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Ovary Development and Maturation in *Nezara viridula* (L.) (Hemiptera: Pentatomidae)

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Abstract

Ovary development and maturation of *Nezara viridula* (L.) were evaluated by examining ovariole morphology and the alterations in the biochemical (protein synthesis related to reproduction) composition of the hemolymph. Quantitative and qualitative protein analyses were performed and ovary structural alterations for the pre-reproductive and reproductive stages were recorded. Total concentration of proteins in female hemolymph gradually increased until the end of the pre-mating stage, remaining unaltered thereafter. Proteins linked to reproduction (vitellogenins) appeared in the hemolymph 10 days after adult emergence and indicated the end of the pre-mating stage. After mating, total protein concentration in the hemolymph was lower compared to virgin females; vitellogenin levels were similar during most of the observation period. Oocyte development and maturation were gradual and age dependent. Ten-day-old females had chorionated oocytes ready for fertilization. Mating did not stimulate oocyte development in *N. viridula,* but the lack of mating activity appeared to have stimulated oocyte resorption in 17-day-old females.

Introduction

Insect vitellogenesis is a heterosynthetic process occurring primarily in the fat body, in which the synthesis of the main component of the egg yolk, the vitellogenins, occurs. The absorption of extra-ovarian proteins released in the hemolymph by the fat bodies is mediated by specific receptors, dividing the vitellogenesis process in two well-defined phases, synthesis and uptake, which are both under hormonal regulation (Raikhel & Dhadialla 1992, Raikhel *et al* 2005, Swever *et al* 2005, Ziegler & Antwerpen 2006).

Vitellogenins are a group of proteins synthesized outside the ovary, originating the main egg yolk component, the vitellins (Hagedorn & Kunkel 1979, Swever *et al* 2005). They perform an important nutritional role during embryogenesis, acting as a source of amino acids, lipids and carbohydrates for the developing embryo (Masuda & Oliveira 1985).

The process of vitellogenesis is regulated by hormones, such as juvenile hormone (Engelmann 1979, Wyatt 1988), ecdysteroids (Hagedorn & Kunkel 1979, Hagedorn 1985), and neurosecretory hormones (Raabe 1986). In Heteroptera, vitellogenesis is mainly regulated by the juvenile hormone, while ecdysteriods have a discrete role (Dittmann & Biczkowski 1995, Davey 1997). The juvenile hormone regulates not only vitellogenin (Vg) synthesis, but also Vg uptake (Martinez & Garcera 1987, Hirai *et al* 1998).

Vg synthesis and uptake depend on the ovary development strategy of each particular species. Insects have different reproductive strategies depending on nutrient allocation and use, which are acquired in pre-imaginal and adult stages (Alcock & Gwynne 1991,

Huffaker & Gutierrez 1999, Jervis *et al* 2005). Therefore, synovigenic insects invest the nutritional resources acquired at the immature stage for the development of the soma and nutritional reserves, requiring a later source of nutrient at the adult stage to sustain the reproductive activities. These nutrients may derive from reserves stored in the fat bodies, but are mainly obtained from adult feeding activities (Adams 1997, Davey 1997). There are also cases in which nutrients will be acquired through the use of male seminal fluids (Wheeler 1996, Poiani 2006). On the other hand, provigenic species will prioritize their rapid reproduction using nutrients from the immature stage to develop their reproductive apparatus, producing adults which can reproduce soon after emergence (Jervis & Ferns 2004).

Studies on the development and maturation of the reproductive apparatus of female *N. viridula* are rare. Therefore, the objective of this study was to evaluate the process of female reproductive maturation during the ovarian maturation and reproduction of *N. viridula* by observing morphological alterations in the ovary and by analyzing the biochemical composition of the hemolymph regarding the synthesis of proteins linked to reproduction.

Material and Methods

Collection and maintenance of the N. viridula *colony*

A *N. viridula* colony was set in the laboratory from adults collected on soybean at the DuPont Experimental station (Paulínia, SP, Brazil, 22°45'0"S/47°10'0"W). Field-collected insects were taken to the laboratory and kept under controlled conditions (25 ± 1°C; 60 ± 10% RH; 14 photophase) in screened cages (50 x 50 x 70 cm). The stinkbugs were fed peanut seeds glued to paper strips (15 x 3 cm) and *Ligustrum lucidum* fruits (glossy privet) as supplemental food (Corrêa-Ferreira 1985, 1993). Water was offered on hydrophilic cotton inside plastic dishes. Eggs were collected daily and kept under controlled conditions until eclosion. Nymphs were kept in polypropylene boxes (11 x 11 x 3 cm) until the 4th instar when they were transferred to rearing cages. Nymphs were fed the same diet as the adults.

