away from the longitudinal axis of the tibia without much deviation in the horizontal axis (i.e., not curved in the direction of either the anterior or posterior face of the tibia; Figures 3(b) and 3(c)). In fact, the small size and lack of color contrast often obscure the spur's curvature when the tibia is examined directly from a dorsal or ventral perspective. In these cases, the anterior spur may superficially appear shortened relative to the posterior spur until the segment is rotated (or higher magnification/better lighting/etc. is utilized). The posterior spur remains straight and generally in line with the longitudinal axis of the male tibiae. This same orientation of the tibial spurs is present on the metathoracic tibiae of males. However, the curvature of the anterior spur on the metathoracic tibiae of the males is less extreme than the curvature of the spur on the mesothoracic tibiae [21]. Only images of mesothoracic spurs on males are provided here because their sharper curvature relative to the metathoracic spurs enables more rapid determination of sex.

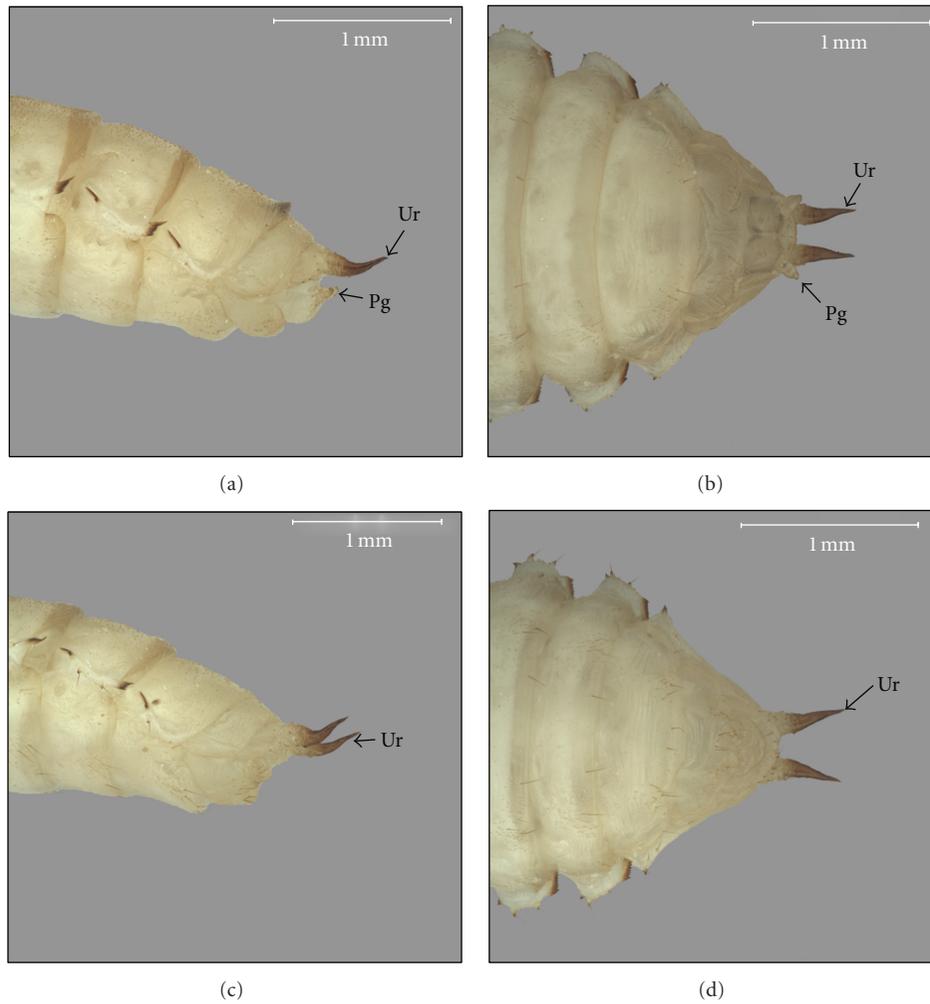


FIGURE 2: External posterior characteristics for determining sex of *A. diaperinus* pupae: female with urogomphus and prominent pygopods ((a) lateral view and (b) ventral view) and male with urogomphus, lacking prominent pygopods ((c) lateral view and (d) ventral view). Pg, pygopods; Ur, urogomphus.

