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Male chemical cues mediate the female preference for egg deposition site in *Rhynchophorus ferrugineus*

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Abstract

The choice of a suitable oviposition site is critical for the reproductive success of many animals. In insects, oviposition site choice is often driven by chemicals: oviposition-deterring pheromones are well known for many insects, whereas oviposition-stimulating pheromones have only been reported for a few species. Here, we investigate which cues trigger the choice of a deposition site in the Red Palm Weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera Dryophthoridae), the worst palm pest. Our experiments clearly showed that females prefer to lay eggs in a substrate already used for the same purpose. To provide evidence for cues mediating this preference, we carried out a series of bioassays following a binary-choice test paradigm. Our results showed that neither the presence of eggs nor the polar or apolar compounds extracted from the egg surface affected female preference. Moreover, experiments in which the body of adults was rubbed on the substrate allowed us to exclude that body surface compounds are involved in this process. Bioassays preventing male-substrate contact suggested a role of the male in determining the female's choice of the oviposition site. Our results suggest the male aggregation pheromone as the best candidate to mediate such female preference. Further studies will be necessary to clarify whether female preference represents a simple by-product of the aggregation pheromone effect or is due to a specific compound of the pheromone that triggers the female behaviour.

Key words: oviposition site, oviposition-stimulating pheromones, palm pest, red palm weevil.

Introduction

The choice of a suitable oviposition site is critical for the reproductive success of many animals and it is expected to be subject to strong selective pressures (Resetarits, 1996). Females of oviparous organisms can deposit their eggs in a wide range of habitats that differ in ecological characteristics. Selection for sites free from predators and pathogens is fundamental for egg survival and development, which, in many species, requires habitats with a narrow range of temperature-humidity conditions. Moreover, the very first post-hatching stages of several animals consist of helpless individuals with limited movement abilities that do not allow them to select the better conditions for their survival. Thus, the choice of the most suitable oviposition sites represents a crucial factor also for offspring growth.

Since number, size and rate survival of offspring influence fitness, they have been traditionally used to give reproductive success quantification. However, although phenotypic traits (i.e. behavioral ones) related to oviposition site strategy directly affect the aforementioned characters, they have received less attention (but see Hilker and Meiners, 2002).

Choosing a suitable oviposition site is fundamental to assure offspring survival of many insects (Anderson, 2002). A considerable number of studies have shown that insect female choice is influenced by chemicals. Insects may produce substances able to deter parasites, predators or conspecifics, or pheromones to attract conspecifics (Corbet, 1973; Saini *et al*., 1995; Anderson and Löfqvist, 1996; Picket and Woodcock, 1996; Rai *et al*., 1997; Torto *et al*., 1999; Seenivasagan and Vijayaraghavan, 2010; Sloggett *et al*., 2011). Semiochemicals are released by adults, larvae or eggs onto the substrate chosen for oviposition or in its proximity. Adults perceive these pheromones through the chemical receptors of antennae and legs or the area around the mouth (Hilker and Meiners, 2002).

Pheromones may be used by females to advise conspecifics that a patch is occupied (Addesso *et al*., 2007), therefore dissuading females from further oviposition in overcrowded sites and reducing competition for subsequent immature stages (Prokopy, 1972). Conversely, pheromones could provide cues for the conspecific to oviposit in the same site (reviewed by Wyatt, 2003): the use of semiochemicals to promote collective/aggregative oviposition may help to overcome host plant defences and protect offspring from pathogens and predators (Wertheim *et al*., 2003; Desurmont and Weston, 2011). *Culex* mosquito females, for example, are attracted by egg rafts of congeneric females and 1,3-diacylglycerols washed from the eggs elicit preferential oviposition (Starratt and Osgood, 1972; 1973; Picket and Woodcock, 1996), even if the active substance consists of a single compound, erythro-6-acetoxy-5-hexadecanolide (Laurence and Pickett, 1982; 1985). Similarly, desert locust *Schistocerca gregaria* (Forskal) females aggregate for oviposition in soils where eggs have already been laid (Saini *et al*., 1995; Rai *et al*., 1997; Torto *et al*., 1999). The selection of appropriate oviposition sites is vital for phytophagous insects, especially when the immature stages are unable to leave an unsuitable plant species and move to another host. Many studies have shown that such selective process is influenced by pheromones secreted by either eggs or adults of both sexes (for a review see Anderson, 2002).

