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Morphology of the female reproductive system and physiological age-grading of *Megamelus scutellaris* (Hemiptera: Delphacidae), a biological control agent of water hyacinth

Elizabeth Mattison[1], Ted D. Center[1], Michael J. Grodowitz[2,], and Phillip W. Tipping[1]*

Abstract

The morphology of the female reproductive system in *Megamelus scutellaris* Berg (Hemiptera: Delphacidae), a biological control agent of *Eichhornia crassipes* (Mart.) Solms (Pontederiaceae), was examined with standard light microscopy techniques. Ovaries extracted from individuals dissected in phosphate buffered saline were examined with both stereo and compound microscopy. This species was typical of other delphacid planthoppers in that the reproductive system was composed of 2 ovaries with each consisting of 11 to 13 tubular ovarioles. The lateral oviduct from each ovary led to a common oviduct. Ovaries were of the telotrophic meroistic type wherein nurse cells were within the distal germarium. an enlarged sac-like bursa copulatrix opened to the common oviduct just posterior to the spermatheca. The bursa copulatrix contained globular structures that we interpreted to be spermatophores. More mature follicles contained a yolk-filled oocyte with a distinct germinal vesicle or enlarged nucleus present in the distal region of the follicle. Each oocyte was surrounded by a single layer of cells, termed the follicular epithelium, and the entire ovariole was surrounded by a thin membranous layer, the ovariole sheath. A unique structure, surrounding the most distal portion of the lateral oviduct and appearing as a loose grouping of cells that opened proximally, was visible at the base of each ovariole in reproductive females. This structure, which we termed the "collar," did not appear to be attached to the ovarioles or the lateral oviduct tissues and has not previously been reported in other planthopper or insect species. The continuum of ovarian development was divided into 3 nulliparous and 3 parous stages based on ovariole differentiation, presence and appearance of follicular relics, and collar length, which increased linearly with increasing numbers of ovulations. Taken together, these characteristics can be used to estimate the reproductive condition and number of ovulations of this biocontrol agent.

Key Words: *Eichhornia crassipes*; ovary; ovariole; follicular relics

Resumen

Se examinó la morfología del sistema reproductor femenino en *Megamelus scutellaris* Berg (Hemiptera: Delphacidae), un agente de biocontrol de *Eichhornia crassipes* (Mart.) Solms (Pontederiaceae), con técnicas de microscopía óptica estándar. Se examinaron los ovarios extraídos de individuos disecados en solución salina tamponada con fosfato con microscopía estereoscópica y compuesta. Estos fueron típicos de otros plantófilos delphacid en que el sistema reproductor estaba compuesto de 2 ovarios con cada uno consistente de 11 a 13 ovarioles tubulares. El oviducto lateral de cada ovario condujo a un oviducto común. Los ovarios eran del tipo meroísta telotrófico donde las células enfermeras estaban dentro del germario distal. Un bursa copulatrix de tipo saco ampliado se abre al oviducto común justo posterior a la espermateca. La bursa copulatrix contenía estructuras globulares que interpretamos como espermatóforos. Los folículos más maduros contenían un ovocito lleno de yema de huevo con una vesícula germinal distinta o un núcleo ampliado presente en la región distal del folículo. Cada ovocito estaba rodeado por una sola capa de células, denominada epitelio folicular, y el ovariole entero estaba rodeado por una fina capa membranosa, la vaina ovariole. Una estructura única que rodeaba la porción más distal del oviducto lateral y que aparecía como un grupo suelto de células que se abrían proximalmente era visible en la base de cada ovariole en hembras reproductivas. Esta estructura, que denominamos el cuello, no parecía estar unida a los ovarioles ni a los tejidos laterales del oviducto y no ha sido reportada previamente en otras especies de saltamontes o insectos. Se dividió el continuo del desarrollo ovárico en 3 estadios nulíparos y 3 parosos, basados en la diferenciación ovariole, la presencia y aparición de reliquias foliculares y la longitud del collar, que aumentó linealmente con el aumento del número de ovulaciones. En conjunto, estas características se pueden utilizar para estimar la condición reproductiva y el número de ovulaciones.

Palabras Clave: *Eichhornia crassipes*; ovario; ovariole; reliquias foliculares

An understanding of the reproductive biology of a plant-feeding insect used as a biological control agent is useful for interpretation of host range studies and performance of the agent under field conditions (Center & Dray 2010; Center et al. 2014). The degree and extent of ovarian development can figure prominently in the response of these insects to proffered test plant species during biological control efficacy and host specificity experiments. Poor performance on a putative host plant may be associated with poor host quality or an incorrect associa-

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tion of the insect with the host species. Likewise, if host quality relates to reproductive performance of the insect, it is important that this is comparable to all test species to ensure valid experimental conditions. In addition, establishment and population increase in the field or lack thereof can relate to host quality as manifested in reproductive condition. For these reasons, we studied the reproductive morphology of *Megamelus scutellaris* Berg (Hemiptera: Delphacidae) as well as progressive changes associated with increasing frequencies of oviposition (i.e., physiological age-grading).

