Ovarian development and influence of copulation in the maturation of the ovaries of *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae)

Ovarian development and analysis of mating effects on ovary maturation of *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae)

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**ABSTRACT**

The many other insects with a restricted diet, the citrus psyllid *Diaphorina citri* Kuwayama, the vector Causing Which agent of the *HLB*, the bacterium *Candidatus* spp Liberibacter., is intimately Associated with symbiotic microorganisms. These mutualistic symbionts play a key role on Their host nutritional ecology, and are vertically Transmitted to the progeny. However, Despite The role symbionts play on host development and reproduction, and Opportunities of the growing exploitation of the insect-symbiont association to control insect-vectored pathogens, there are very few studies on the reproductive biology and host symbiont on the transovarial transmission. Therefore, we Aimed at Analyzing the ovary During
Insects have different reproductive strategies, which influence the allocation and utilization of nutrients acquired in immature and adult (Alcock & Gwynne 1991, Huffaker & Gutierrez 1999). Thus, the nutritional resources acquired in the immature stage may be used for development and the sum of nutritional reserves, requiring the acquisition of nutrients in adulthood to support breeding activities. They can also be used for the development of the reproductive tract, giving rise to adults able to reproduce soon after emergence. Females using nutrients from adulthood to sustain their reproductive activities may benefit from nutrient molecules and / or regulators present in seminal fluid, which are transferred during copulation. The components can be transferred to the female role in the nutritional support of metabolic activities of these or be used directly as food resources for support of embryonic development of progeny. Seminal fluid also may contain regulatory molecules that will induce physiological changes in females, leading them to begin the process of vitellogenin synthesis and maturation of ovarian and / or change their behavioral responses (Adams 2000, Liu & Kubla 2003, Jervis & Ferns 2005).

The variation found on the maturation of the ovary of insects has implications adaptive matters for the reproductive success of females. Thus, females that exhibit high reproductive capacity soon after emergence potentiate the deposition of the largest possible number of eggs in early adult life, but reduce the chances of responding to temporal limitations on the availability of suitable hosts. On the other hand, females have a reduced number of mature eggs to emerge, have the limiting factor for their reproductive success after the fact with only a fraction of their overall reproductive capacity and was subject to future opportunities for the production and deposition of fractions Additional eggs to reach their full reproductive capacity (Heimpel & Rosenheim 1998, Jervis et al 2001, Ellers & Jervis 2003). Besides the implications of these different strategies on diversity and evolution of different modes of insect life, the pattern of ovarian maturation of oocytes and development brings important information about the reproductive strategy of insects and therefore the population dynamics of these (Wheeler 1996, Papaji 2000, Vanderkerkhove et al 2006).
The citrus psyllid, *Diaphorina citri* Kuwayama, became one of the most serious threats to national citrus industry because of its relationship with the bacterium causing the *HLB*. Thus, knowledge of the reproductive strategy of this insect would provide relevant information for understanding their population dynamics, assisting in monitoring and controlling. Still, information on the reproductive strategy of this insect, and consequently the maturation of their ovaries, would give grants to study the process of transovarial transmission of the symbionts associated with it. The symbiotic relationship of mutualism show and its host, being essential for the proper growth and development of its host, ensuring their reproductive success. The contribution of symbionts to the biological fitness of the host has been exploited in the development of techniques for control of pathogens associated with insects (Beard *et al* 1998, Douglas 2007, Feldhaar & Gross 2009). It is also presented as a possible alternative to control the bacterium *Candidatus spp Liberibacter.*, causing the *HLB* in citrus.

Thus, this study aimed to evaluate the maturation of the ovaries of *D. citri* throughout his adult life and its response to mating, as an initial step in determining the reproductive strategy used by this insect, which is part of transovarial transmission of endosymbionts to the host key.

**Material and Methods**

Specimens of *Diaphorina citri* Kuwayama used in the experiments were from the population kept in net cages under controlled conditions (28 ± 2 °C, 60 ± 10% RH and photophase 14h), using seedlings of orange jasmine, *Murraya paniculata*, as rearing substrate (Tsai & Liu 2000, Nava *et al* 2007).