The red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera Dryophthoridae) (hereafter RPW), is a large insect native to southern Asia and Melanesia and one of the most important pests of several palm species. Today, RPW is distributed in Europe, Africa, Oceania, and Asia (e.g. Wattanapongsiri, 1966; Zhang *et al*., 2003; Al-Ayedh, 2008; Li *et al*., 2009; Giblin Davis *et al*., 2013; Rugman-Jones *et al*., 2013) and it is rapidly spreading in the Mediterranean basin through *Phoenix dactylifera* L. and *Phoenix canariensis* Hort. ex Chabaud date palms.

Due to the threats RPW poses to human economy, several studies have been carried out to find effective strategies to manage this species. However, information concerning life cycle and some basic aspects of its biology, such as mating and egg laying behaviour, are scarce and need further investigation (but see Ince *et al*., 2011; Inghilesi *et al*., 2015).

The biological cycle of this species is strictly linked to the host plant. RPW males produce an aggregation pheromone (Hallett *et al*., 1993) whose most important compounds are ferrugineol (4-methyl-5-nonanol) and ferrugineone (4-methyl-5-nonanone). Both compounds are reported to attract individuals of both sexes. Female usually lays each egg separately in a small hole previously dug by the rostrum at the base of palm fronds, especially in damaged plants. After egg-laying, the female produces a deposition plug to seal the holes in which the eggs have been laid (Gindin *et al*., 2006; Dembilio *et al*., 2012). A female in a good condition is able to lay up to 500 eggs during its life (Wattanapongsiri, 1966), however the average number of eggs laid by a female per day is variable according to time, substrate, temperature and female age (Ince *et al*., 2011; Dembilio and Jacas, 2011; Dembilio *et al*., 2012). Recently, Dembilio *et al*. (2012) have reported a temperature of 15.45 °C as the lower threshold for oviposition in this species. Females of RPW lay eggs irregularly, also in the same period, as already reported in two congeneric species, *Rhynchophorus phoenicis* (F.) and *Rhynchophorus palmarum* (L.), and can suspend oviposition for some days even during the periods of oviposition peak (Thomas *et al*., 2004). Moreover, it has been reported that RPW females can lay eggs at any time throughout the day (Ince *et al*., 2011). After emergence, the larvae burrow into the fresh plant tissue where they feed for 2-4 months and at the time of pre-pupation eventually migrate to the decayed region (e.g. Rajapaksha and Kanagratnam, 1988; Poorjavad *et al*., 2009). The habitat of this insect makes it quite difficult to apply pesticides for its control. Consequently, integrated pest management programmes (including sanitation, trapping, chemical and biological control, Sterile Insect Technique and public awareness) have been proposed (Faleiro, 2006; El-Mergawy and Al-Ajlan, 2011; Giblin-Davis *et al*., 2013; Mazza *et al*., 2014; Ávalos and Soto, 2015; Mazza *et al*., 2015).

Expanding the knowledge of the olfactory communication system of this pest could lead to various practical applications. For example, the discovery of other attractants, such as oviposition-stimulating pheromones, might help in trapping gravid females (Faleiro *et al*., 2003). We observed that RPW females prefer to lay eggs on substrates where conspecific eggs have already been laid suggesting the presence of oviposition pheromones in this species. The aim of this paper is to investigate the origin and the nature of cues that drive the female choice during egg deposition.

Materials and methods

Insect collection and maintaining

Adults (*n* = 819: 401 males and 418 females) of unknown age were collected from *P. canariensis* infested plants or from traps baited with the aggregation pheromone Rhyfer 220 (Intrachem Bio Italia S.p.A.) during spring and summer of 2009-2012 in Sicily, southern Italy.

Once in the laboratory, the insects were separately maintained in glass cages (15 × 15 × 15 cm) at T: 25 ± 5 °C, 80-100% RH, under a natural light:dark (L:D) cycle regime (about 14:10), with apple (Golden Delicious variety) slices as both food and oviposition substrate. Apple is usually used as the substrate for laboratory maintaining of this species (e.g. Fanini *et al*., 2014; Montagna *et al*., 2015). The insects were kept in the laboratory and fed with apple *ad libitum* at least one week before the experiment.