Megamelus scutellaris has recently become of interest as a prospective biological control agent for water hyacinth (*Eichhornia crassipes* (Mart.) Solms) (Pontederiaceae) (Center et al. 2002; Sosa et al. 2004; Sosa et al. 2007a). It was described by Berg in 1883 in Argentina but remained little known until efforts to discover biological control agents of this floating aquatic weed resumed during the late 1990s (Center et al. 2002). A related species attacking *E. crassipes*, *Megamelus electrae* Muir, was briefly studied in Trinidad for use as a biological control agent by Cruttwell (1973), but further studies were never pursued. Interest in additional agents waned after the introduction of the weevils *Neochetina eichhorniae* Warner (Coleoptera: Brachyceridae) and *Neochetina bruchi* Hustache, and the pyralid moth *Niphograpta albiguttalis* (Warren) (Lepidoptera: Crambidae) into North America during 1972, 1974, and 1977, respectively. These agents performed well in some systems, but were largely ineffective in water bodies subjected to intensive herbicidal management due to the periodic, although often temporary, elimination of the host plant. This led to the realization that additional agents were needed, particularly vagile species capable of producing large populations rapidly and thereby able to thrive in the unstable circumstances associated with weed management activities. Therefore, additional surveys were undertaken in South America where *M. scutellaris* was soon recognized as a species of promise (Center et al. 2002; Sosa et al. 2007b). It was subsequently redescribed (Sosa et al. 2004) and preliminary studies done in South America confirmed its narrow host range and potential efficacy (Sosa et al. 2007a). As a result, *M. scutellaris* was imported into US quarantine during 2006 for further host range evaluation and was subsequently released in the US in 2010 (Tipping et al. 2011). Field establishment has been confirmed (Patrick Moran, United States Department of Agriculture [USDA] Agricultural Research Service [ARS], personal communication; Tipping et al. 2014) though failures to establish have been reported for sites in southern Louisiana (Grodowitz et al. 2014) and California (Patrick Moran, USDA-ARS, personal communication). The causes of these failures remain unknown but lack of reproduction due to inadequate host quality is a possibility.

Materials and Methods

Planthoppers used in this study were from colonies maintained in quarantine at the USDA-ARS Invasive Plant Research Laboratory located in Fort Lauderdale, Florida. The colonies were derived from individuals collected during Mar 2006 at Otamedi Natural Preserve, Buenos Aires Province, Argentina. Females were selected randomly from a single cohort within a few hours of emergence. They were provided *E. crassipes* leaf petioles as an oviposition substrate. Individuals were followed for over 1 mo and then dissected as described below. Terminology of the female reproductive system follows that of Mochida (1973) and Heady and Wilson (1990) with minor modifications as noted.

Adult females were collected from the colonies and pinned ventral side up in a wax-lined dish. Two pins were inserted through the thorax, which anchored the planthopper to the wax. The specimen was then covered with a phosphate-buffered saline solution (BupH™ Phosphate Buffered Saline Packs, pH 7.2, Thermo Scientific, Rockford, Illinois). Legs were removed with fine tipped iridectomy scissors while holding the left side of the specimen with fine-tipped jeweler's forceps.

Dissections were done while viewing the specimen through a stereo microscope. Valvifers covering the ovipositor were removed with 2 pairs of fine-tipped jeweler's forceps. The insect was held at the base of the ovipositor while separating the 7th and 8th segments to create a flap on both sides of the ovipositor. Flaps were then spread laterally to reveal the body cavity. The hind gut was grasped with forceps at the base of the anus while holding the ovipositor and pulled to remove the digestive system. The 9th sternite was pinned to the side and the genital capsule was removed. Attached tracheoles were separated with the hooked end of a minuten pin while pulling on the genital capsule. Ovarioles were pulled posteriorly until the suspensory filaments became visible, which were then cut anterior to the ovaries. Extracted reproductive organs were placed on a microscope slide in a drop of buffered saline solution and manipulated with forceps and minuten pins on the stage of a stereo microscope to separate structures for examination. The slide was then viewed and photographed with a compound microscope with an attached camera. Images were captured by either a Leica M216 or a Leica DMLB2 compound microscope (Leica Microsystems Inc., Buffalo Grove, Illinois) with a SPOT INSIGHT Firewire Diagnostic Instrument 14.3 3-SHOT COLOR camera (SPOT Imaging Solutions, a division of Diagnostic Instruments, Inc., Sterling Heights, Michigan).

Ovary condition was noted and photographed, and numbers of ovulated eggs in lateral oviducts and in leaf petioles were enumerated. An oocyte was considered an egg after being ovulated as defined by passage into the lateral oviduct. Follicular relic condition and collar length were determined by image analysis. Relationships between female age and collar length to numbers of ovulations were determined by correlation analysis (Statistica 64 version 12; Statsoft 2014).

