

Overstinging by hymenopteran parasitoids causes mutilation and surplus killing of hosts

Ruth Cebolla¹ · Pilar Vanaclocha^{1,2} · Alberto Urbaneja¹ · Alejandro Tena¹

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Abstract An appraisal of the regulatory role of natural enemies on target pests requires the identification of the mechanisms/traits that enhance the ability of an organism to control the density of its prey/host. After stinging herbivore hosts with their ovipositor, hymenopteran parasitoids tend to reject them without ovipositing or host-feeding. Termed pseudoparasitism, the frequency and consequences of this type of attack (hereafter overstinging) have been largely disregarded in the hymenopteran parasitoid literature. We choose the parasitoids *Aphytis melinus* and *A. chrysomphali* and their common host *Aonidiella aurantii* as a model system to study this behavior. Using field and laboratory observations, we showed that overstinging is a common behavior in the wild. Under controlled conditions, overstinging occurred more frequently than host-feeding, a behavioral trait that is used to evaluate the potential of parasitoids as biological control agents. Overstinging reduced the fecundity and survival of the herbivore host. When we compared between parasitoid species that attack the same host species, the virulence and frequency of this behavior depended on parasitoid species. These results demonstrate that overstinging should be incorporated in the models of host–parasitoid interactions

to analyze population dynamics as well as in the future selection of parasitoids for biological control.

Keywords *Aphytis* · *Aonidiella aurantii* · Behavioral ecology · Biological control · Host-feeding · Overkilling · Physiological entomology

Key message

- After stinging their hosts, hymenopteran parasitoids tend to reject them without ovipositing.
- The frequency and consequences of this type of attack (overstinging) have been largely disregarded.
- Using parasitoids of genus *Aphytis* and its host *Aonidiella aurantii*, we demonstrate that overstinging is a common behavior, even more than host-feeding.
- The frequency and virulence depended on parasitoid species.
- These results prove that overstinging should be incorporated in the selection of parasitoids for biological control.

Introduction

Entomologists and ecologists interested in biological control have long sought insights to guide the selection of effective natural enemies because many natural enemies have important limitations as potential regulators of herbivorous pests (Jervis 2005). However, an appraisal of the regulatory role of natural enemies requires the identification of the mechanisms/traits that enhance the ability of an organism to control the density of its prey/host. Parasitoids

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✉ Alejandro Tena
atena@ivia.es

¹ Unidad Asociada de Entomología UJI-IVIA, Centro Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA), Carretera CV–315, Km 10.7, 46113 Moncada, Valencia, Spain

² Southwest Florida Research and Education Center, University of Florida, Immokalee, FL, USA

are the most important and successful group of natural enemies used in the biological control of insect pests (Godfray 1994; Jervis 2005), and their efficacy depends on the behavioral decisions of females when they search for and find a host (Mills and Wajnberg 2008). Generally, when a female parasitoid encounters a host, she either (1) lays eggs in/on the host and the larvae then feed on the host, and/or (2) she feeds on the hemolymph of the host and uses it to produce additional eggs (i.e., host-feeding); both behaviors eventually kill the host. To obtain information about the suitability of a potential host, the female parasitoid inserts her ovipositor and, in some cases, (3) then rejects the host (Heimpel and Collier 1996; Heimpel et al. 1998; Hopper et al. 2013). This behavior is common in hymenopteran parasitoids and is known as “probe/sting and rejection” or “overstinging.” However, despite its prevalence, the effect of this behavior on hosts has been largely disregarded in the parasitoid literature, but it might be an important trait in the selection of parasitoids for biological control programs.

