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RESEARCH REPORT

The effect of *Leptomastix dactylopii* parasitism and venom injection on host *Planococcus citri*

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Abstract

One of the major alterations observed in mealybug *Planococcus citri* parasitized by *Leptomastix dactylopii* is a strong reduction of laid eggs, which is evident soon after parasitization. Venom injection in unparasitized hosts determines a drastic reduction of fecundity indicating that this female secretion injected at the oviposition plays a key-role in host regulation. In order to assess the impact of parasitism and venom injection on host reproductive tissues, ovaries were dissected at different time intervals after these treatments and observed by light and transmission electron microscopy. The developing eggs showed clear symptoms of degeneration, already half an hour after parasitization or venom injection. Heat and protease treatments of venom nearly suppressed its effects on host reproduction, indicating that proteins are likely responsible for the observed alterations. The electrophoretic profile of venom proteins covers a wide range of molecular masses between 15 to 200 kDa but five major bands having a molecular mass of about 27, 30, 40, 90 and 120 kDa respectively were more evident. Moreover, to establish any parasitoid preference in host selection, among the adult female mealybugs at different stages of maturation and a possible relation with fecundity reduction in the host, the parasitoid behavior was observed.

Key Words: parasitoid wasp; venom proteins; Hymenoptera; Homoptera; microinjections

Introduction

Leptomastix dactylopii (Hymenoptera: Encyrtidae) is a primary solitary endoparasitoid of the citrus mealybug, *Planococcus citri* (Homoptera: Pseudococcidae) (van Baaren and Nénon, 1997), a polyphagous species known from all zoogeographic regions (Williams and Watson, 1988; Islam *et al.*, 1997). It is a pest of citrus and many other orchard crops and ornamental plants in temperate and tropical countries, both outdoors and in greenhouses (Blumberg *et al.,* 1995; Islam *et al.,* 1997). *L. dactylopii* has been successfully used as biological control agent of *P. citri*, often in combination with other natural enemies (Doutt, 1952; Kole *et al.,* 1985; Summy *et al.,* 1986; Hennekam *et al.,* 1987; Tingleand Copland, 1988; Spicciarelli et *al.,* 1992; Noyes and Hayat 1994; Blumberg, 2001). The female of this species parasitizes the third instars (pre-adult stage) and young adult females (Zinna, 1959) inducing the reduction or total inhibition of host oviposition and a strong reduction of host wax production (Zinna, 1960). The foraging parasitoid examines the potential host through antennae before deciding its acceptation or rejection. The final decision is generally based on host size (Zinna, 1959; de Jong and van Alphen, 1989). *L. dactylopii* female does not attack the smallest hosts (on first and second instars) probably because they do not supply of a good fitness value. This behavior could also be explained taking into account that *L. dactylopii* avoids competition with *P. citrus* parasitoids that prefer host with smaller size (de Jong and van Alphen, 1989). However, host size selection is decisive for the different offspring gender allocation as well: the host size class corresponding to pre-ovipositing and ovipositing females is more often used than the third instars to produce the largest female offspring (de Jong and van Alphen, 1989). Moreover, for solitary parasitoids like *L. dactylopii* the recognition of already parasitized hosts is determining as well in order to avoid superparasitism (van Alphen *et al.,*1987).

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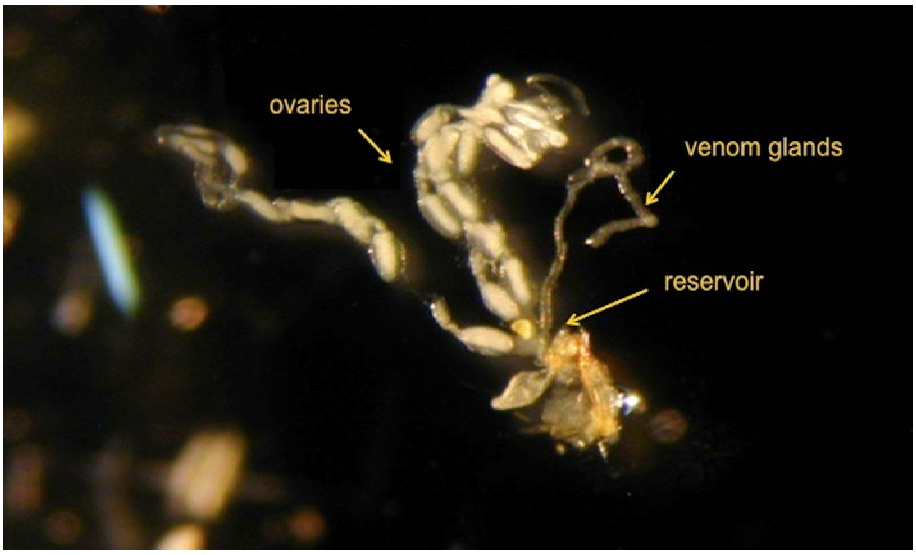

Fig. 1 Reproductive apparatus of *Leptomastix dactylopii* female.

It is known that endoparasitoids of the order Hymenoptera have developed strategies to finely regulate the host physiology in order to provide a suitable amount of nourishment and a favorable environment for its offspring. The host parasitization success is usually determined by regulating factors injected by the female wasp along with the egg and/or the ones progeny produced during development (Vinson and Iwantsch, 1980; Thompson, 1993; Jervis and Copeland, 1996). The maternal secretions generally include the venom and the ovarian fluid which, in certain endoparasitoids of Lepidopteran larvae, also contains a symbiotic virus belonging to the *polydnaviridae* (PDV) family (Webb *et al*., 2000; Webb and Strand, 2005). These factors play a key role in the induction of the host physiological alterations, the host regulation, (Vinson and Iwantsch, 1980; Pennacchio and Strand, 2006) which include the suppression of the host immune response (Er *et al*., 2011), the misregulation of the host endocrine system, often associated with altered patterns of development and reproduction (Digilio *et al.,* 2000).