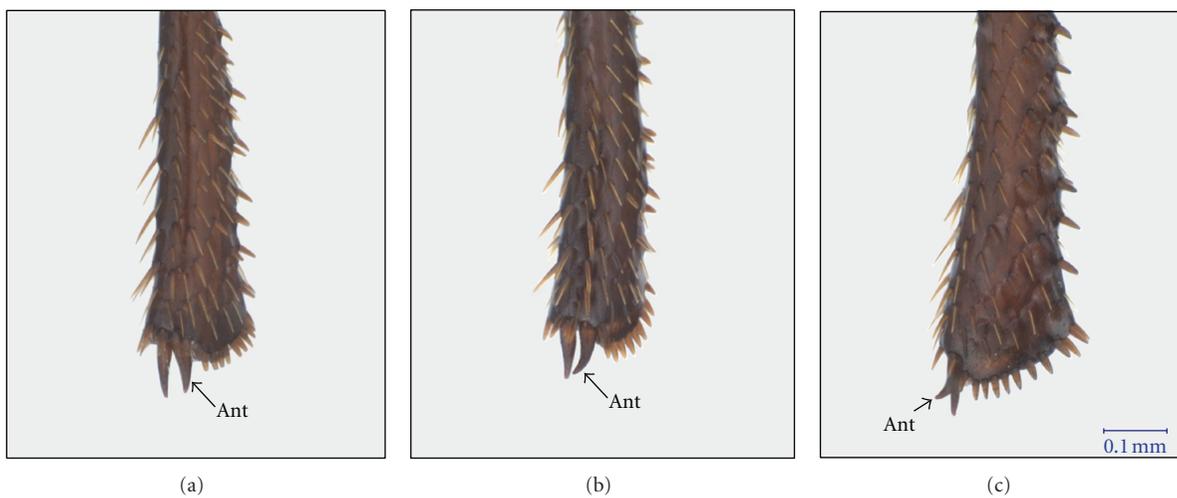


FIGURE 3: External characteristics of mesothoracic tibiae for determining sex of *A. diaperinus* adults: (a) anteroventral view of parallel apical spurs on females; (b) and (c) anteroventral and anterior views, respectively, of apical spurs on males, note curvature of anterior spur. Ant, anterior spur; scale bar applicable to all frames; tibiae from left legs shown.