In the few studied cases, the consequences of overstinging vary from reduced fitness of the wounded hosts (mutilation) to host death (Abdelrahman 1974; Jones 1985; Jones et al. 1986; Brown and Kainoh 1992). This variability might depend on the stage of the host being stung by the female parasitoids; older and larger hosts may be more resistant to overstings than younger and smaller hosts (Salt 1968; Vinson 1976; Beckage and Gelman 2004). Therefore, as most species of parasitoids attack hosts of different sizes and even instars, we hypothesize that small hosts will be more likely to die after these attacks. If these hosts die, the stings represent a case of surplus killing or overkilling as the female parasitoid will be killing more hosts than needed for parasitism or host-feeding. Surplus killing by parasitoids might be another useful trait in the identification and evaluation of their potential as biocontrol agents, as it is for predators (Johnson et al. 1975).

The frequency of these attacks (overstings) has also been poorly researched, and it might vary among parasitoid species. Generally, parasitoids with low egg loads and high life expectancies (i.e., egg limited) might oversting more frequently than species with high egg loads and low life expectancy (i.e., time limited) because the former will reject more hosts as having low suitability for their progeny (Heimpel and Collier 1996; Heimpel et al. 1998; Hopper et al. 2013). The probability of overstinging may also depend on the geographical origin of the species involved. In this sense, overstinging might be more frequent in non-coevolved parasitoids when compared with coevolved parasitoids because host evaluation by the parasitoid might be decoupled from the suitability of the host species for the immatures, as a result of a lack of shared evolutionary history (sensu Schlaepfer et al. 2005).

Here, we chose the parasitoids of the genus *Aphytis* (Hymenoptera: Aphelinidae), which attack the California red scale, *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), in citrus, as a model system to evaluate and compare i) the occurrence and frequency of overstinging in two parasitoids under laboratory and field conditions and ii) the differences in the effects of overstinging on different instars of their common host. In the Mediterranean Basin, California red scale became a key citrus pest at the end of the last century, and it was rapidly parasitized by the native parasitoid *Aphytis chrysomphali* (Mercet) (Hymenoptera: Aphelinidae). Later, its coevolved parasitoid *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) was introduced in a classical biological control program, and it has since displaced *A. chrysomphali* in most areas (Sorribas et al. 2010). Both parasitoids tend to reject hosts after stinging according to laboratory observations (Abdelrahman 1974; Casas et al. 2004), but the consequences of these stings on the hosts have never been examined. All of these factors make this a highly suitable model system to study the frequency and effect of overstinging by hymenopteran parasitoids; and determine whether overstinging should be considered when evaluating the efficacy of parasitoids as biological control agents as well as modeling the population dynamics of parasitoids and hosts.

Materials and methods

Experimental insects

Aonidiella aurantii were reared on lemons from a laboratory colony at the Instituto Valenciano de Investigaciones Agrarias, IVIA (Montcada, Valencia, Spain). This colony was initiated in 1999 from scales collected from citrus fields in Alzira (Valencia, Spain) and renewed every 2–3 years with scales from the field (Tena et al. 2013). We followed the methodology described in (Pina 2007) for rearing *A. aurantii*. Briefly, $\sim 2/3$ of the surface of each lemon was covered with red paraffin around the mid-section to retard desiccation; the red paraffin was prepared with a mixture of 1 kg of paraffin pearls (Parafina USP Perlas; Guinama S.L., Alboraya, Spain) and 1 g of red pigment (Sudan III; Panreac Química S.A., Castellar del Vallés, Spain). The remaining surface area (approx. 24 cm²) of the lemons was infested by exposure to gravid female scales in the *A. aurantii* colony for 48 h. Once infested, the lemons were maintained at 26 ± 1 °C, $70 \pm 5\%$ RH and darkness until the female scales reached the second (9–11 days) and third (19–22 days) nymphal instars.

Aphytis melinus and *A. chrysomphali* were obtained by exposing third-instar *A. aurantii* on lemons to parasitism by

insectary-reared adult wasps maintained in the laboratory at 26 ± 1 °C, $60 \pm 5\%$ RH and LD 16:8 h. The *A. melinus* colony was initiated in 2008 and the *A. chrysomphali* colony was initiated in 2013 from scales collected in citrus fields from Alzira and Moncada (Valencia, Spain), respectively. Both colonies are renewed yearly with parasitoids collected in the field.