Venom of wasp parasitoids is produced in a pair of specialized glands, the venom glands, and stored in a reservoir connected to the common oviduct where it is injected into the host body at the oviposition (Asgari, 2006). It is a very important source of host regulation factors which carry out different functions. Hymenopteran parasitoid venom composition can be rather variable among the species (Leluk *et al.,* 1989; Skinner *et al*., 1990; Sanchez *et al*., 1994; Asgari and Rivers, 2011). Recent studies have demonstrated that parasitoid wasp venom is generally a very complex blend of high and low molecular weight molecules with a dominant component of proteinaceous nature (Leluk *et al.,* 1989; Noyes and Hayat, 1994; Rivers *et al*., 2002).

This complexity in venom composition enables parasitoids to avoid variations in the susceptibility of the host to a single component and/or to adapt for a wide range of hosts (Moreau and Guillot, 2005). So the effects of hymenopteran parasitoid venom generally vary among the host species and their stages (Drenth, 1974; Rivers *et al*., 1993; Digilio *et al*., 1998; Gupta and Ferkovich, 1998).

The extensive determination of some virulence factors of wasp parasitoids, already undertaken for a number of species, (Asgari *et al*, 2003; Asgari, 2011; Colinet *et al.,* 2014) is showing that they diverge much more rapidly than that of housekeeping genes, as shown for *polydnavirus* encoded genes but also for venom proteins. For these reasons the wasp parasitoid venoms analyzed until now cannot be considered as general models for other unknown ones, even when parasitoids are phylogenetically very close; on the contrary it is essential to analyze the venom composition of each parasitoid species and the specific effects on their hosts. Anyway, it is possible to observe some common strategies in exploitation of host resources by using factors of parasitoid origin sometimes converging in unrelated organisms. Either alone or in combination with other maternal and/or embryonic factors, parasitoid venom is known to actively suppress host's immune system (Luckhart and Webb, 1996; Li *et al.,* 2007). In parasitoids associated with PDVs, venom increases the effects of viruses or calyx fluid (Davies *et al*., 1987; Tanaka, 1987; Gupta and Ferkovich, 1998; Beckage and Gelman, 2004; Asgari, 2006) however, in parasitoid species that are devoid of PDVs or virus-like particles (Parkinson and Weaver, 1999; Thompson, 1999; Rivers *et al*., 2002; Cai *et al*., 2004; Ergin *et al*., 2006; Wu *et al.*, 2008), venom perturbs host immune defenses, development and reproduction and may complement or replace the functions of other

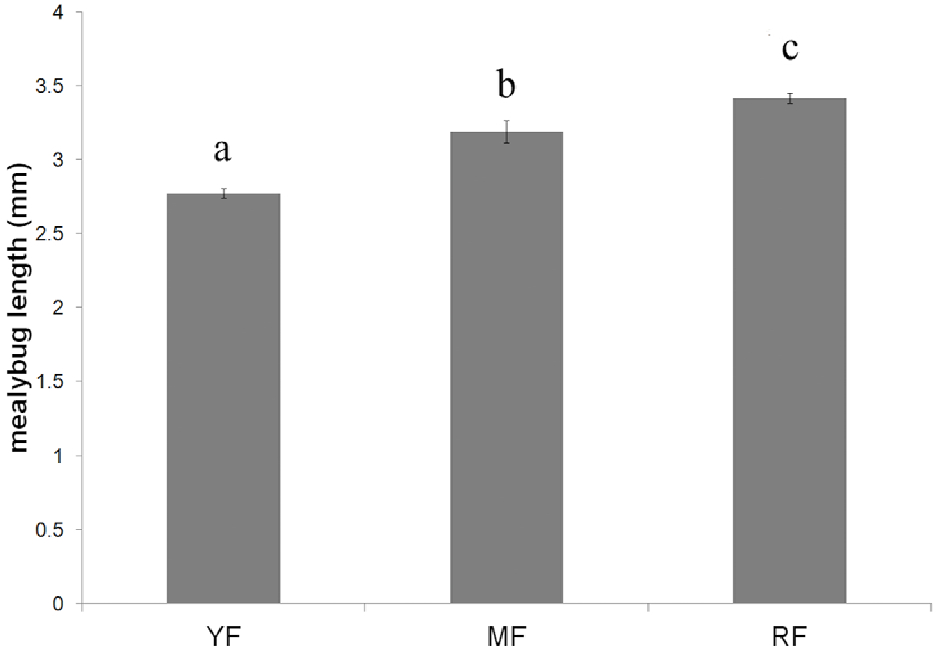

Fig. 2 Mealybugs body length (Mean ± SE); YF (Young Females, 15 days after egg hatching), MF (Mature Females, 20 days after egg hatching), RF (Reproductive Females, 24 days after egg hatching). Means denoted with different letters are significantly different (Tukey's test, α = 0.05).

maternal factors (Digilio *et al.,* 2000; Beckage and Gelman, 2004; Wu *et al*., 2008).

L. dactylopii injects venom into the host body at oviposition (Zinna, 1959). Here we evaluate the host regulation effect of *L. dactylopii* venom, its involvement in reduction or inhibition of oviposition in parasitized hosts (Zinna, 1960) and the possible relation with parasitoid acceptance and/or preference for mealybug females at different stages of maturation. For this purpose, observations on the parasitoid behavior regarding the host selection were performed and the reduction in the number of laid eggs by the females of parasitized mealybugs compared to healthy ones has been noticed. Furthermore, a microinjection method has been developed to evaluate *in vivo* the biological effects of venom through injections into unparasitized mealybugs compared with synchronous parasitized hosts and synchronous unparasitized hosts injected with saline solution. The effect of venom in modifying the reproductive capacity of parasitized/injected hosts was related to structural and ultrastructural alteration of host ovarioles.