Morphological development of N. viridula *ovary*

Nezara viridula ovaries were extracted during pre and post mating periods. Females of *N. viridula* have a pre-mating period which can vary between 7 and 13 days (Mitchell & Mau 1969, Singh 1972, Costa 1991). Newly-emerged females were individualized and placed in two groups. One group was allowed to mate when they were 10 days old, while the other group remained virgin. Females were dissected in an anti-coagulant solution (98 mM NaOH, 0.15 M NaCl; 1.7 mM EDTA; 46 mM citric acid; pH 4.5) (Masashi Hotta & Okuda 2001) and the ovaries removed 1, 5, 10, 12, 15, 17, 20 and 23 days after emergence, including the periods of pre- (1, 5 and 10 days) and post-mating (12, 15, 17, 20 and 23 days). In the case of mated females, the post-mating sample periods coincided with the periods after the first mating (12 days) and first oviposition (15 days), between the 1st and 2nd ovipositions (17 days), after the 2nd oviposition (20 days), and between the 2nd and 3rd ovipositions (23 days).

General ovarian morphology and oocyte development were observed. At each sample period, 10 ovaries were evaluated (one female = one ovary = one replicate). Ovariole maturation was ranked as follows: 0 = no oocyte development; 1 = developing oocyte at the germarium; 5 = non-chorionated oocyte at the vitellarium; and 10 = chorionated oocyte (Adams 2000). The total number of oocytes and the number of vitellogenic and chorionated oocytes in each ovariole were evaluated.

Alterations in the biochemical composition of N. viridula *hemolymph*

Samples of female hemolymph were collected during pre- and post mating periods. Newly emerged females were individualized and hemolymph samples taken at the same stages of development as described earlier.

Hemolymph was collected through an incision in the female tegument, and immediately mixed with an anti-coagulant buffer solution (Masashi Hotta & Okuda 2001) kept in ice and centrifuged (2000*g* x 2 min) to remove haemocytes. The supernatant was recovered and stored at -20°C for quantitative and qualitative protein analyses (Cônsoli *et al* 2005).

Quantitative evaluation of hemolymph proteins

The total concentration of hemolymph proteins was evaluated using a commercial formulation of the Coomassie reagent (Coomassie Plus Protein, Pierce Biotechnology, Inc., Rockford, IL) (Bradford 1976). The total protein concentration of each sample was determined by comparing the absorbance observed at 595 nm for the sample with that from the standard curve using albumin as the standard.

Qualitative evaluation of the hemolymph proteins

The qualitative composition of *N. viridula* hemolymph was evaluated by electrophoresis in discontinuous polyacrylamide gel in a denaturing medium (SDS-PAGE), using the system described by Laemmli (1970). Samples collected in TBS buffer (20 mM Tris, 0.15 M NaCl, 5 mM EDTA, pH 7.5) were mixed in a 1:1 ratio with 2x SDS buffer (0.25g SDS, 1.26ml 0.5M Tris-Cl, pH 6.8; 0.84 ml 75% glycerol, 0.50 ml β-mercaptoethanol, 0.40ml 0.005% bromophenol blue; 2 ml Milli-Q water, 2% NaCl), and diluted to a final concentration of 30 µg/20 µl with a 1x SDS buffer. Later, the samples were heat treated on a dry

block (100ºC x 5 min), quickly centrifuged, loaded on a 7.5% polyacrylamide gel (30 µg of proteins/sample), and the proteins were separated at a constant voltage (200V) (Protean II, Bio-Rad, Hercules, CA) until the front marker reached 1.0 cm from the gel base (Cônsoli *et al* 2001). The protein bands were revealed after staining with Coomassie R-250 (GelCode, Pierce Company) for 1.5h and destaining by immersing the gel in water for 12-16h, and constant shaking. The two vitellogenins released to the hemolymph of stinkbug females were identified as described by Cônsoli *et al* (2001) and their quantities determined using the GelQuant versão 2.7 (Bio-Imaging Systems) capture and analysis system.