Results and Discussion

The female reproductive system (Fig. 1) consisted of a pair of ovaries each bearing multiple tubular ovarioles that connected to lateral oviducts by thin pedicels. Lateral oviducts joined to form the common oviduct, which led to the vagina. Each ovary consisted of 11 to 13 ovarioles (mean 11.7 ± 0.29 SE, n = 9) joined toward the anterior end of the body by terminal filaments that fused to form a suspensory filament presumably attached to the fat bodies (Tsai & Perrier 1993; Ammar 1985). An enlarged sac-like bursa copulatrix opened into the common oviduct just posterior to the spermatheca (Fig. 2a). The bursa copulatrix sometimes contains globular structures, which we interpreted to be spermatophores. The spermatheca consisted of an elongate irregularly-shaped tube-like structure with a yellowish to dark orange medial portion at mid length becoming progressively lighter distally and then terminating at an ellipsoidal spermathecal gland (Fig. 2a). The spermathecae often contained motile sperm. The spermatheca entered the common oviduct just distal of the junction of the 2 lateral oviducts. The ovarioles were of the telotrophic meroistic type, wherein nurse cells in the distal germarium provide nutrients to developing follicles contained in the vitellarium (Chapman 1998). The vitellarium contained from 3 to 5 developing follicles (Fig. 2b); parous females with functioning ovaries usually contained only 1 mature oocyte at any given time. Each lateral oviduct was divided into 2 distinct portions (Fig. 1), a thinner proximal segment that expanded into a calyx where the pedicels attached and an enlarged distal portion that connected to the common oviduct. *Megamelus scutellaris* ovarian morphology was similar to other delphacid planthoppers (McMillian 1963; Mochida 1973; Szklarzewicz et al. 2007) and other hemipteran species (Becker 1980; Lalitha et al. 1997; Hummel et al. 2006; Szklarzewicz et al. 2007).

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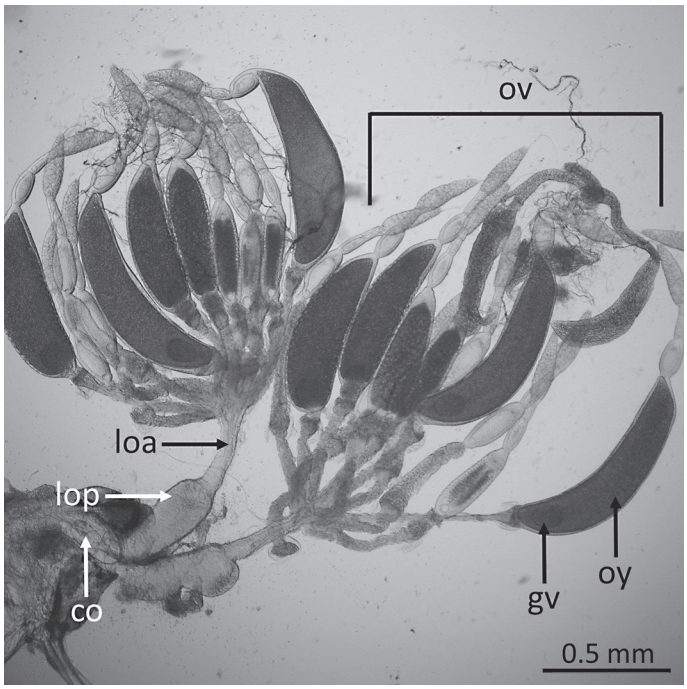


Fig. 1. Photomicrograph of the female reproductive system of *Megamelus scutellaris* showing ovaries (ov), common oviduct (c), anterior and posterior portions of the lateral oviduct (loa and lop, respectively), and overall structure of a follicle including the germinal vesicle (gv) and oocyte with yolk (oy).

Individual follicles contained a yolk filled oocyte (in more mature follicles) with a distinct germinal vesicle or enlarged nucleus present in the distal region of the follicle (Fig. 3a). The oocyte was surrounded by a single layer of cells, the follicular epithelium (Fig. 3a, b). An oocyte was considered an egg after being ovulated as defined by passage into the lateral oviduct (Fig. 3b). The entire ovariole was surrounded by a thin membranous layer, the ovariole sheath. In reproductive females, a unique structure was visible at the base of the ovariole surrounding the most distal portion of the lateral oviduct (Fig. 3a, b). It appeared to be a loose covering of cells that opened distally. This structure, which we termed the "collar," does not seem to be attached or part of the ovariole or the lateral oviduct as observed in higher magnification images (Fig. 3b). It has not been described previously in other planthopper species or other insect groups.

A collection of cellular debris was evident at the base of the follicles in reproductively active females (Fig. 4). This material was typically yellow in color and appeared to increase in volume as successive ovulations occurred. These follicular relics apparently arise from a variety of sources. A group of cells present at the base of the ovarioles encloses the mature oocyte in the follicular chamber and prevents it from passing into the pedicel (Snodgrass 1935). This plug degenerates when the ovum is ready to be released, thus allowing the egg to pass into the pedicel and thence into the lateral oviduct (Snodgrass 1935). The vacated follicular chamber collapses (Fig. 3b) and the accompanying follicular epithelium along with cellular debris from the degenerating plug accumulates in the pedicel at the base of the ovarioles, forming what is referred to as a corpus luteum, yellow body, or follicular relic (Tyndale-Biscoe 1984). As more and more ovulations occurred, accumulated material in this region increased and became more pronounced and in some cases produced a bulge in the lateral oviduct (Fig. 4). The appearance and quantity of follicular relics have often been used to determine reproductive activity (Tyndale-Biscoe 1984; Grodowitz et al. 1987; Grodowitz et al. 1997; Lenz et al. 2007; Perez-Mendoza et al. 2004).