Materials and methods

Insect rearing

The parasitoid *Leptomastix dactylopii* was reared on *Planococcus citri*, maintained on etiolated potato sprouts (*Solanum tuberosum*), cv. Desirèe, and pumpkin (*Cucurbita pepo*) cv. Butternut. Citrus mealybug culture was kept in an environmental chamber, at 24 ± 1 °C, 60 ± 5 % RH and with a dark photoperiod. *L. dactylopii* was reared in an environmental chamber, at 24 ± 1 °C, 60 ± 5 % RH and at 16 h photoperiod.

Host selection

P. citri eggs were allocated on sprouted potatoes (*S. tuberosum*) and mealybugs were reared until adult stage at 24 ± 1 °C and 60 ± 5 % RH. Mealybugs were checked daily and morphological features were used to identify instars (Gullan, 2000). Mealybugs underwent the last molting after 15 days from eggs hatching. The young females (YF) with ovaries still immature (fourth instars) continued growing and matured the first ovarioles (Mature Females = MF) 20 days after eggs hatching. Females had already deposited several eggs in a cottony-waxy egg sac 24, 25 days after eggs hatching (Reproductive Females = RF). Host selection behavior of *L. dactylopii* females was studied to assess the preference towards adult mealybug females at the different stages of maturity mentioned above. For this purpose, three individuals, one for each stage (YF, MF and RF), fixed to a 5 cm potato sprout, were exposed to one parasitoid female inside a Petri dish (60 mm in diameter). Each observation ended with the parasitism of one of the three insects, then mealybugs were removed and replaced with new individuals. Mealybugs size (body length) was measured before testing by using a stereo microscope (magnification 25×). Parasitized individuals were maintained on a potato shoot at a temperature of 24 ± 1 °C and 60 ± 5 % RH until parasitoids emergence. New adults were sexed. A total of 60 tests, distributed in three days were performed.

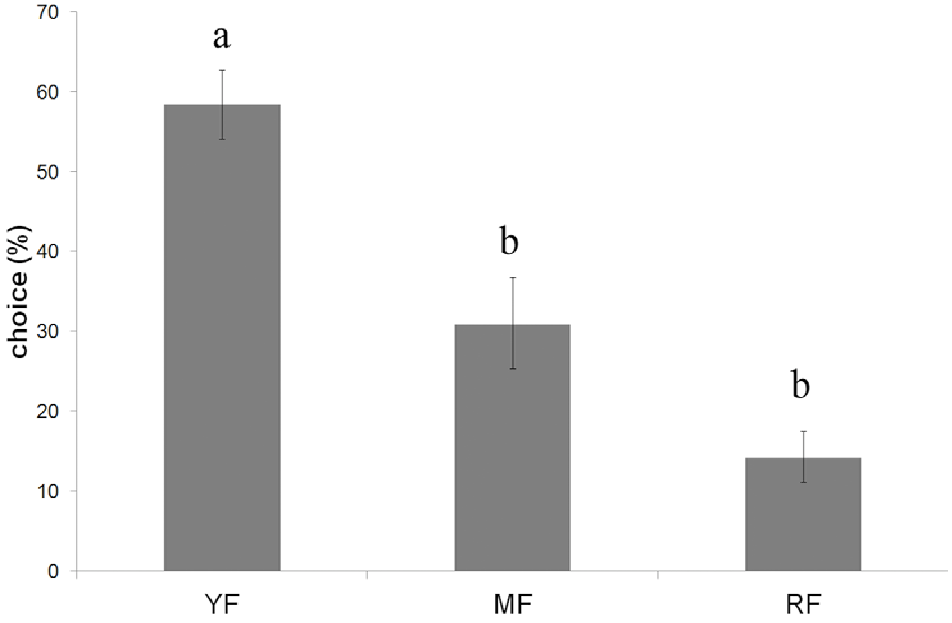

Fig. 3 Frequencies with which the mealybugs of different ages were chosen (Mean ± SE). YF (Young Females, 15 days after egg hatching), MF (Mature Females, 20 days after egg hatching), RF (Reproductive Females, 24 days after egg hatching). Means denoted with different letters are significantly different (Tukey's test, α = 0.05).

Macroscopic effects of parasitization on mealybugs reproduction

To evaluate the effects of *L. dactylopii* parasitization on the reproductive activity of *P. citri,* both, 52 YF and 51 MF mealybugs were individually parasitized by *L. dactylopii* mated female and reared on sprouted potatoes at 24 ± 1 °C and 60 ± 5 % RH. The same number of synchronous non parasitized mealybugs was allocated on separate sprouted potatoes and reared in the same condition as control. Every day the eggs laid by each mealybug were removed and counted. Observations ended with mealybug natural death or parasitoid pupation.

Venom collection and electrophoresis

L. dactylopii females of mixed age, collected directly from the rearing, were used to explant the venom glands and reservoir. The whole reproductive tract of adult females (Fig. 1), previously anaesthetized on ice, was pulled out by grasping the ovipositor with a pair of forceps, while keeping the abdomen in Pringle's saline solution (Pringle, 1938). Venom glands and reservoir obtained from each female were pooled in 20 µl of ice-cold Pringle's solution and gently opened with dissecting needles. 20 reservoirs were pooled per each drop, the crude extract was centrifuged at 5,000*g* for 5 min, at 4 °C, and the supernatant was recovered, transferred in a new tube and immediately used at the appropriate dilution obtained with saline solution for the injection of non-parasitized host or stored at -80 °C until use. Electrophoresis of crude venom extract was performed with precast 10 % Bis-Tris gel (NuPAGE, Invitrogen, Carlsbad, CA, USA) in MES buffer and run under 200 V constant-current conditions. The gel was stained O/N in Coomassie Brilliant Blue G 250 and destained with several washes with an aqueous solution of 45 % methanol, 10 % acetic acid.