General experimental procedure

We used a binary choice test paradigm to assess the female preference for an oviposition substrate by simultaneously offering, in the same cage used for rearing, two equally sized apple slices of about 45 g (cut from the same apple) to a pair of weevils (a male and a female) or to a female alone. One of the two slices was subjected to one of various treatments (see below) and then used as the experimental substrate while the other one was used as the control substrate. Treatment and control substrates were placed at the maximum distance from each other in the cage (about 18 cm). As an index of female choice, we used the number of eggs laid in each substrate after one day from the start of the experiment. Clean entomological tweezers were used to collect eggs both on the surface and in depth of the apple, trying not to seriously damage the experimental apple; however the same treatment was applied to the control slice.

1) Female preference to lay eggs in a substrate already used for oviposition

Each pairs (*n* = 20) was isolated in a cage where male and female were allowed to mate. The day before the experiment, each cage was provided with an apple slice as the substrate for oviposition (experimental substrate). At the same time, for each pair, another apple slice (the control substrate) was drilled with a clean glass capillary to favour the same degradation process occurring in the experimental substrate due to the weevils' activity and it was kept outside the cage. On the second day, the eggs laid in the experimental substrate were counted and the control substrate was added to each cage. On the next day, the number of eggs laid in both substrates was counted for each pair.

2) Role of eggs or egg-deposition behaviour in eliciting female oviposition substrate preference

a) Testing of eggs presence

To test whether the presence of eggs was responsible for the substrate preference by females, we removed the laid eggs from their deposition holes in apple slices. We then provided 24 pairs with two new apple slices, both drilled with a glass capillary, in each cage. One of them was the control substrate while the other was used as the experimental substrate where the fresh previously collected eggs were inserted into the holes made with the drilling procedure. The number of inserted eggs for each experimental slice was counted (from two to fourteen eggs per pair). On the next day, the number of eggs laid in both substrates was counted for each pair.

b) Testing egg surface compounds

To test whether the compounds on the egg surface were responsible for the female preference of a substrate, we extracted apolar and polar fractions from the surface of the eggs (see below for extraction procedure) and used these extracts in subsequent bioassays.

b1) Substrate with polar and apolar substances extracted from the eggs:

We collected fresh eggs (laid in the previous 24 h) from substrates in all the cages and then inserted 10 (first trial) and 30 (second trial) eggs into each glass vial and washed them in 1 ml of an apolar (pentane) or a polar (methanol) solvent for 3 min to extract respectively apolar or polar compounds from the egg surface (Baracchi *et al*., 2012). The solution was collected from each vial and the apple slice (the experimental substrate) was treated with it. The control substrate was treated with 1 ml of pure solvent as control. To test the female oviposition preference, we offered to each pair an experimental substrate with either apolar ($n = 20$ for both trial) or polar ($n = 20$ for both trial) extract and a control substrate. On the next day, the number of eggs laid in both substrates was counted for each pair.

b2) Substrates with concentrated apolar and polar substances extracted from the eggs:

We collected fresh eggs (laid in the preceding 24 h) from substrates in all the cages and then inserted 10 eggs into each glass vial and washed them in 1 ml of either apolar (pentane) or polar (methanol) solvent for 3 min to extract apolar or polar compounds from the egg surface. The solution was collected from each vial, air dried overnight and re-suspended the following day in 100 µl of solvent (pentane or methanol) in order to concentrate the extract with respect to the previous experiment.

We offered to each pair an experimental substrate with concentrated apolar ($n = 27$) or polar ($n = 23$) extracts of the egg surface and a control substrate with 100 µl of solvent only. On the next day, the number of eggs laid in both substrates was counted for each experimental pair.

c) Testing "oviposition plugs"

To test whether some substances present in the deposition plug were responsible for the female preference of a substrate, we provided in each cage of 21 pairs two new apple slices: 10 plugs (five for each side of the apple slice) were placed on one slice (experimental substrate) while the other slice was not treated (control substrate). On the next day, the number of eggs laid in both substrates was counted for each experimental pair.