The availability of proteins linked to reproduction in the hemolymph throughout the maturation and reproduction processes was compared with the morphological data obtained in order to verify the relationship between the release of vitellogenins and the ovarian maturation process.

For all tests, the two treatments (virgin and mated females) 10 replicates were set in a completely randomized design for the two treatments (virgin and mated females). Data were analyzed with analysis of variance and means compared using the *t* test (Proc t test, SAS Institute Inc. 2001) at 5% significance.

Results

Ovary morphological development

A pair of ovaries was observed on *N*. *viridula*, each one with seven ovarioles. Each ovariole contained an apical filament, the trophic region, the vitellarium and the pedicel. The apical filament extends up to the middle of the surface of the tergosternal muscle of the mesothorax, where it is anchored. Oocyte development in ovarioles was synchronous during the successive developmental stages. More developed oocytes are present in the region next to the ovarioles and distant from the germarium region. *Nezara viridula* ovary is classified as meroistic teletrophic with nurse cells located at the apex of each ovariole, in the germarium, containing long filaments (trophic cord) that connect them to the developing oocytes. In the trophic region, also called the germarium, there are nurse cells, a trophic chamber and the oocytes in an early developmental stage.

Nezara viridula females are synovigenic since they are born without any developed oocytes (Figs 1a, 2). The ovarioles of newly emerged females have no oocytes in the germarium. After five days of imaginal development, oocytes could be seen developing in the germarium and in the upper region of the vitellarium. During the pre-mating period (up to 10 days old), oocytes gradually matured, passing through vitellogenin uptake and the deposition of the chorion as they moved in the ovariole towards to the lateral oviduct and became ready for fertilization. The oocytes occupied the whole region of the vitellarium (Fig 1a).

After oviposition, the ovarioles only contained non-chorionated oocytes undergoing vitellogenesis in the upper region of the vitellarium, while vitellogenic and choriogenic oocytes were observed between ovipositions (Figs 1b, c, 2).

The total number of oocytes (non-chorionated and chorionated) varied according to the age and mating status

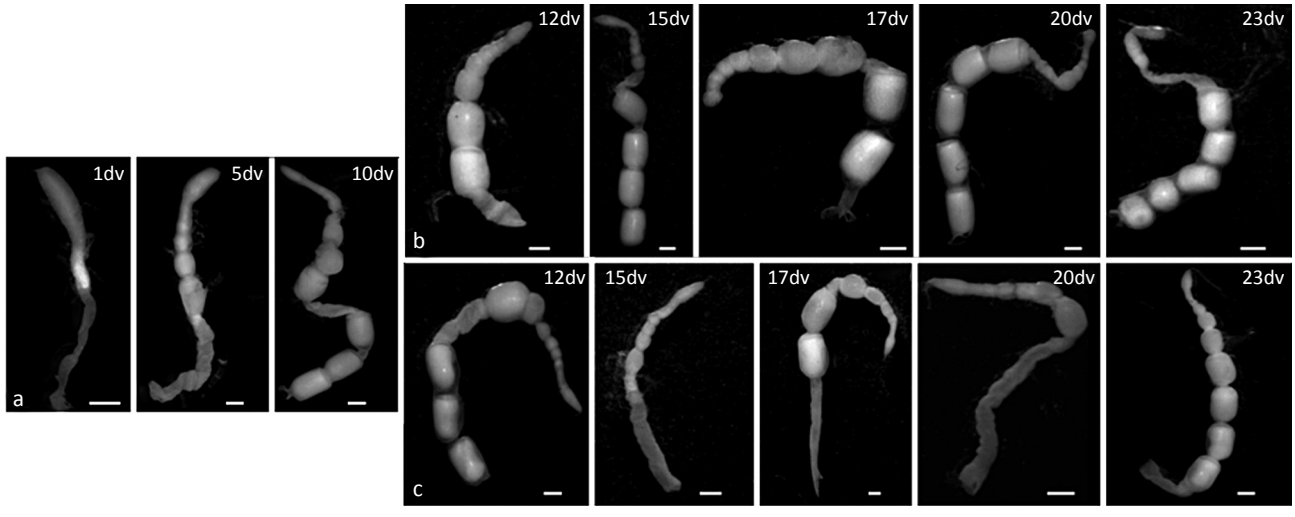

Fig 1 Ovarioles of *Nezara viridula* at different ages and stages of ovarian maturation. a) Ovarioles of virgin females in pre-mating period; b) Ovarioles of virgin females; c) Ovarioles of mated females (12dc – newly mated; 15dc – after first oviposition; 17dc – between first and second ovipositions; 20dc – after second oviposition; 23dc – between second and third ovipositions) (d = days; c = mated; v = virgin). Scale = 500 µm.