Inactivation treatments

Two inactivation protocols were performed in order to assess the sensitivity of the venom extract to heat or protease treatments. The heat inactivating treatment was performed by boiling the extract for 5 min. Then, the crude extract was centrifuged at 13,000*g* for 5 min, at 4 °C, the supernatant was recovered and immediately used. The enzymatic digestion treatment was carried out by using active proteases linked to acrylic beads (P0803, Sigma-Aldrich,St. Louis, MO, USA), which were incubated with the venom extract, at 37 °C, for 3 h. The digested extract was separated from the beads by centrifugation, 1 min, 13,000*g*, 4 °C, and immediately used.

Injection bioassay

Injections of crude or inactivated venom were performed on non-parasitized mealybugs MF. Experimental insects were anaesthetized on ice, washed in ethanol 70 % and then in distilled water and the injection performed through the basal membrane of a hind leg, by using a glass micro capillary tube (P0549, Sigma-Aldrich, St. Louis, MO, USA) with a very fine tip, shaped with an automatic puller for microelectrodes (Puller PC 10, Nikon, Tokyo, Japan). The injected volume was 0.1 µl/mealybug in 0.4 sec in all experimental treatments, for a total amount of about 0.1 female-gland equivalent/mealybug. The microcapillary tube was loaded by capillarity and the fluid gently

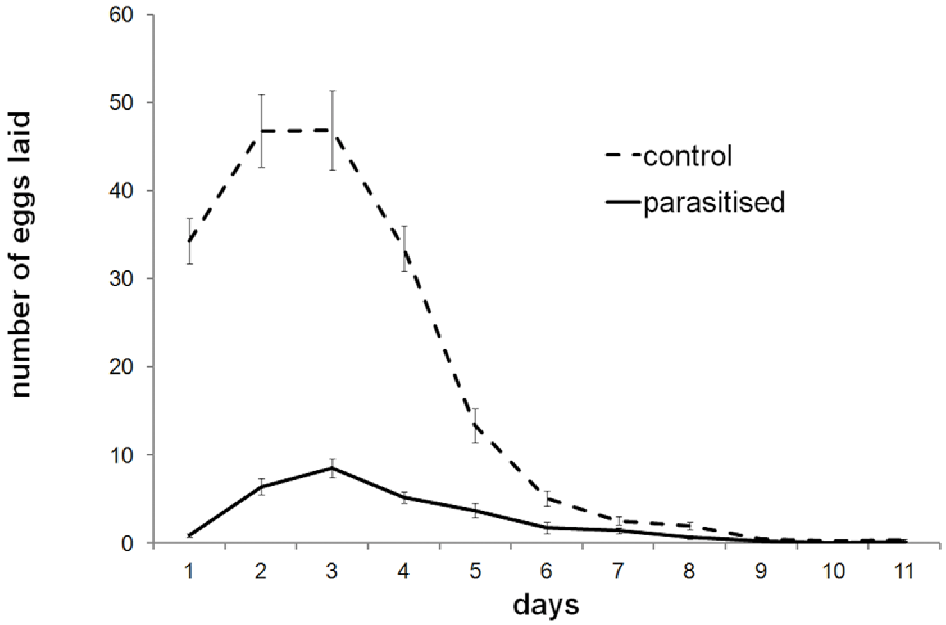

Fig. 4 Oviposition trend (number of eggs laid per female per day) in parasitized and non-parasitized mealybug MF (Mature Females, 20 days after egg hatching).

injected by providing the required pressure with a small electric pump, regulated by a potentiometer (FemtoJet 5247, Eppendorf Hamburg, Germany). Control mealybugs were injected with 0.1 µl of Pringle's saline. After injection, citrus mealybugs were kept on sprouted potatoes at 24 ± 1 °C and eggs laid were checked and removed daily. Individuals died in the 24 h after the injection were not considered in the analysis. A total of 134 MF (net of the dead in the first 24 h) were injected by using crude venom, 37 MF were injected by using heat inactivated venom and 37 MF were injected by using protease inactivated venom, 79 MF were injected with Pringle's saline, and 40 MF with boiled Pringle's saline. Fifty parasitized and fifty unparasitized mealybugs were used to compare eggs deposition after the injections with that in natural conditions. Due to the high mortality of YF injected with saline solution it was no possible to test the venom effects on this developing stage.

Light, transmission and scanning electron microscopy

Mealybugs that had been parasitized or injected as described above, along with synchronous non-parasitized, or Pringle's injected, were dissected already half an hour after each treatment and their ovarioles were isolated. Dissections were carried out in PBS 1X and the isolated ovarioles transferred into a tubes, washed twice with PBS 1X for 7 min, 400*g* at 4 °C. Samples for transmission electron microscopy were fixed in 4 % glutaraldehyde, washed in 0.1 M cacodylate buffer pH 7.4 and post-fixed with 1 % osmic acid in cacodylate buffer, pH 7.4. After standard dehydration in ethanol series, samples were embedded in an Epon-Araldite 812 mixture and sectioned with a Reichert Ultracut S ultratome (Leica, Nussloch, Germany). Semithin sections were stained by conventional methods (crystal violet and basic fuchsine) and observed with a light microscope (Nikon Instruments Europe BV, Amsterdam, Netherlands). Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 electron microscope (Jeol, Tokyo, Japan). Ovarioles for SEM were fixed and dehydrated as described above, treated with hexamethildisilazane and mounted on stubs. Next, the samples were air-dried and covered with a 9 nm gold film by flash evaporation of carbon in an Emitech K 250 sputter coater (Emitech, Baltimore, MD, USA). Specimens were then examined with a SEM-FEG Philips XL-30 microscope (Philips, Eindhoven, Netherlands).

Statistical analysis

Mean comparisons were performed by one-way analysis of variance (ANOVA) and mean separation was carried out by Tukey's test. Data were analyzed by statistical software SigmaStat ver. 3.1. Oviposition trends of parasitized and unparasitized mealybugs were analysed by applying a full factorial model analysis of variance (ANOVA), including parasitisation and the days after parasitisation as main fixed effects. This analysis was performed with R 2.13.1 (www.r-project.org).