The plugs were gently collected one day before the experiment directly from the surfaces of apples already used for oviposition; we avoided the collection of those plugs which were strongly linked to their egg to exclude any contamination of the plug by substances from the eggs.

d) Testing for adult compounds possibly released by adults before oviposition

To test whether substances possibly released by the adults in the deposition hole before egg-laying could be responsible for the female preference, we applied the same procedure as in experiment 1 to 15 pairs except for the removal of eggs from the experimental substrate on the second day before the control substrate was added. On the next day, the number of eggs laid in both substrates was counted for each experimental pair.

3) Role of adults in eliciting oviposition substrate preference

a) Testing adult abdominal cuticular substances

To test whether the substances covering the cuticle of adults were responsible for the female preference, we simultaneously offered two apple slices to each pair ($n = 14$). The experimental substrate was rubbed for 30 seconds with the abdomen of a male on one side and with the abdomen of a female on the other. Unlike eggs and small larvae, the abdominal surface of both RPW sexes presents the same polar substances with antimicrobial activity (Mazza *et al*., 2011); these compounds could favour egg survival when placed on the substrate prior to egg laying. The control substrate was an untreated apple slice. On the next day, the number of eggs laid in both substrates was counted for each experimental pair.

b) Testing the female or male rostrum compounds

To test whether the compounds present and/or released on/by the rostrum of adults were responsible for the female preference for a substrate, we simultaneously offered two apple slices to each pair. Females of this species use the rostrum to drill the substrate before oviposition while males use it to diffuse aggregation pheromones, similar to *R. palmarum* (Sánchez *et al*., 1996). We drilled the experimental substrate (10 holes) with the rostrum of a female ($n = 22$) or a male ($n = 15$) by forcing the weevils' rostrum inside the substrate; the control substrate was drilled (10 holes) with a glass capillary (with the same diameter as the rostrum). On the next day, the number of eggs laid in both substrates was counted for each experimental pair.

c) Testing the male role in oviposition substrate preference

c1) Substrate used only by females:

To test whether the male was responsible for the female preference, we followed the same procedure as experiment 1 except that we used a single females alone ($n = 17$) instead of a pair (male and female).

c2) Substrate used only by males:

To test whether substances released by males were responsible for the female preference, we simultaneously offered two apple slices to females ($n = 24$). The experimental substrate was used by males ($n = 24$) for one day while the control apple slice, drilled as in the previous experiments, was kept outside the cage. On the second day, the two substrates were added to each cage with a female. On the next day, the number of eggs laid in both substrates was counted.

c3) Effect of the aggregation pheromone:

To test whether the aggregation pheromone was responsible for the female preference, we simultaneously offered two apple slices to each pair ($n = 26$). A single grain of the synthetic aggregation pheromone Rhyfer 220 (about 0.15 mg of pheromone) was inserted in the surface of the experimental substrate. The control substrate was an untreated apple slice with only a single hole of the same dimension of the grain above. On the next day, the number of eggs laid in both substrates was counted for each experimental pair.

4) Female preferences over time and in space

a) Testing the effect over time

To test if the experimental substrate is preferred by females at different time lags, we carried out three contemporaneous assays as follows. Pairs were allowed to use the experimental substrate for one day. The control apple slice, drilled as in the previous experiments, was kept outside the cage. In the 1st assay ($n = 23$), we performed the procedure of experiment 1. In the 2nd assay ($n = 24$), we kept both the experimental and control apple slices outside the cage on the second day, and then we offered them to the female on the third day (time lag = 24 h, i.e. the experimental slice was not used for 24 hours). In the 3rd assay ($n = 23$), we kept both the experimental and control apple slices outside the cage on the second and third day, and we offered them to the female on the fourth day (time lag = 48 h). For these assays, the number of eggs laid were counted on the day after both substrates was offered to the female.

b) Testing effects in space

To test whether females ($n = 20$) maintain the preference for a substrate already used for oviposition in a more extensive space than that just tested in the previous experiments, we conducted experiment 1 in a glass larger cage (59 × 42 × 8.5 cm).

Statistical analyses

The non-parametric paired-samples Wilcoxon signed rank test (statistic: z) was used to compare the numbers of new eggs laid on the experimental and control substrates by tested females (SPSS®, Statistical Package for Social Sciences, 13.0 for Windows).

Results

1) Female prefer to lay eggs in a substrate already used for oviposition

The first experiment showed that females lay significantly more eggs in a substrate already used for oviposition (by a pair) than in a control substrate ($z = -3.76$, $n = 20$, $p = 0.0001$).