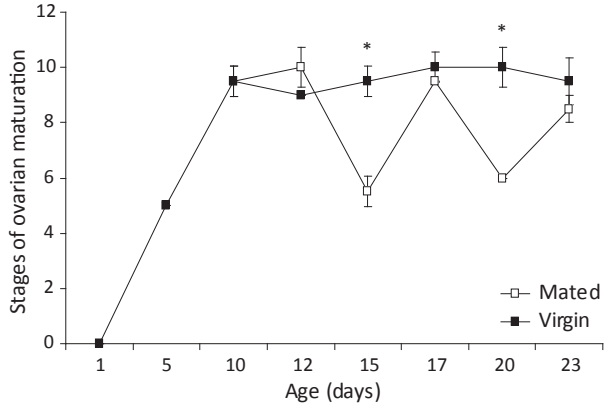

Fig 2 Development of virgin and mated female ovaries of *Nezara viridula* according to the developmental stage of oocytes in their ovarioles, where: 0 – no oocytes; 1 – oocyte present in germarium region; 5 – non-chorionated oocyte present in the vitellarium region; 10 – chorionated oocyte present (mated females: 12d – after first mating; 15d – after first oviposition; 17d – between first and second ovipositions; 20d – after second oviposition; 23d – between second and third ovipositions). The means of rankings for the virgin and mated females were compared using a *t* test at 5% significance.

of *N. viridula* females. The total number of oocytes in virgin females gradually increased up to 12 days, decreasing at 15 days to reach its maximum value at 17 days of age, with a gradual reduction thereafter (Fig 3). The total number of oocytes was greater for virgin females on the 15th, 17th and 20th days after emergence compared to mated females. However, the opposite was observed on day 23 (Fig 3).

After emergence, females invested in egg production and there was a large increase in the number of non-chorionated oocytes until the period before the first mating (12 days) (Fig 4a). During this period, the oocytes underwent vitellogenesis resulting in a large increase in size. After the first oviposition (15 days), and between the 2nd and 3rd ovipositions (23 days), the total number of non-chorionated oocytes in mated females was significantly higher than in virgin females. However, between the 1st and 2nd ovipositions (17 days), mated females had fewer non-chorionated oocytes (Fig 4a).

In the pre-mating period, virgin females had no chorionated oocytes. These oocytes were only seen after the 10th day of emergence, with their availability reduced in 17- and 23-day-old virgin females, even in the absence of oviposition (Fig 4b). Only non-chorionated oocytes were observed in female ovarioles after oviposition at days 15 and 20, with all chorionated oocytes in the ovary being deposited in the same egg mass (Fig 4b).

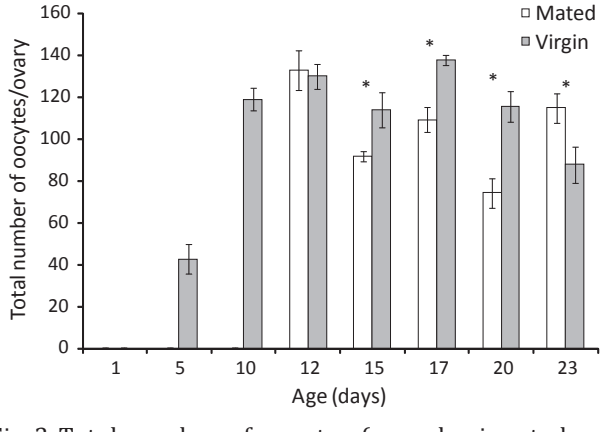

Fig 3 Total number of oocytes (non-chorionated and chorionated) in ovaries of virgin and mated females of *Nezara viridula* at different ages (mated females: 12d – newly-mated; 15d – after first mating; 17d – between first and second ovipositions; 20d – after second oviposition; 23d – between second and third ovipositions). The means of total number of oocytes of virgin and mated females were compared using the *t* test at 5% significance.