Results

Host selection

P. citri adult females begin to lay eggs only a few days after molting in fourth instar. In time lapse that at 24 °C lasted 5, 6 days, the mealybug increases in

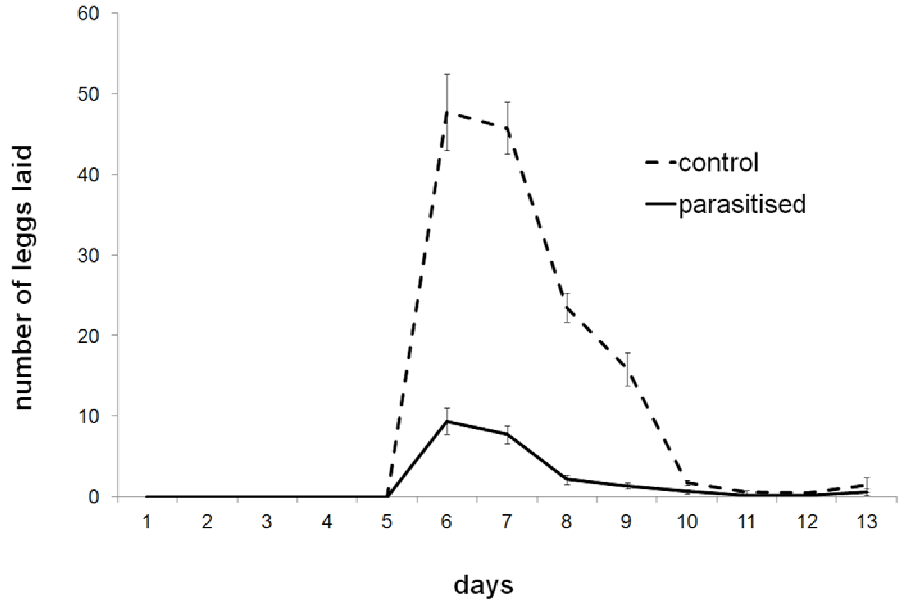

Fig. 5 Oviposition trend (number of eggs laid per female per day) in parasitized and non-parasitized mealybug YF (Young Females, 15 days after egg hatching).

size and matures the ovaries. *P. citri* adult females of different ages, YF (15 days after egg hatching), MF (20 days after egg hatching), RF (24 days after egg hatching), had the average size significantly different ($F = 40.903$, $df = 2$, $p < 0.001$), with a progressive increase in size from YF (smaller) to RF (larger) (Fig. 2), however, all individuals were within the class size 2.77 to 3.42 mm, suitable for the deposition of female eggs (Noyes and Hayat, 1994).

Host selection behavior of *L. dactylopii* females was studied to assess the preference towards adult mealybug females at the different ages mentioned above, which correspond to different stages of maturity: immature (YF), maturing the first ovarioles (MF), and laying eggs in the cottony-waxy egg sac (RF). The mean frequencies with which the mealybugs of different ages were chosen were significantly different ($F = 22.6$, $p = 0.002$) (Fig. 3).

YFs were preferred to the other ages tested (Fig. 3), while, between MFs and RFs, basically those with egg sac (RF) were chosen less frequently, although the difference was not statistically significant.

Sex ratio of parasitoid progeny was strongly female-biased, without any significant difference among the three host stages.

Macroscopic effects of parasitization on mealybugs reproduction

To evaluate the effect of parasitization on host deposition capacity, YF of 15 days old and MF of 20 days old were parasitized and kept at 24 °C. Every day the laid eggs from each mealybug were removed and counted.

The MF laid the majority of eggs during the first six days of observation but the oviposition continued until the eleventh day. The oviposition trends in parasitized and non-parasitized hosts were quite similar but the parasitized mealybugs laid a significantly lower egg number than the non-parasitized ones (Fig 4). The effects of both factors "parasitization" ($F_{1,961} = 379.6$; $p < 0.001$) and "day" ($F_{11,961} = 72.8$; $p < 0.001$) were highly significant, as well as the interaction "parasitization" × "day" ($F_{11,961} = 42.7$; $p < 0.001$).

The same trend was observed in case of parasitized and non-parasitized YF but the oviposition started with a delay of four days (Fig. 5). Also in this case, the effects of both factors "parasitization" ($F_{1,1013} = 229.0$; $p < 0.001$) and "day" ($F_{12,1013} = 110.1$; $p < 0.001$) were highly significant, as well as the interaction "parasitization" × "day" ($F_{12,1013} = 50.3$; $p < 0.001$). Overall these data demonstrated that parasitization of *P. citri* YF and MF significantly reduces the fecundity of mealybugs. However, there were not significant differences in the total number of eggs laid by parasitized YF and MF nor between the respective non parasitized control.

Electrophoresis and effects of venom

The electrophoretic profile of *L. dactylopii* venom proteins showed various components with a molecular mass ranging between 15 and 200 kDa and of five relatively more abundant proteins with an approximate molecular masses of 120, 90, 40, 30 and 27 kDa, respectively (Fig. 6), confirming, as in several wasp parasitoids, that most of venom proteins are of high molecular weight (Leluk *et al*., 1989).

Crude venom injection, in *P. citri* MF, reproduced the same alterations observed in the parasitized host, inducing a significant reduction in

the number of laid eggs compared to the synchronized controls injected with Pringle's saline or inactivated venom, that regularly laid and reproduced, ($F_{6,426}$ = 80.4; $p \leq 0.001$) (F = 52,62; g.l. = 2; p = ≤ 0.001). The injection of *L. dactylopii* venom prevented the mealybug oviposition while the mealybugs injected with Pringle's saline regularly laid and reproduced (Fig. 7).

Heat and protease treatment both eliminated *L. dactylopii* venom biological activity. Fecundity (number of deposited eggs) of injected MF mealybugs with treated venom in both cases was not significantly different from those determined by the injection of Pringle's solution (F = 73.780; df = 4; $p < 0.001$) (Fig. 7). These results allowed to recognize that molecules determining host castration are proteins.