2) No role of eggs or egg-deposition behaviour in eliciting female oviposition substrate preference

a) No effect of eggs presence

There was no significant difference in the number of eggs laid in the substrate with experimentally transplanted eggs or in the control substrate ($z = -1.88$, $n = 24$, $p = 0.06$). This indicates that it is not the physical presence of eggs that elicits female oviposition substrate preference and suggests that substances deposited on the substrate by the eggs themselves or by adults could mediate the female preference.

b) No effect of eggs surface compounds

In both trials (10 and 30 eggs), the number of eggs laid in the experimental substrate with apolar ($n = 20$) or polar ($n = 20$) egg surface extracts was not significant different from those laid in the control substrate (10 eggs: apolar $z = -1.16$, $n = 20$, $p = 0.24$; polar $z = -0.50$, $n = 20$, $p = 0.61$; 30 eggs: apolar $z = -0.71$, $n = 20$, $p = 0.48$; polar $z = -0.67$, $n = 20$, $p = 0.50$). The same results were obtained using concentrated extracts of apolar ($n = 27$) or polar ($n = 23$) fractions ($z = -0.57$, $n = 27$, $p = 0.57$; $z = -0.13$, $n = 23$, $p = 0.90$, respectively). This excludes that the female preference depends on substances from the egg surface.

c) No effect of "oviposition plugs"

There was no significant difference in the number of eggs laid in the substrate with transplanted deposition plugs or in the control substrate ($z = -0.31$, $n = 21$, $p = 0.76$).

d) Effect of adult compounds possibly released before oviposition

Females laid significantly more eggs in the substrate where eggs were previously removed than in the control substrate never used for egg laying ($z = -2.34$, $n = 15$, $p = 0.02$). This suggests that the female preference for an oviposition site could be mediated by some substances emitted by adults before/during oviposition.

3) Role of adults in eliciting oviposition substrate preference

a) No effect of adult abdominal cuticular substances

There was no significant difference in the number of eggs laid in the substrate rubbed with the abdomen of an adult (both male and female) or in an untreated control substrate ($z = -0.93$, $n = 14$, $p = 0.35$).

Table 1. Summary of the results of the bioassays. Median (± 1st, 3rd quartiles) of eggs laid (per day per female) in the control and in the experimental substrates for each trail was given. Results of Wilcoxon non-parametric test are reported; *n* is the number of females tested for each experiment. Significant values are denoted in bold.

Experiment	no. of eggs laid in the control	no. of eggs laid in the experimental	*z*	*n*	*p*
1) female preference to lay eggs in a substrate already used for oviposition	0 (0, 0)	3 (2, 4)	−3.76	20	**0.0001**
2) effect of egg-deposition behaviour or eggs:					
a) egg presence	1 (0, 1.5)	2 (0.75, 4)	−1.88	24	0.06
b) egg surface compounds					
- apolar diluted (10 eggs)	2.5 (0.75, 4)	2 (0.75, 10.75)	−1.16	20	0.24
- polar diluted (10 eggs)	1 (0, 3)	1 (0.75, 3)	−0.50	20	0.61
- apolar diluted (30 eggs)	1.5 (0, 4)	1 (0, 3)	−0.71	20	0.48
- polar diluted (30 eggs)	1 (0, 3.25)	2 (0, 5.25)	−0.67	20	0.50
- apolar concentrated	1 (0.5, 3)	2 (1, 3)	−0.57	27	0.57
- polar concentrated	1 (0, 2)	2 (0, 3.5)	−0.13	23	0.90
c) “oviposition plug”	1 (0, 3)	1 (0, 5)	−0.31	21	0.76
d) possible compounds released by adults before oviposition	1 (0, 1.5)	2 (1, 3)	−2.34	15	**0.02**
3) to test the effect of adults:					
a) adult abdominal cuticular substances	0.5 (0, 1.75)	1 (1, 2)	−0.93	14	0.35
b) compounds present and/or released on/by the rostrum					
b1) female rostrum	0 (0, 1.75)	1 (1, 2)	−1.51	22	0.13
b2) male rostrum	1 (0.5, 3)	1 (1, 4)	−0.32	15	0.75
c) substrate used					
c1) only by females	1 (0, 2)	1 (0, 2)	−0.73	17	0.46
c2) only by males	1 (0, 2.25)	4 (1, 7)	−3.20	24	**0.001**
c3) with aggregation pheromone	2 (0.25, 3.75)	4.5 (3, 6)	−3.41	26	**0.001**
4) Experiments to test female preferences over time and in space					
a) effect over time					
- 1st assay	3 (1, 3)	45 (2, 9)	−2.70	21	**0.01**
- 2nd assay	0 (0, 1)	7 (3, 9)	−3.84	23	**0.001**
- 3rd assay	1 (0, 3)	7 (4, 12)	−3.15	23	**0.001**
b) effects in space	0 (0, 1)	2.5 (1, 5.5)	−2.06	20	**0.04**