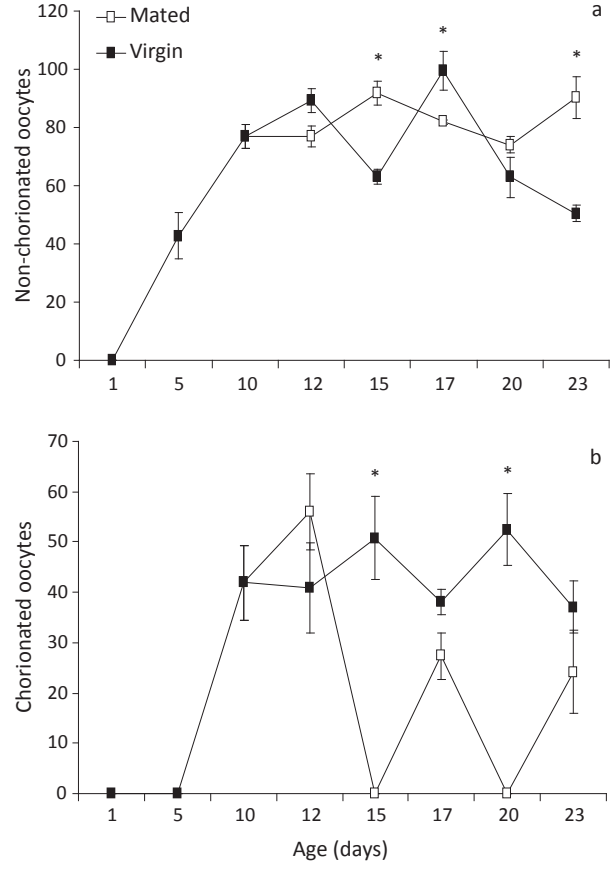

Fig 4 Number of oocytes of virgin and mated females of *Nezara viridula* in different reproductive stages. a) Non-chorionated oocytes; b) Chorionated oocytes. (mated females: 12d – after first mating; 15d – after first mating; 17d – between first and second ovipositions; 20d – after second oviposition; 23d – between second and third ovipositions. The means of non-chorionated and chorionated oocytes in virgin and mated females were compared using the *t* test at 5% significance.

Alterations in the Biochemical Composition of Hemolymph

Quantitative protein composition of the hemolymph

The quantitative analysis of the total proteins in available the hemolymph of *N. viridula* females showed variations in concentration according to ovarian development and female mating experience. Newly emerged females had low protein concentrations in the hemolymph, which greatly increased after 10 days, as the ovaries matured. After this period, protein concentration remained constant in virgin females, while decreasing for mated and reproductively active females (Fig 5).

Qualitative protein composition of the hemolymph

The electrophoretic pattern of the proteins in the hemolymph of *N. viridula* females at different ages and reproductive stages showed that vitellogenins appeared on the 10th day after emergence and remained available throughout the whole reproductive process (Fig 6). The availability of vitellogenins in mated females slightly increased after the 1st mating, while the abundance of these proteins in the hemolymph of virgin females decreased during the same period (Figs 6, 7). Mated females also showed a slight decrease of vitellogenin in the hemolymph at days 17 and 20, remaining constant thereafter. In virgin females, there was an initial drop in vitellogenin concentration from day 10 to 15 followed by an increase in day 17, and by a steep decrease up to day 23 (Figs 6, 7).

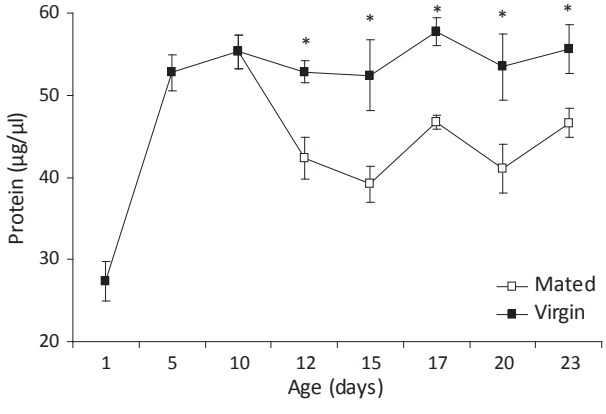

Fig 5 Concentration of total proteins (µg/µl) available in the hemolymph of virgin and mated females of *Nezara viridula*. Mated females: 12 days (after first mating); 15 days (after first oviposition); 17 days (period between first and second ovipositions); 20 days (after second oviposition); 23 days (between second and third ovipositions) (*t* test at 5% significance).