Light, scanning and transmission electron microscopy

In response to *L. dactylopii* parasitism, the whole architecture of the host ovarioles is lost and the same effect was obtained when *L. dactylopii* venom was injected into non parasitized mealybugs already after half an h from both treatments (Fig. 8).

The most evident symptom was the general degeneration of the follicle cells in parasitized and venom-injected mealybugs compared with those unparasitized (Figs 8 A - C). This was already evident by 30 min after parasitization or venom injection (Figs 8 D - I) in contrast with synchronous non-parasitized controls and with mealybugs which received an injection of saline solution (Figs 8 A - C). After parasitization or venom administration cells show clear signs of degeneration due to the appearance of large vacuoles (Fig 8G) - in the cytoplasm. Additionally, a rapid initiation of cell degeneration took place and cells showed autolysosomes and damaged nuclei (Fig 8F).

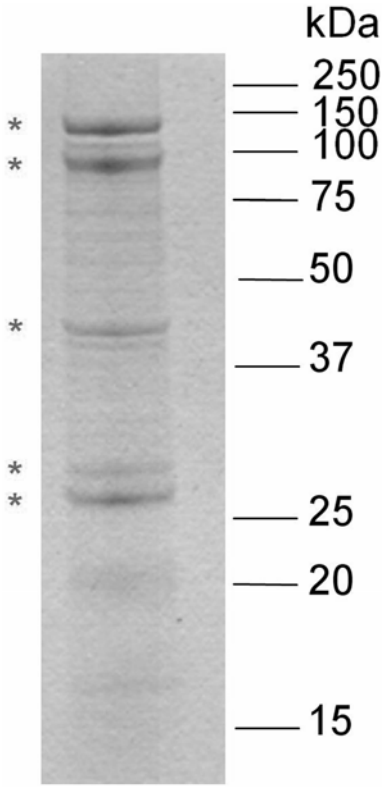

Fig. 6 SDS-PAGE analysis of *Leptomastix dactylopii* venom proteins. Stars indicate the more expressed proteins.

Discussion

Insect parasitoids have developed different strategies to exploit host resources for their offspring survival. Parasitoid wasps achieve this goal by altering host metabolism, mobilizing stores of nutritional resources, and/or disabling metabolic sinks such as metamorphosis, reproduction, or both (Pennacchio and Strand, 2006). The suppression of host reproductive activity and redirection of host nutritional physiology occur in aphid parasitoids such as *Aphidius ervi* (Hymenoptera, Braconidae). Indeed, the ovary degeneration is observed in all *Acyrthosiphon pisum* stages when parasitized by *A. ervi*. Parasitized pea aphids show various degrees of castration: total castration of aphids is normally observed only when hosts first or early second instars are parasitized (Digilio *et al.,* 2000). The reduction of fecundity was also reported for *P. citri* parasitized by *Anagyrus pseudococci*, an encyrtid parasitoid which attacks all mealybug instars with preference for the third instar and adult female (Islam *et al.,* 1997). Parasitization induced cessation of fecundity of *P. citri* and the number of eggs produced by parasitized pre-ovopositing and reproductive mealybug adults were significantly different as compared to number of eggs produced by unparasitized adult females (Islam *et al*., 1997). Moreover, the reproductive period was much longer in the non-parasitized mealybugs as compared to parasitized ones.

L. dactylopii parasitizes the third instar and the adult female and selectively ignores the first and the second instars (de Jong and van Alphen, 1989) of its natural host *P. citri*. Larger hosts, bigger than 2 mm in length, corresponding to pre-ovipositing and ovipositing females, are preferred. Moreover, in *L. dactylopii*, host size affects sex allocation and parasitoid offspring size, that is not obvious in koinobiont parasitoids. Our results suggest that, within a certain class of dimensions, a bigger host is not always better for foraging *L. dactylopii* females, since young females (YF) were preferred in the choice test. This result confirms that host suitability doesn't solely depend on host size (Colinet *et al*., 2005; Trotta *et al*., 2014) and, as a consequence, host acceptance relies on a complex series of external and internal cues (Rehman and Powell, 2010). *L. dactylopii* and related species, such as *Leptomastix epona* and *Epidinocarsis lopezi*, seem to use mainly the antennae to examine the host (Zinna, 1959; van Baaren *et al*., 1993; Karamaouna and Copland, 2000). Two types of antennal contact take place during host antennation, a longer one, lasting several seconds, with the anterior part of the antenna, and a short one made with the distal end of the antenna (van Baaren *et al*., 1993). Antennal