The continuum of ovarian development can be divided into 2 distinct categories each with characteristic morphological features. The nulliparous (i.e., no offspring produced) category was characterized by ovarioles with no follicular relics and typically a limited number of mature follicles (Fig. 5). Three distinct stages were found within the nulliparous category, which we term N1, N2, and N3 af-

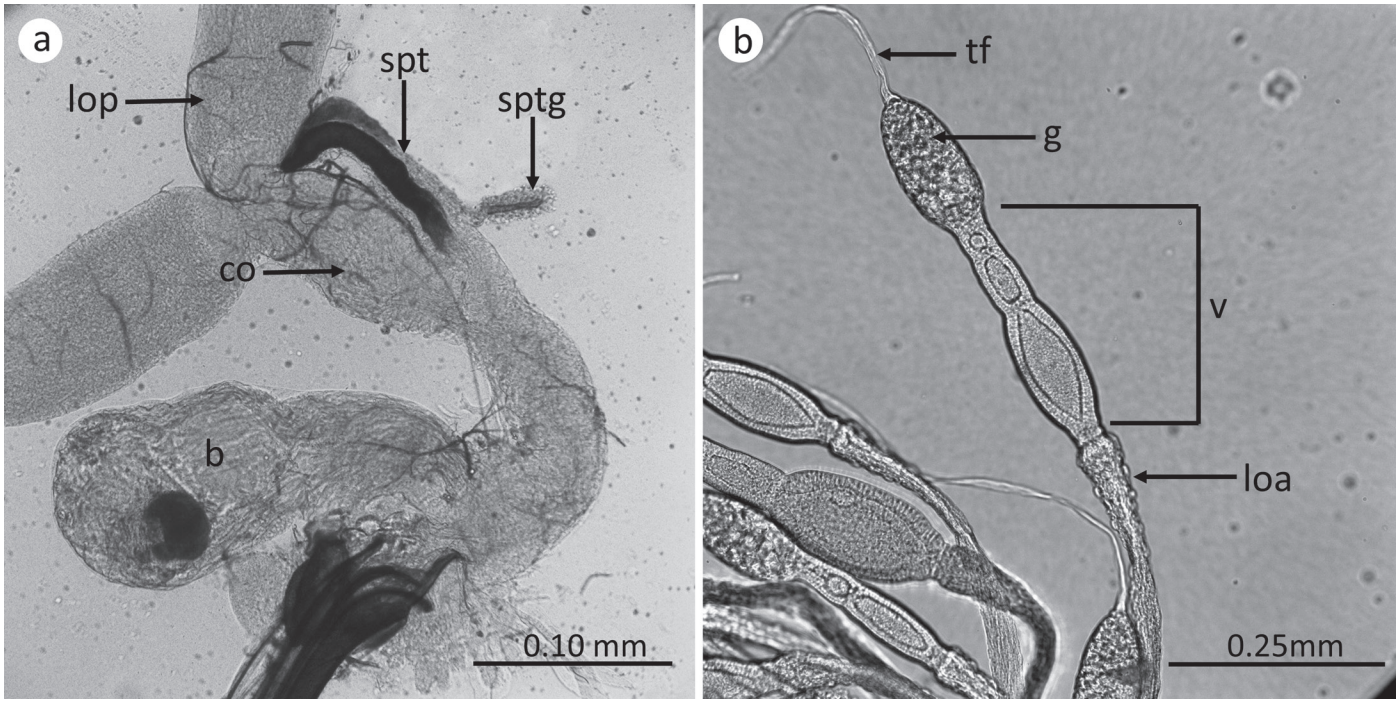


Fig. 2. Photomicrographs of the female reproductive system of *Megamelus scutellaris* showing a) distal portion of the ovary showing the distal lateral oviduct (lop), common oviduct (co), bursa copulatrix (b), and spermatheca/spermathecal gland (spt and sptg, respectively), and b) close-up of ovariole morphology (b) showing the anterior lateral oviduct (loa), germarium (g), vitellarium (v), and terminal filament (tf).