Ovarian development and influence of copulation in the maturation of the ovaries of *D. citri*. Adults of *D.citri* newly emerged ( < 12 h) were sexed, males and females being kept separate in myrtle plants inside cages made of polyethylene (30 cm height x 10 cm diameter). According Wenninger & Hall (2007), *D. citri* becomes able to copulation around 2-3 days after emergence. However, preliminary tests showed high heterogeneity with respect to pre-mating insect, opting for allowing copulation only ten days after emergence. Thus, males and females were sexed, based on the dimorphism of terminalia, and placed in cages (ratio 1♂ : 1♀ ) With the same dimensions as above, allowing it to mate for 24 hours, after which the males were removed. For the evaluation of ovarian development and the effect of mating on the maturation of ovary of *D. citri*, virgin females copulated were dissected after mating (the tenth day after emergence) at intervals of 48 hours, until the eighteenth day after emergence. The confirmation of the occurrence of mating was obtained verifying the successful transfer of sperm, since the appearance of the spermatheca, which, when extended, indicating the occurrence of successful copulation. To evaluate the development of the ovary during the reproductive period of *D. citri*, was considered the degree of maturation of ovarioles, if i) pre-vitellogenic, ii) or vitellogenic coriogênicos and iii) post-oviposition.

**Collection of ovaries.** Females of *D. citri* and newly-emerged in the pre-vitellogenesis and vitellogenesis, determined in preliminary tests, were dissected in
Ringer’s solution buffered to insects (CaCl₂ 0.2 H 2 O 3 mM KCl 182 mM, 46 mM NaCl, 10 mM Tris base, 1N HCl to adjust pH 7.2) (CSH Protocols 2007), under a stereomicroscope Motic SMZ-168. The ovaries were transferred immediately after extraction to the fixative solution conducive to further processing.

**Preparation of ovaries for analysis in whole mounts.** Ovaries *D. citri*, previously fixed in AFATD (75.0 ml 96% ethanol, 10.0 ml 40% formaldehyde, acetic acid 5.0 ml, 10.0 ml DMSO, 1 g trichloroacetic acid) for 2 hours at room temperature, were hydrated in decreasing series of ethanol (96%, 80%, 70%, 50% and 30% - 5 min each) and distilled water (5 min) and subsequently hydrolyzed in 2.5 N hydrochloric acid (5 min) (Martínez 2002). After hydrolysis, the ovaries were treated with Schiff (30 min), washed with distilled water (5 min) and stained in green light (1 min) (Martínez 2002). Then the material was washed in distilled water (5 min), absolute ethanol (2x - 10 min each) and cleared in xylene (2x - 10 min each) mounting Entelan ® (Merck) on a microscope slide. The SEM and photographic documentation were performed under light microscope Zeiss Axiostar Plus, equipped with a camera to capture images and software Motic Image Advanced 2000 3.2 (Motic Co.).

**Preparation of ovaries for histological analysis.** Immediately after dissection, the ovaries were immersed in fixative solution PFA-TBS (5% paraformaldehyde, 1% Tween20, 0.1% in phosphate buffer, pH 7.2) or Karnovsky (3% glutaraldehyde, 3% paraformaldehyde in 50 mM cacodylate buffer, pH 7.2, containing 5 mM CaCl₂) for 24h. After fixation, were washed in the same buffer (3x - 10 min) and dehydrated in increasing concentrations of ethanol (1x - 30%, 50%, 70% and 90%, 3x - 100%, 10 min each). Further, the material was infiltrated in a solution of hystoresin-absolute ethanol (1:1) for 24h, followed by pure hystoresin for 24h at 4 °C. Soon after, the ovaries were included in a solution of inclusion HistoResin Leica ® and kept at room temperature for 48 hours for polymerization of the substrate.