b) No effect of female or male rostrum compounds

The number of eggs laid in the substrate drilled with the rostrum of both sexes was not significantly different from the number of eggs laid in the control substrate (female: $z = -1.51$, $n = 22$, $p = 0.13$; male: $z = -0.32$, $n = 15$, $p = 0.75$).

c) Effect of the male in eliciting oviposition substrate preference

The number of eggs laid in the substrate already used for oviposition was not significant different from the number of eggs laid in the control substrate never used for egg laying ($z = -0.73$, $n = 17$, $p = 0.46$). This suggests that female preference for an oviposition substrate depends on the male presence. Indeed, the females preferred to lay eggs in a substrate previously used by a male ($z = -3.20$, $n = 24$, $p = 0.001$) and in a substrate with the aggregation pheromone ($z = -3.41$, $n = 26$, $p = 0.001$) than in the control substrate.

4) Female preference persists over time and in space

The experimental substrate remained attractive over time for females, at least for the tested time lags (1st assay: $z = -2.70$, $n = 21$, $p = 0.01$; 2nd assay: $z = -3.84$, $n = 23$, $p = 0.001$; 3rd assay: $z = -3.15$, $n = 23$, $p = 0.001$) and the females were able to discriminate the experimental substrate in a more extensive space ($z = -2.06$, $n = 20$, $p = 0.04$).

The results of all the experiments are summarized in table 1.

Discussion and conclusion

Our results clearly demonstrate that RPW females prefer to lay eggs in a substrate already used for oviposition (experiment 1) and that this preference is not related to the physical presence of eggs nor to the substances on the egg surface (experiment 2).

Therefore, eggs do not seem to be indicators of a suitable substrate for oviposition. Unexpectedly, Salem *et al.* (2012) suggest, for the same species, that substances on egg surface can discourage females from further oviposition in already used site. These results are in contrast with our findings: first, females lay more eggs in an already laid substrate (experiment 1), and second, eggs do not have any role in favouring or impeding females’ oviposition (experiment 2). We believe that the opposite results reported by Salem *et al.* (2012) could be due to the different experimental procedure used in the two studies, but unfortunately the method section of their paper is not detailed enough to identify any potential bias (e.g., the number of performed trails, as well as the details about the extraction methods are not given). Moreover, in Salem *et al*. (2012), there is an unexpected finding: the extracts prepared with polar and apolar solvents elicited the same response, although such solvents are used to extract different classes of compounds (polar and apolar compounds).

We have additional concerns regarding the prolonged time (24 h) chosen by Salem *et al*. (2012) for the extraction procedure (in our experiments we used a 3 min extraction) that probably caused the extraction of internal compounds from the eggs, i.e. the oleic acid. Furthermore, oleic acid is a compound frequently found in the extracts from insect material (see Dani *et al*., 2003) and it is difficult to prove its actual function as an insect pheromone. Some studies (Ayasse and Paxton, 2002; Purnamadjaja and Russell, 2005) reported this compound as a necrophoric pheromone; thus, we suspect that the oleic acid, suggested by Salem *et al*. (2012) as potential surface signaling compound, could be an internal compound extracted from the eggs due to the prolonged extraction time. Oleic acid is produced by decaying insect corpses and this could explain the deterrent effect on oviposition found by Salem *et al*. (2012). The oleic acid scent may actually dissuade females to use a site for deposition.