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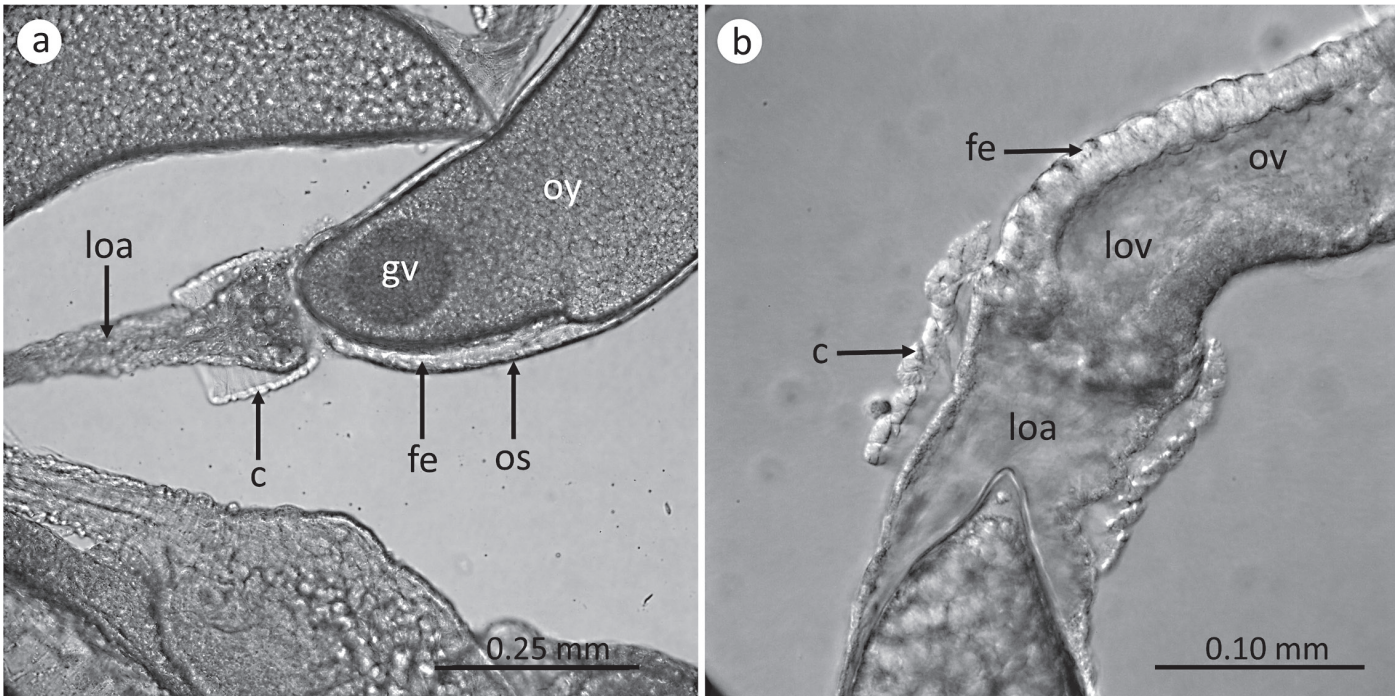


Fig. 3. Photomicrographs of the female reproductive system of *Megamelus scutellaris* showing a) close-up of the distal portion of an ovariole showing the anterior lateral oviduct (loa), follicular epithelium (fe), ovariole sheath (os), germinal vesicle (gv), oocyte with yolk (oy), and collar (c), and b) distal portion of an ovariole showing a newly ovulated egg (e) into the anterior lateral oviduct (loa), ovary (ov), ovariole (lov), and the collar (c).

ter Grodowitz et al. (1987, 1997). Unlike many insect species, the N1 stage was not found in adult females of *M. scutellaris* (Fig. 5a). This species has 5 instars, N1 females were found in the 4th instar. At that point, ovarioles were small undifferentiated tubes without distinguishable follicles. The N2 stage was found initially in the 5th instar, which possessed ovarioles that were fully differentiated into 1 to 2, rarely 3 developing follicles (Fig. 5b). The N2 stage continued through adult emergence. The N3 stage, only found in adults prior to the first oviposition, was characterized by having ovarioles fully differentiated (typically having 3 to 4 developing follicles) with multiple ovarioles containing mature follicles, which had not yet ovulated as indicated by a lack of follicular relics in the distal portion of the lateral oviducts (Fig. 5c, d). Mature follicles were recognized as being enlarged, containing a well-defined germinal vesicle, and darkened, i.e., the darkening arising from the formation of yolk in the ovum.

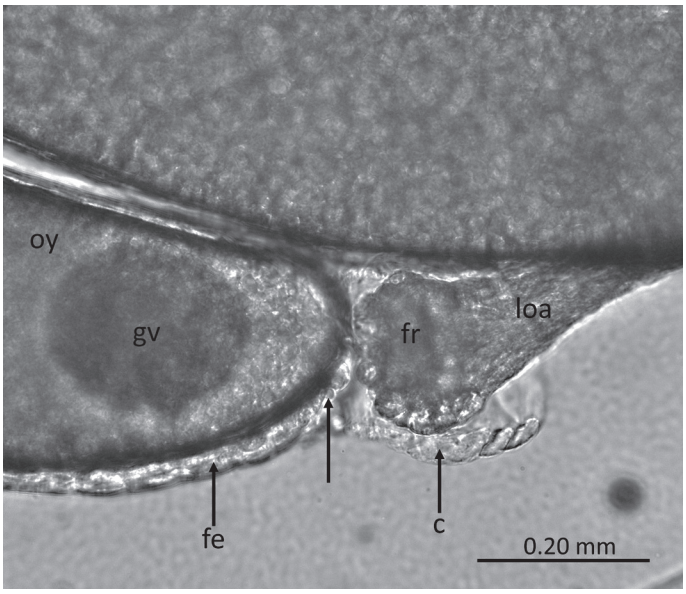


Fig. 4. Follicular relic formation and appearance in the distal area of an ovariole and anterior lateral oviduct (loa) with the germinal vesicle (gv), oocyte with yolk (oy), follicular epithelium (fe) beginning to slough off into the ovariole base (as shown by the arrow), follicular relics (fr), and collar in *Megamelus scutellaris*. Note the granular appearance of follicular relics having a high enough density to begin to expand or bulge the sides of the lateral oviduct.