Semi-thin slices (1 to 4 mm) were obtained in ultra-microtome Leica Ultracut UCT, stained in aqueous toluidine blue (1% toluidine blue, sodium borate, 1% w / v) or Azan-Mallory Heidenhain (0.5% aniline blue, orange G 1%, 3% acid fuchsin, phosphotungstic acid 1% w / v) for 1 min (Behmer et al. 2003). After staining, sections were quickly washed in distilled water in order to remove excess dye, to be dried at 60 °C for 20 min before mounting Entelan ® (Merck) on a microscope slide. The analysis and photographic recording of the processed material were performed under light microscope Zeiss Axioskop 2 equipped with Axicam MRc camera (Carl Zeiss ®).

**Scanning electron microscopy.** spermathecae and ovaries were dissected in Ringer’s solution for physiology, fixed in Karnovsky (3% glutaraldehyde + 3% paraformaldehyde in 50 mM cacodylate buffer, pH 7.2, containing 5 mM CaCl₂) for 24h at 4 °C. Then the material was washed in 50 mM cacodylate buffer plus 5 mM CaCl₂ (pH 7.2) (three baths of 10 min each), postfix in osmium tetroxide 1% in the same buffer (60 min) and dehydrated in increasing concentrations of acetone (30, 50, 70, 90, 100%) before critical point drying (Balzers CPD-030, BAL-TEC) mounted, "Stubbs" and sputtering with gold (SCD-050 Sputter Coater, BAL-TEC). The analysis and photographs were performed in a scanning electron microscope Zeiss LEO 435 VP (Carl Zeiss ®).
**Results**

Significant differences were observed regarding the maturation of the ovaries of virgin females copulated. Virgin females remained immature appearance of the ovaries, while mated females, characterized by an increase in volume and turgidity of the spermatheca due to the transfer of sperm (Figs 1, 3) showed rapid development and maturation of their ovaries (Figs 2, 4).

![Bar chart](image.png)

Fig 3 Aspecto morfológico da spermatheca de fêmeas de *Diaphorina citri* virgens e acasaladas, como indicativo de cópula e utilização do fluido seminal no decorrer do respectivo ciclo oogênico.
In some of the females copulated was possible to observe the ovaries of post-oviposition aspect and spermatheca empty but still dilated 48 hours after copulation (Fig. 1). However, this condition became more frequent after the fourth day after copulation, lasting until the eighth day (= last day examined) (Figs 3, 4). Only females had copulated maturation of ovarioles (Table 1). The ovaries after oviposition, observed two, four, six and eight days after mating, similar to those previtellogenic ovaries, but have vitellarium trophic chamber and higher than those observed in ovarioles virgins. Moreover, they have conspicuous basal region, corresponding to vitellarium and pedicel, where it was the last oocyte growth.

Discussion

The development of eggs after emergence may result from the use of nutrient reserves acquired in the immature stage, which are stored in adipose tissue, but mainly acquired via nutrient supply (Hammack 1999, Adams 2000, Lemos et al 2005). Another common source of nutrients used by females sinovigênicas is seminal fluid produced by the male (Friedel & Gillott 1977, Hayashi & Kamimura 2002). Thus, the role of mechanical stimulation and hormonal copulation can present, the transfer of fluids from the seminal vesicles in male mating system may represent an essential source of nutrients to sustain the basic metabolic activities of the female as well as the development of their reproductive system and support the embryonic development of progeny to be produced (Wheeler 1996, Gillott 2003).

The importance of the role of mating and thus the transfer of seminal fluids to sustain the process of reproductive females may be perceived in polyandrous species.
or in need of performing multiple copulations to maximize their reproduction (Fox 1993, Wheeler 1996, Wolfner 2002).

The condition of the immature ovary of *D. citri* and post-emergence ovarian development in females copulated only indicate the importance of mating in the reproductive process of the insect. The results regarding the process of egg development, which was observed in the complete use of the contents of the spermatheca (sperm and seminal fluids) at the end of the development and deposition of a cycle oogonia, combined with the absence of a second cycle until the end of experiments indicate that females of *D. citri* are polyandrous. Thus, this insect appears in need of physical stimuli (distention of the spermatheca) and/or chemical (signaling molecules, nutrients) produced via sexual intercourse, for the development of new cycles oogênicos. But the nature of the factors involved in this stimulation still requires detailed investigation.

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**References**