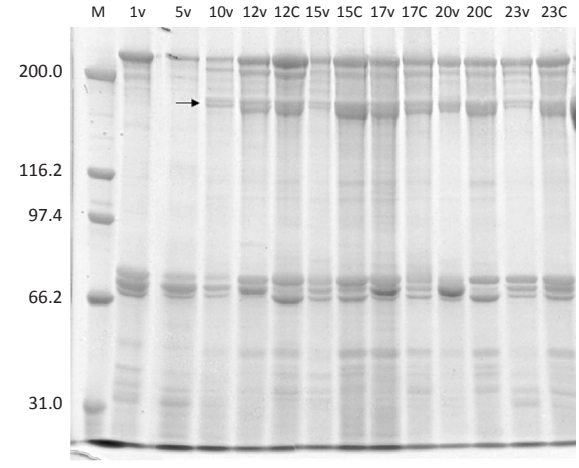

Fig 6 7.5% SDS-PAGE protein pattern of hemolymph of *Nezara viridula* females. Molecular marker (M) ("Broad-range, Bio-Rad Cat. 161-0317: 200 kDa - myosin; 116.25 kDa - ß-galactosidase; 97.4 kDa - phosphorylase b; 66.2 kDa – serum albumin; 31 kDa – carbonic anhydrase). Hemolymph extracted from females at 1, 5, 10, 12, 15, 17, 20 and 23 days. V = virgin female; C = mated female. Couples formed 10 days after emergence. For mated females, day 15 = after the first oviposition; day 17 = between first and second ovipositions; day 20 = after second oviposition; 23 – between first and second ovipositions.

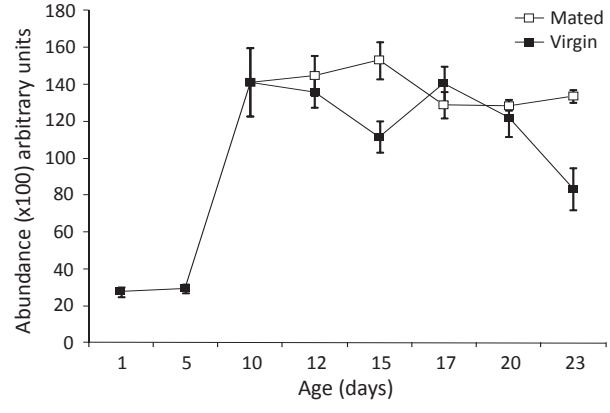

Fig 7 Vitellogenin abundance in hemolymph of virgin and mated *Nezara viridula* females. Mated females: 12 days (after first mating); 15 days (after first oviposition); 17 days (period between first and second ovipositions); 20 days (after second oviposition); 23 days (between second and third ovipositions) (*t* test at 5% significance).

Discussion

The absence of oocytes in the ovarioles of newly-emerged females of *N. viridula* and the appearance of the first oocytes as a result of the availability of vitellogenins in the hemolymph only on the 10th day after emergence, show that the first days of adulthood are used for the pre vitellogenesis

phase, when nutrients that will be accumulated in the developing oocyte are produced (Raikhel & Dhadialla 1992, Nijhout 1994, Chapman 1998).

The morphology of the ovary of newly-emerged *N. viridula*, as well as the morphological alterations observed, which are associated with biochemical alterations in the protein composition of the hemolymph, characterize this ovary as being merostic teletrophic, very similar to other heteropterans (Büning 1994, Adams 2000, Lemos *et al* 2005, Esquivel 2009).

Insect ovariole number can vary considerably from one, in Coleoptera, to thousands, in termites (Nijhout 1994, Chapman 1998). The number of ovarioles observed in *N. viridula* is the same (8 to 14 ovarioles) as that described for other Heteroptera (Büning 1994). The variation in the number of ovarioles is common even within the same species and may be seasonal or influenced by the insect's reproductive strategy. The variation in ovariole number can have implications in progeny development, such as the production of different-sized eggs or it may affect female reproductive capacity (Price 1973, Wellings *et al* 1980, Tschinkel 1987, Stewart *et al* 1991). In *Habrobracon hebetor* (Say), the nutrients to be used in the composition of the vitellum of the parasitoid acted as a limiting factor in the development of extra oocytes in females with a larger number of ovarioles (Petters & Grosch 1977).