The parous (i.e., offspring produced) category was characterized by females with fully differentiated ovarioles each containing 3 to 5 maturing follicles and the presence of distinct follicular relics. Changes in morphology, mainly in the appearance and quantity of follicular relics and collar appearance and length, were used to divide the parous stage into 3 classes; i.e., P1, P2 and P3; after Grodowitz et al. (1987, 1997). The P1 class was characterized by follicular relics that were light in coloration indicating a low density of accumulated cellular debris. Relics did not always completely encircle the distal portion of the lateral oviduct and had not yet accrued sufficiently to cause a bulge (Fig. 6a, b). In the P1 class, follicular relics existed in a limited number of ovarioles. The collar was small, not easily seen, and did not exceed the length of the accumulation area of the follicular relics. The P2 class was characterized by follicular relics being present in all ovarioles with bulging apparent (Fig. 6c, d). The collar was longer and typically extended past the follicular relic accumulation area. The P3 class was highly variable, although follicular relics were usually present in all ovarioles (Fig. 6e, f). Density was variable and bulging not always obvious. The collar was significantly longer than the area where follicular relics accumulate. Although the quantity of follicular relics

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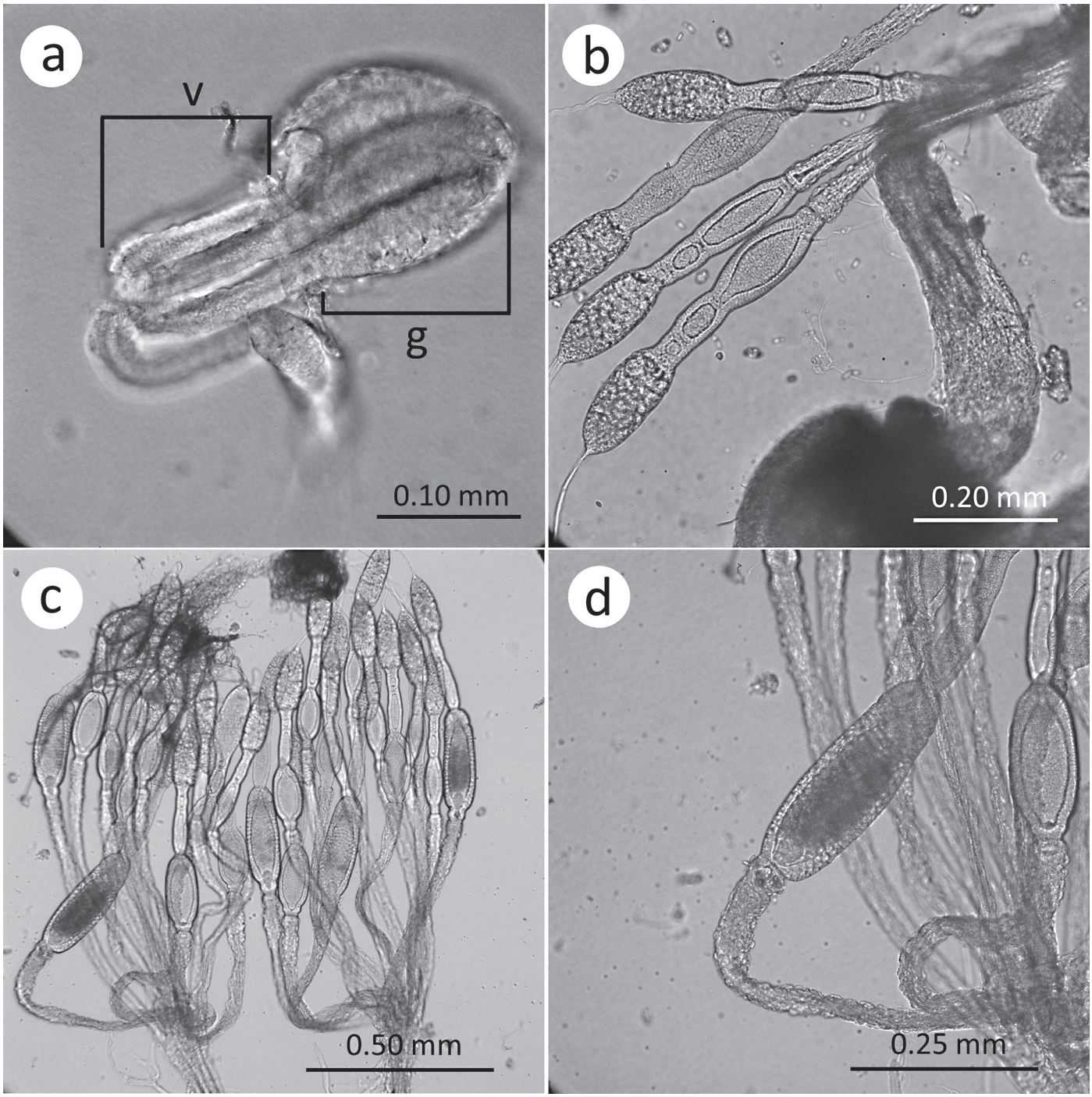


Fig. 5. The 3 nulliparous stages of *Megamelus scutellaris*. a) N1—Note the lack of differentiation in the vitellarium (v) and large size of the germarium (g) in comparison to the vitellarium. b) N2—In this stage the ovarioles are fully differentiated, no fully mature follicles, and no follicular relics. c and d) N3—In this stage the ovarioles are fully differentiated, no follicular relics are present, and at least 2 follicles are mature and ready to be ovulated as indicated by darkening of the interior of the oocyte by yolk deposition.

would be expected to increase linearly with increased ovulations, especially in the P3 class, this is typically not the case (Tyndale-Biscoe 1984; Lenz et al. 2007). Instead, follicular relics can be flushed out of the ovariole, become highly compressed with subsequent ovulations, or absorbed, thereby changing their density.