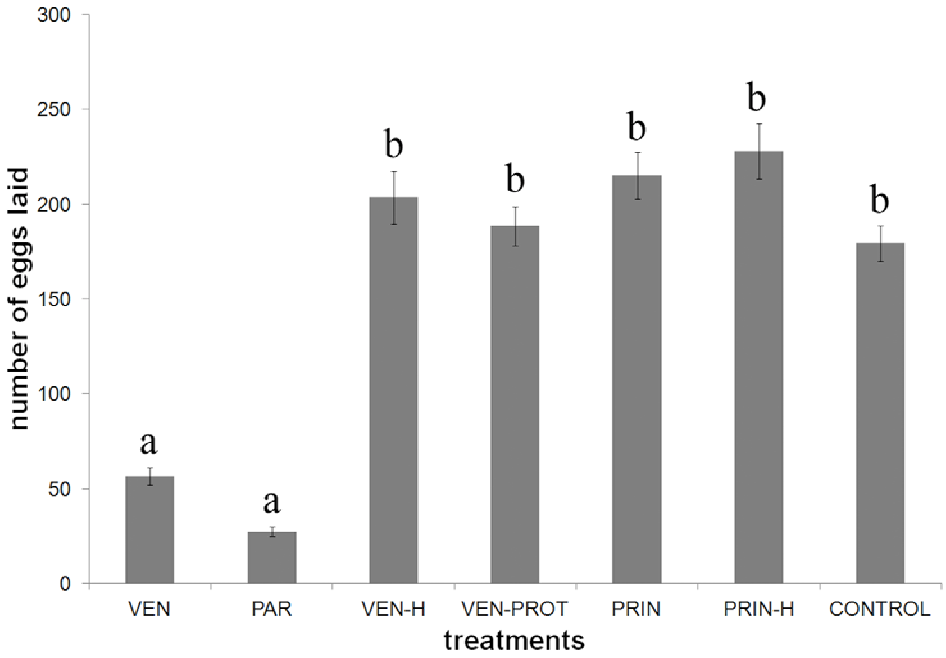

Fig. 7 Total number of eggs (Mean ± SE) laid by *Planococcus citri* pre-ovipositing mature females (MF), after the injection of *Leptomastix dactylopii* venom (VEN), venom after heat inactivating treatment (VEN-H), venom after enzymatic digestion (VEN-PROT), Pringle's saline (PRIN), and boiled Pringle's saline (PRIN-H); Means denoted with different letters are significantly different (Tukey's test, α = 0.05).

sensilla involved in host antennation have been described (van Baaren *et al*., 1996) and they include a relatively high proportion of olfactory sensilla. The ovipositor may be also involved in host acceptance and receptors have been described in some cases (Larocca *et al.*, 2007). The decision to accept or reject a host may then follow the insertion of the ovipositor by parasitoid female (Pennacchio *et al*., 1994; Karamouna and Copland, 2000). This does not seem to be the case of *L. dactylopii*, at least according to our observations, since all hosts probed with the ovipositor were actually found to be parasitized.

L. dactylopii preference for young mealybug fourth instar (YF) may be related with host physiological state. *P. citri* adult females begin to lay eggs only a few days (5 - 6 days at 24 °C) after molting in fourth instar. During this period of time the mealybug increases in size and mature the ovaries. Both young (YF) and mature pre-reproductive females (MF), when parasitized, laid a reduced number of eggs. The difference between the two stages of maturation was only temporal, not quantitative, as YF and MF produced the same total number of eggs. How and if the observed time difference in host reproduction affects the host - parasitoid physiological interaction and parasitoid fitness is not clear.

In the present paper we demonstrate that *L. dactylopii* parasitization induces a strong reduction of reproductive activity (reduction in fecundity) related to the ovarioles degeneration of the host. Host castration is already evident 24 h after the parasitization by *L. dactylopii,* in *P. citri* MF and 6 and 7 days after parasitization in YF. The host castration, as observed in other parasitoids attacking adults (Pennacchio and Strand, 2006), results the main strategy for a biochemical redirection of host metabolism in favor of the adequate nutrition of the developing parasitoid progeny. The disruption of host reproductive activity is the strategy described at molecular level in previous work regarding the host-parasitoid association *A. pisum* - *A. ervi*. In this case, *A. ervi* venom induces the host castration through the *germaria* and the young apical embryos degeneration. This activity is reproduced by the action of a gamma glutamyltranspeptidase contained in the venom, (*Ae*-γGT) which triggers apoptosis in the germarial cells of the ovarioles (Digilio *et al.,* 2000; Falabella *et al.,* 2007). Venom-mediated castration is also complemented by two proteins released in host hemolymph by teratocytes that mediate the extraorally digestion of host reproductive tissues and the transport of host fatty acids to the parasitoid larva (Tremblay and Iaccarino, 1971; Falabella *et al.*, 2000, 2005, 2007, 2009). Therefore overall the host castration generated by the maternal and embryonic parasitoid factors is associated with increases in protein, amino acid, and acylglycerol levels in the hemolymph that coincide with the exponential growth phase of the parasitoid larva (Pennacchio *et al*., 1995; Rahbé *et al*., 2002). Apart from *Ae*-γGT (Falabella *et al*., 2007) also observed in the venom of the ectoparasitoid *Nasonia vitripennis* (de Graaf