The total number of oocytes of virgin females of *N. viridula* remained unaltered for most of the reproductive period indicating no resorption in the absence of stimuli for egg deposition at the beginning of the reproductive period. However, the reduction in the total oocyte number of 23-day-old virgin females may have occurred due to oocyte resorption given the steep decline in the number of non-chorionated oocytes at the beginning of the 17th day after emergence, while the number of chorionated oocytes remained constant. Oocyte resorption observed in virgin females of *N. viridula* may have resulted from time limitation for female reproduction while searching to extend the reproductive period (Rosenheim *et al* 2000).

Oocyte resorption is common in some insect groups as a result of unfavourable environmental and physiological conditions (Bell & Bohm 1975, Papaj 2000). However, the ovarian changes that occur during resorption and the regulation mechanism for these changes are still unknown. Besides the fact that oocyte resorption is associated with nutrient recycling provided by the degradation of the vitellogenins and vitellins of the vitellum for later release of peptides and amino acids in the hemolymph , it is thought that this process has an important role in inhibiting egg production before mating (Kotaki 2003, Horton *et al* 2005).

Although fecundity normally decreases with age, there is no indication that oocyte resorption in the ovary of virgin *N. viridula* females can cause a reduction in its potential future reproduction. However, the delay or absence of mating in the cockroach *Nauphoeta cinerea* (Olivier), which reproduces in well-defined cycles, has been related to the cell death (apoptosis) of immature and mature oocytes, with a consequent reduction in this insect's future reproductive capacity (Moore & Sharma 2005, Barrett *et al* 2008).

Mating can function as a necessary stimulus to various female physiological processes, influencing many reproductive aspects, such as the initiation of vitellogenesis, the beginning or acceleration of ovary development, female fecundity and receptivity to new partners (Jin & Gong 2001, Gillott 2003, Uchida *et al* 2003), but little is known on the role of virginity in the resorption of oocytes (Bell & Bohm 1975, Moore & Sharma 2005, Barrett *et al* 2008). Mating in Hemiptera has been shown to be necessary for follicle production and maturation as well as for influencing the speed of ovarian development (Brunt 1971, Horton *et al* 2005). However, *N. viridula* does not appear to need mating derived stimuli to begin and maintain vitellogenin synthesis and uptake, since the ovary maturation of virgin and mated females was similar, as already observed for other stinkbug species (Masner 1966, Wightman 1973, Davey *et al* 1986, Adams 2000). For these stinkbugs, mating would appear to be a key for female oviposition, since virgin females never laid their eggs. The effects of mating on egg development and oviposition appears to be variable in Heteroptera or even within some specific genera. Even when virgin females lay eggs, alterations in the ovarian developmental period, oocyte size and composition and/or number of eggs laid, may occur (Odhiambo & Arora 1973, Horton *et al* 2005).

The gradual increase in total protein concentration at the beginning of the adult stage and the availability of vitellogenins only close to the end of *N. viridula* pre-mating were similar to those observed in the stinkbug *Riptortus clavatus* (Thunberg), where ovary development coincided with vitellogenin availability in the hemolymph (Shinoda *et al* 1996).

The greater availability of total proteins in the hemolymph of virgin *N. viridula* females when compared to mated females did not correspond to vitellogenin availability. These differences with regard to the mating status of *N. viridula* were not observed in *Plautia stali* Scott, where protein concentration did not vary between virgin and mated females (Kotaki 2003). The greater relative abundance of vitellogenins in hemolymph of mated female after the first oviposition indicates a greater activity of protein synthesis in these females since vitellogenins uptake from hemolymph into the oocyte occurred continuously, considering the cyclical maturation of new oocytes. This fact indicates the existence of a mechanism to regulate protein synthesis which is related to egg laying, as observed in other insects where a mechanism of endocrine regulation is

associated with oviposition and controls the beginning of a new egg maturation cycle (Schal *et al* 1997, Yin & Stoffolano 1997).

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