Changes in the morphology of parous ovaries including follicular relic appearance and quantity, and collar length correlated with the number of ovulations. With low numbers of ovulations (i.e., <45), follicular relics were barely encircling the ovariole base (Fig. 6a) or, if encircling, then no bulging was observed, which is an indication of increased quantity of follicular relics (Fig. 6b). The collar was present, although small (≤0.06 mm), barely visible and not extending past the region of follicular relic accumulation (Fig. 6b). This was the P1 class. With increased ovulations (>45 but <120) follicular relics encircled the ovariole base and increased to produce obvious bulging (Fig. 6c, d). The follicular relics appeared dense, darker, and were usually distinctly yel-

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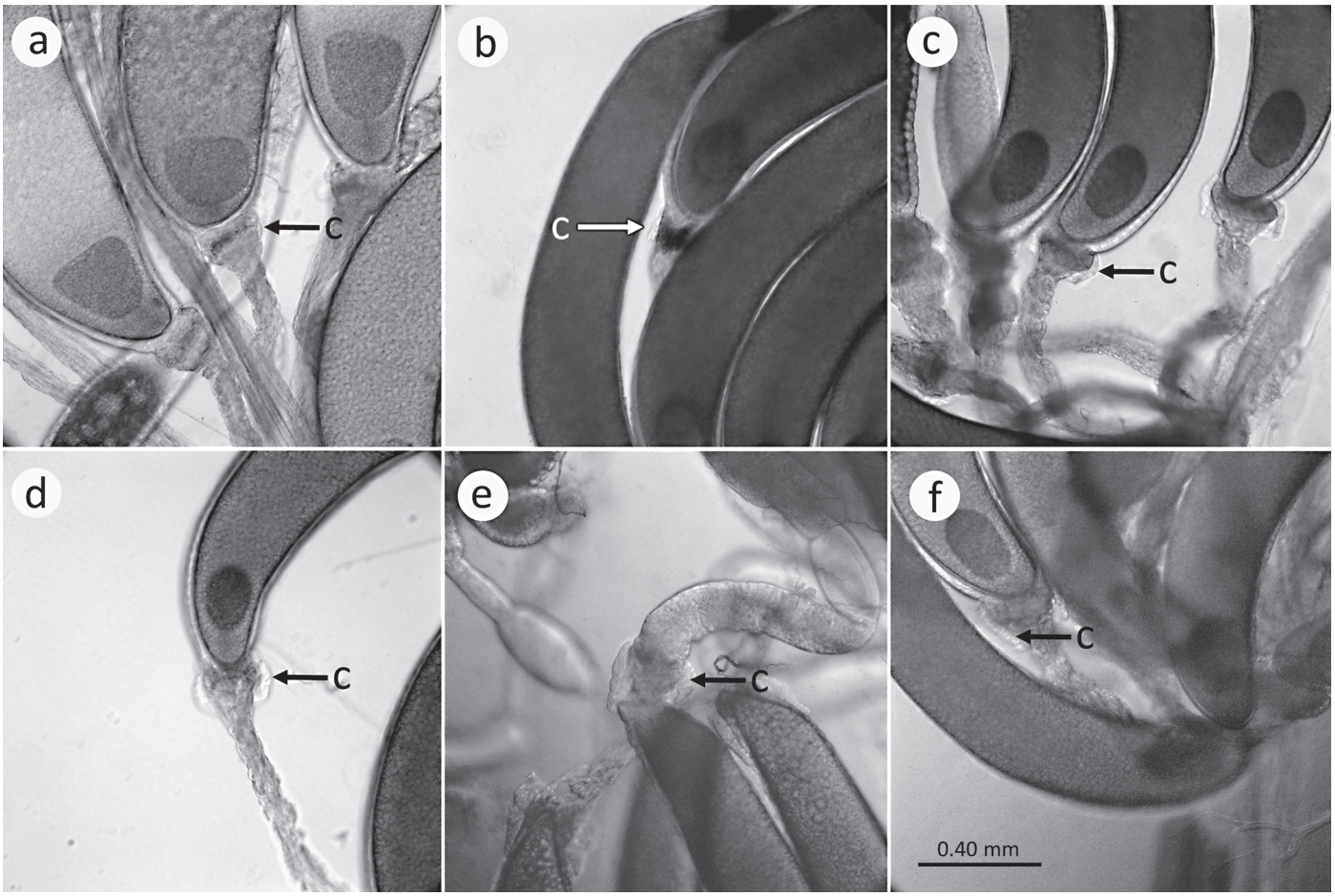