The female tendency to lay eggs in sites where eggs had already placed by conspecifics is in accordance with the biology of this species. The RPW life is characterized by an aggregative behaviour; when males find suitable damaged palms, they release a pheromone which makes the weevils aggregate on the same trees probably to overcame the plant defences, as reported also for the bark beetles (Byers, 2004); moreover, within the palm a large number of adult individuals and immature stages can be found contemporaneously in the same trunk area and many eggs are laid in a narrow space. This characteristic overcrowding of RPW inside the palm does not support the findings of Salem *et al*. (2012) that would lead to spacing rather than to aggregating individuals. At the same time, there are several reasons to justify this female behaviour. First of all, while the palm material could be considered a limiting factor for RPW feeding in its introduced range, this is not true in its native range, where the behavioural repertoire of the species has been selected. Secondly, the RPW action generates a suitable microhabitat for pathogens and parasites inside the trunk that could prevent the development of RPW immature brood. As highlighted in Cito *et al*. (2014) and Mazza *et al*. (2014), there are some pathogens, such as the indigenous ones, that are able to overcome the host insect's immune defence system and cause insect death. However, a previous study of our research group (Mazza *et al*., 2011) demonstrated that both the larval and adult body of RPW individuals is covered by substances with antimicrobial activity, suggesting a good reason for laying eggs on an already used substrate where such substances are probably spread. The female strategy to lay eggs in an already used substrate could protect eggs from microbial attacks and assure their survival during the first period after hatching (according to the same authors, egg surface and small larvae were not covered by antimicrobial substances).

Finally, since the lower temperature threshold for egg hatching is 13.95 °C (Dembilio *et al*., 2012), ovipositing in the same site (and thus the formation of adult aggregations) should favour a higher temperature inside infested palms (mainly due to the activity of the grubs on the interior of the palm) that could not limit egg hatching. Regarding the origin of cues driving female choice during egg deposition, our data exclude that the female preference depends on substances from the egg surface and suggest that the female preference for an oviposition substrate might be induced by substances produced by the adults before or during oviposition. However, RPW body portions, such as the ventral face of the abdomen and the rostrum of both sexes, have no effect on the female oviposition preference. In addition, our experiments provide evidences that male presence has a role in the female preference for an oviposition substrate. We hypothesize that the aggregation pheromone could also indicate a good substrate for laying eggs. Experiments carried out with substrates treated with aggregation pheromone trigger the same female response. For the moment, it is not easy to say whether female preference is a simple by-product of the aggregation pheromone or is elicited by a specific compound of the aggregation pheromone (i.e. an oviposition pheromone). Nevertheless, our study highlights a possible additional function of aggregation pheromone of this species. Further studies will be necessary to clarify this point and to identify the potential active compound eliciting this behavioural response. Experiments will also be necessary to determine if the aggregation pheromone has a double effect, as hypothesised in accordance with the "semiochemical parsimony" theory (Blum, 1996). However, some indications provided by other authors support our hypothesis: mated RWP individuals of both sexes increased their response to ferrugineol, the aggregation pheromone (Poorjavad *et al*., 2009) and mated females searching for an oviposition substrate showed a stronger response to the aggregation pheromone than unmated females (Poorjavad *et al*., 2009); a study conducted in India showed that most of the females trapped with the use of the pheromone were gravid or were starting egg development (Kalleshwaraswamy *et al*., 2005).

RPW females mate on the host palm after their emergence from cocoons (Avand-Faghih, 1998), thus, it is likely that the high sensitivity to the aggregation pheromone ensures that females find mates and suitable oviposition sites, while avoiding host palms of low quality (Poorjavad *et al*., 2009). In *R. palmarum*, the aggregation pheromone is secreted both from the mouth and in the faeces. Once secreted from the mouth, the pheromone flows to a depression on the dorsal part of the rostrum and is collected by a complex hairy structure that diffuses it (Sànchez *et al*., 1996). However, our experiment in which we tested the effect of the male rostrum did not yield significant results. This might be due to the absence of secretion activity during our manipulation; moreover, the aggregation pheromone is typically released in response to particular odours (Sànchez *et al*., 1996), lacking in our experiment setup.

Our findings show that the compounds responsible for female preference are active for at least two days and in both a restricted and a larger space, suggesting their volatile nature, as reported for the aggregation pheromone.

In conclusion, our findings on the chemoecology of red palm weevil oviposition open perspectives for new tools in the monitoring and controlling of this pest using integrated management strategies.

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