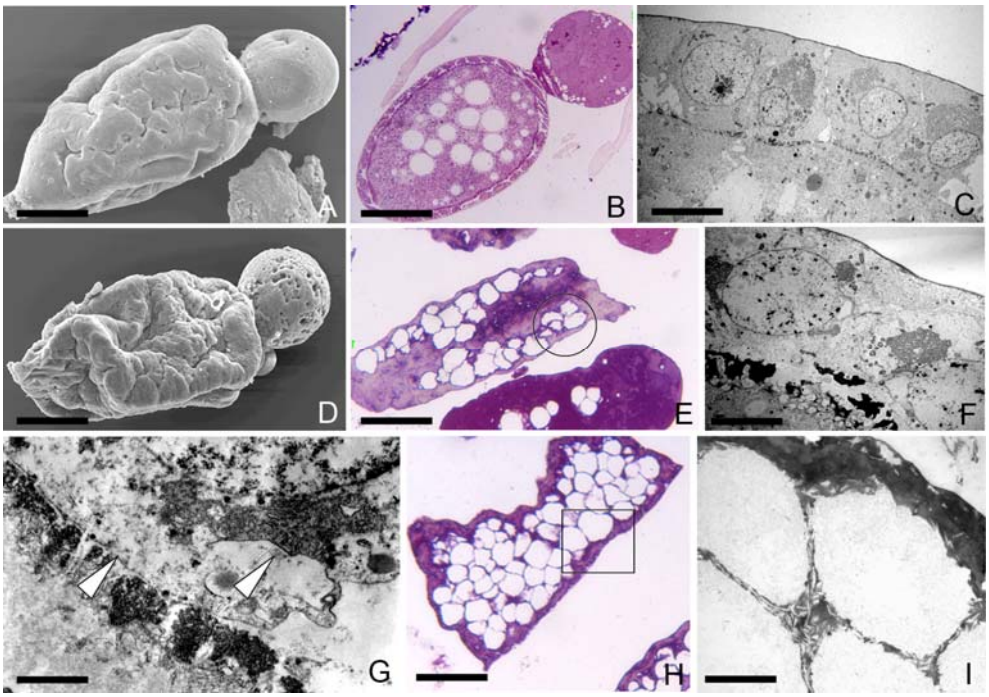

Fig. 8 Ovariole structure in unparasitized and parasitized/venom injected *Planococcus citri* MF mealybugs. Optical and ultrastructural (SEM, TEM) analysis: unparasitized MF mealybugs (A - C); parasitized/ venom injected MF mealybugs (D - I). Just half an hour from parasitization or venom injection (D - I), deep changes in the overall organization of the ovariole are evident. The whole structure collapses to the loss of internal organization (compare A and D). The radical change appears to be due to depletion of the tissue as a result of events relevant to cell death (compare C with F and G). Very often only the resistant cell structure such as the endoplasmic reticulum membranes remain (arrowhead). The inner part of ovariole (E, F, H, I) is characterized by de-structured tissues as visible in light microscopy (E, H) and in TEM photograph (F, I). Scale bars: A, 40μm; B, 50 μm; C, 6 μm; D, 40 μm; E, 40 μm; F, 6 μm; G, 3 μm; H, 30 μm; I, 5 μm.

et al., 2010), in a recent study (Colinet *et al.,* 2014) 16 putative proteins were identified as the main components of *A. ervi* venom. Among them, two new γGTs, cysteine-rich toxin like peptides, serine protease homologues, Serpins and other proteins unique to *A. ervi* with unknown functions were identified. Similarly to *A. ervi, L. dactylopii* would seem to put in place a strategy to provide the best nutritional condition to the progeny, strongly reducing host reproductive activity (Digilio *et al.,* 2000; Falabella *et al.,* 2007*,* 2009). This could be a common adaptive mechanism consisting in host metabolism redirection by parasitoid of sucking insects with a typical reproductive ecological strategy, obtained with the injection of maternal secretions that may have different composition and action mechanism but often converging in same goal: the parasitism success.

In our experimental model system the host ovarioles are directly targeted and the parasitoid venom appears to be the only host regulation factor responsible for their degeneration. The venom injection in MF mealybugs induces the same effects of natural parasitization, determining a strong reduction of the egg number laid by the host. Moreover, all the described effects are evident in parasitized host before the parasitoid egg hatches demonstrating that venom is the major component responsible for the host castration. However, we cannot completely exclude the possible occurrence of embryonic or larval regulation factors of parasitoid origin. In previous study Barbier and co-authors (1987) described the presence of spherules, arising from ventral follicular cells of *L. dactylopii* and following the egg into the host. These spherules are thought to be able to produce a protein layer that is supposed to be involved in the prevention of the host immune response, egg incapsulation in particular. The role of both venom and spherules injected at oviposition in the regulation of host physiology, especially for what concerns the suppression of host reproduction, has not been investigated in this species.

The histological observation by light and electron microscopy of adult female ovaries half an hour after parasitization or venom injection showed evident signs of degeneration with damages at ultrastructural level that fully justify the loss of their activity in reproduction.

The oviposition trend in *P. citri* young female both in parasitized and unparasitized were very similar to the observed trend in mature parasitized and unparasitized female, but the oviposition, evaluated at 24 °C, started four days after. This

observation allowed us to hypothesize that venom components were not rapidly degraded in host hemolymph.

Our preliminary characterization of the bioactive component(s) of *L. dactylopii* venom allows us to conclude that the molecules responsible for the physiological alterations observed in mealybugs receiving a venom injection are proteins. In fact, heat and protease treatment eliminated the biological activity of venom and the injection of inactivated venom did not induce host castration. Moreover, in the complex venom protein profile we observed some proteins more abundant and it is reasonable to assume that they could carry out a key role in the alteration of host physiology. This is not surprising since parasitoid venoms are mainly composed of proteins with conserved enzymatic domain that in some cases have been characterized (Asgari and Rivers, 2011). The venom of two endoparasitoid ichneumoids belonging to *Pimpla ssp, Pimpla hypocondriaca and Pimpla turionella,* contains a wide range of enzymes including laccase, trehalase, reprolysin-type metalloprotease, phospholipase, phenoloxidare and serin protease (Parkinson *et al.,* 2002a, b, 2003, 2004; Uçkan *et al*., 2004).

In *Cotesia rubecula* venom, a 50 – kDa glycoprotein (Vn50) was found to be a serine proteinase homolog (SPH). This protein (SPH) lacks proteolytic activity and inhibits melanization of the host hemolymph *Pieris rapae,* in a dose-dependant manner (Asgari *et al.,* 2003). The negative impact of venom on host immune response has been detected also in the case of *Pteromalus puparum* (Pteromalidae), which significantly reduces the percentages of spreading plasmatocytes and encapsulated Sephadex beads (Cai *et al*., 2004).

The egg parasitoid trichogrammatid *Trichogramma pretiosum* injects at the oviposition a venom with protease and phosphatase activity that digests yolk and the host embryo (Strand, 1986). As a result, newly hatched *T. pretiosum* larvae feed as saprophytes.

Our data on the biological activity of *L. dactylopii* venom provide knowledge useful to guide the next challenges for our study, consisting in identification and, subsequently, in decodification of the biological function of each venom component and their role in host castration.

The achievement of this goal will provide not only new details in the panorama of heterogeneous composition of parasitoid venoms but also could add information about the possible development of a new set of bioinsecticide molecules acting on sucking insects. On the other hand the host specificity of parasitoid sometimes could be due to the parasitoid behavior in host selection and oviposition more than to the specificity of venom components and each single molecules could be active also on some other pest or non target species. For this reason once identified the *L. dactylopii* venom component further studies will be necessary to elucidate their functional activity on target and non target species, as well as evaluating effective delivery systems for active protein components in hemocel before their possible use as bioinsecticides.

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