Fig. 6. The 3 parous classes of *Megamelus scutellaris*. The P1 class (a and b) is characterized by the presence of follicular relics, which may not be present in some or all ovarioles, may be light in coloration and may or may not encircle the base of the ovariole. The follicular relics do not occur at high enough densities to cause an expansion or bulging. The collar may or may not be visible and does not extend past the follicular relic accumulation area. In the P2 class (c and d) follicular relics are present in all ovarioles and at high enough densities to cause bulging. They are distinctly yellow in coloration and relatively darker in comparison to those found in the P1 class. The collar is easily seen and typically extends past the follicular accumulation area. In the P3 class (e and f) follicular relics are variable, may or may not be in high enough densities to cause bulging, and typically completely encircle the base. The collar length easily surpasses the follicular relic accumulation area.

low. The collar was longer, extending past the region of follicular relic accumulation and ranged in length from 0.07 mm to 0.09 mm. This demarcated the P2 class. With increased ovulations (>125 ovulations) follicular relic appearance was variable, often encircling the base and bulging was common but did not always occur (Fig. 6e, f). Variability in follicular relic quantity and density in individuals with increased ovulations is most likely due to flushing, reabsorption, and compression as discussed previously. Collar length increased (>0.09 mm) and extended well past the area of follicular relic accumulation (Fig. 6e, f). This defined the P3 class.

It is important to realize that the number of ovulations and correlation to changes in ovarian morphology is a continuous process making it difficult to assign discrete stages or classes to this continuum. For example, the most significant correlation to egg number in this experiment was chronological age of the female with an $n = 15$, $r = 0.93$, and $P < 0.0001$ (Fig. 7a). However, chronological age is not known in field collected individuals (Tyndale-Biscoe 1984) and is often not correlated precisely to ovarian condition and reproductive status as this is related to nutritional content of the food material. The highly significant correlation between age and egg number that we observed occurred because all individuals were fed host plants of similar nutritional quality.

There was a significant linearly increasing correlation between egg number and collar length ($n = 15$; $P = 0.001$ and $r = 0.778$; Fig. 7b), which by itself may be a good strategy to use to determine number of ovulations in a field collected individual. Collar length coupled with observations of follicular relic appearance provided an adequate estimate of past reproductive activity (i.e., number of ovulations). However, the number of females with which to correlate egg number, follicular relic appearance and collar length was relatively small. Additional experiments are needed with more individuals as well as under different plant nutritional regimes to better develop regressions that estimate egg number from collar length. Collar length can also be affected by dissection techniques and slide preparations. For example, transferring the ovaries from the abdominal chamber to the slide and teasing of ovarian structures on the slide for viewing can deform the collar causing inaccurate measurements of length.

In summary, the reproductive system of female *M. scutellaris* appeared to be similar to other delphacids as well as other hemipteran species. A structure not described previously was identified near the base of the ovarioles and termed the "collar." The appearance and quantity of follicular relics and collar length may serve as indicators of past reproductive activity; i.e., number of ovulations, though additional experimentation is needed for verification. Access to this physiological age-grading system will allow researchers to assess the reproductive health of field populations or greenhouse colonies providing essential information on establishment failures and suitability of host plants.

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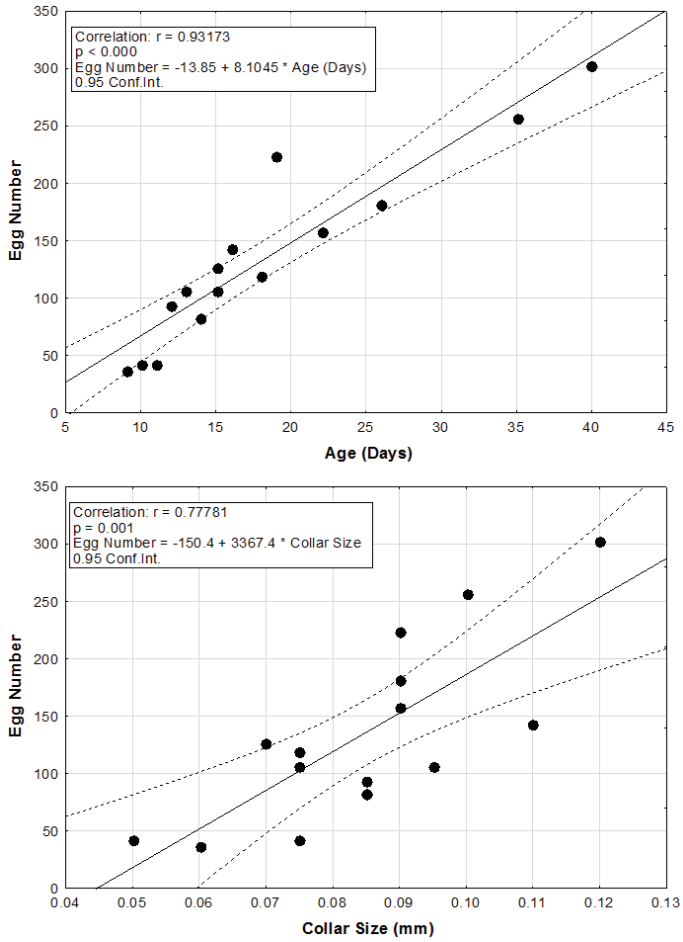


Fig. 7. Number of eggs ovulated by adult females of *Megamelus scutellaris* correlated by a) age (days) and b) collar length (mm). The solid line represents the linear relationship between variables and the dashed lines is the 95% confidence interval (n = 15; P = 0.001 and r = 0.778).

Acknowledgments

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