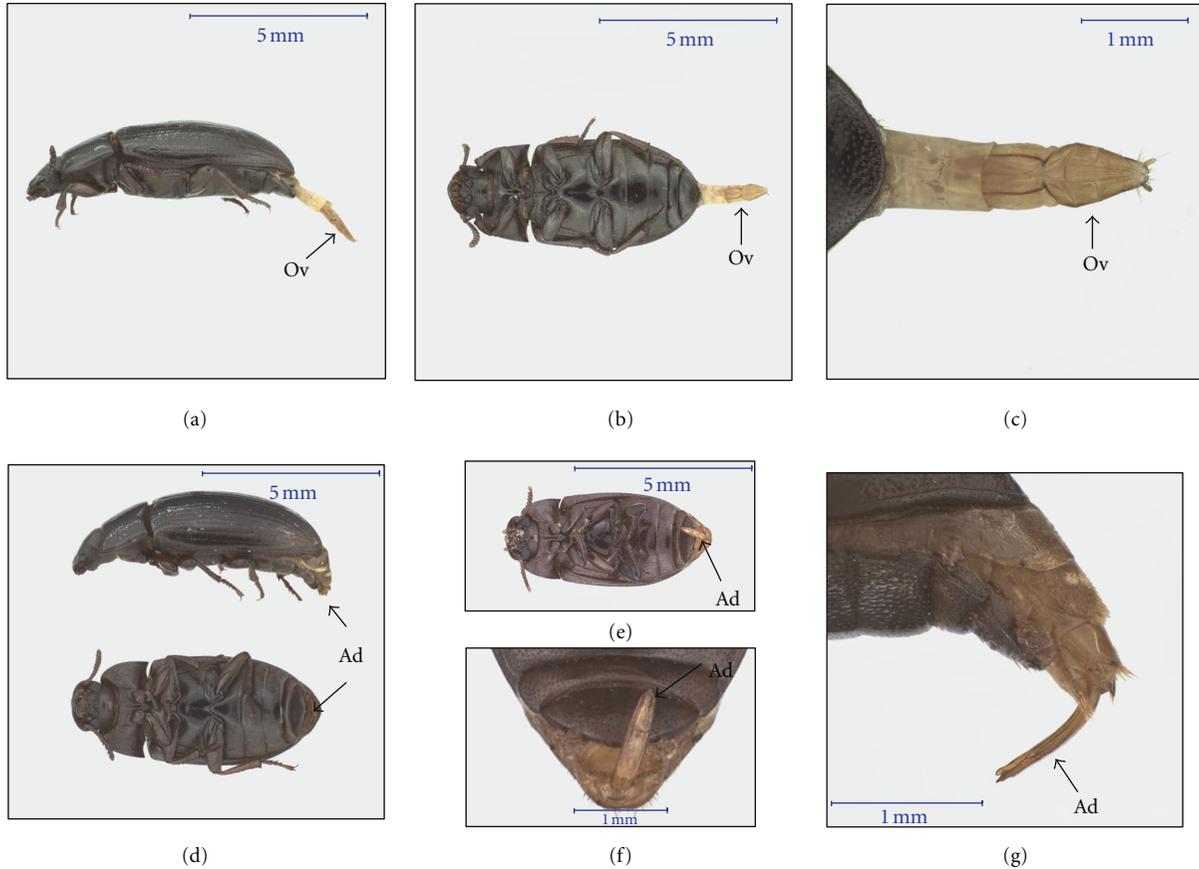


FIGURE 4: Genitalia of *A. diaperinus* adults: female ovipositor:protracted, lateral (a), and ventral views (b, c); male aedeagus:retracted, lateral, and ventral views (d); protracted, ventral (e, f), and lateral views (g). Ad, aedeagus; Ov, ovipositor.

**3.4. Adult Genitalia.** Images of *A. diaperinus* genitalia (Figure 4) improve upon previous drawings [19, 20]. Coloration of the distal end and along the length of the ovipositor indicates sclerotization within the organ membrane (Figures 4(a)–4(c)). Two dark longitudinal lines dorsally and ventrally within the ovipositor suggest sclerotization along the length which may aid in movement of the ovipositor (i.e., protraction and retraction, directionality). In fact, the dorsal lines were observed to aid in the opening and closing of the anus. The paired cerci protruding from the end of the ovipositor each possess a solitary seta, likely to aid in site selection for oviposition. The male aedeagus is a sclerotized organ and, if not readily visible (Figure 4(d)), can be protracted by squeezing of the abdomen (Figures 4(e)–4(g)), although more pressure is required than that used on females. When protracted, the aedeagus may have a curvature to either side of the longitudinal midline of insect body (Figures 4(e) and 4(f)) and is projected away from the body (Figure 4(g)).

#### 4. Discussion

This paper is unique in providing a comprehensive photographic guide for differentiation of sexes in *A. diaperinus*. Further, this paper clarifies that the prominent fleshy

processes observed ventrally on female larvae are pygopods. Although general dorsal, lateral, and ventral views of *A. diaperinus* larvae have been provided [9, 22], sex determination of late-instars has been previously alluded to because the pygopods were reportedly retained from late instars to the female pupal stages [18]. Visual aides were not provided to unequivocally establish the external morphology of late instars; however, our observations indicate determination of sex in the larval stages based on the presence of pygopods is unreliable. The urogomphus has been used to differentiate between species of *Alphitobius* [26], but this is the first record to visually demonstrate the variation of pygopods in *A. diaperinus* larvae.

This paper clarifies the definition of the ventral paired processes in female pupae as remnants of the paired pygopods observed in larvae. The pygopods were previously noted but incorrectly identified as “genital appendages” and “second valvifers” [18, 23]. Line drawings of external characters on pupae for differentiating between sexes [18] fail to show potential coloration of urogomphus and pygopods. Figure 2 allows more clear identification of these external characters, including representation of coloration for these characters. Newly formed pupae are completely white [18]. Coloration of external characters suggests these pupae

(Figure 2) are not newly formed; thus, coloration could potentially be used as an indicator of pupal age. However, the latter was outside the scope of this paper.

Differences in the orientation of tibial spurs of *Alphitobius laevigatus* (F.) have been previously shown [21, 22]. The curvatures of tibial spurs on *A. laevigatus* reportedly [21] resemble those of *A. diaperinus* but comparative illustrations of *A. diaperinus* tibial spurs were not provided. Viewing angle of the specimen is critical when assessing spur orientation to determine sex; more so, if using the metathoracic tibial spurs [22] because the curvature of male metathoracic spurs is “so slight as to be barely noticeable” [21]. Thus, it is recommended that the mesothoracic spurs be used for determination of sexes for adults, in addition to examination of genitalia. Descriptions presented here regarding location and orientation of the spurs are more thorough and images more clearly delineate differences between mesothoracic spurs of respective sexes.

Differing intensity of coloration was observed between ovipositors of different females. Whether coloration intensity of ovipositors reflects age remains to be determined. In sexing dead adults, either the cerci were protruding slightly from the last abdominal segment or the ovipositor was protruding altogether. If neither were evident, application of slight pressure to the abdomen caused the cerci to protrude. Generally, if the ovipositor and cerci are not immediately visible after applying pressure to the abdomen, in all likelihood the insect is a male. However, to confirm male gender, applying more pressure to the abdomen caused protrusion of the aedeagus for confirmation (Figures 4(e)–4(g)). Detailed descriptions and morphometrics of the ovipositor and the aedeagus were previously reported [19, 20].

This comprehensive guide is user-friendly towards novice entomologists and nonentomologists (e.g., microbiologists, pathologists) and will be an invaluable tool for those entering the study area of *A. diaperinus* and pathogen interactions affecting poultry and humans.

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