Between five and ten late-stage pupae of both parasitoids were removed from parasitized scales and held separately in 8-mm-diameter and 35-mm-long crystal vials. At emergence, parasitoids were sexed and held in these vials for one day to obtain mated females of *A. melinus* (*A. chrysomphali* reproduces parthenogenetically) (Gottlieb et al. 1998). One day after their emergence, the females were isolated in the same vials as above and used 2–3 days later. A drop of honey was added to the inside wall of each vial, which were stoppered with a cotton plug. Vials were stored in a climatic chamber (SANYO MLR- 350; Sanyo, Japan) at 25 ± 1 °C, 50–70% RH and LD 14:10 h.

Arena

The arena consisted of a lemon with an approximately 24-cm² surface area covered with a transparent cardboard ring that 5.5 cm in diameter and 4 cm high to prevent the parasitoids from escaping. We used a dissecting microscope with a micrometer to select ten scales from the surface of the lemon, and we removed the rest using an insect pin and a paper towel that had been moistened with water. The selected scales were 9–11 days old and 0.55 ± 0.05 mm² for the second instar and 19–22 days old and 0.85 ± 0.05 mm² for the third instar. To estimate their sizes, photographs of the scales were taken with a Leica EC 3 3.1-megapixel digital color camera (Leica Microsystems GmbH, Spain), and the images were processed with Leica LAS EX imaging software for Windows (Leica Microsystems GmbH, Spain). Measurements from all of the pictures were made with ImageJ, a public-domain Java Image processing program (Rasband 2016). All of the scales were mapped and numbered before the observations began.

Behavioral observations

In each replicate, we continuously observed female behavior using a dissecting microscope at 10× to 50× magnification and used a cool fiber light to illuminate the arena. An observation began when a single female of one of the two species was placed in the arena with the ten host scales, and each female parasitoid was observed until she rested for more than 10 min.

We recorded sequences of behavioral interactions with all of the hosts including behaviors that took place within

the host body. Thus, three separate behavioral events on a host were identified, timed and recorded: (1) overstinging, (2) ovipositing and (3) host-feeding. After drumming the scale with its antenna, a female parasitoid may investigate a host by stinging, which includes using the ovipositor to drill through the scale cover, explore the cavity between the scale body and cover, and pierce the body and explore the hemocoel. The parasitoid may leave the host at any time during this process (hereafter termed oversting) and/or may proceed to oviposit or consume its body fluids (host feed). Vibration of the ovipositor during stinging indicates that an egg has been laid, and host-feeding is recognized by the female parasitoid lowering its head and positioning its mouthparts over the sting immediately after probing (Casas et al. 2004). Additionally, we also mapped the ovipositor insertion points during host stinging. In detail, we distinguished between ovipositor insertions in the center of the scale cover (molt rings) and those ones made in the scale edge (gray skirt).

Aonidiella aurantii fitness and survival

Once the observations ended, the parasitoid was removed, and each lemon was kept in a plastic container (14 × 14 × 8 cm) along with another lemon infested with male and female scales of the same age. Thus, males from this second lemon could mate with the experimental female scales. The plastic container was covered with a piece of muslin fixed in place with a rubber band and kept in the same climatic chamber as above.

To determine the effect of the behavior of each parasitoid on the survival and fecundity of *A. aurantii*, scales were mapped, observed and measured as described above. We considered a scale to be dead when it did not grow, and this was confirmed by removing the scale cover and inspecting the turgency of the body. Hereafter, we use the term “surplus killing” to refer to the mortality caused by the overstings; to our knowledge, there is no existing term in the parasitoid literature to refer to this type of mortality. This term, as well as “overkilling”, is used when predators kill more prey than they eat, so in parasitoids, this term describes females killing more hosts than they eat or parasitize.

To measure the fecundity of the surviving scales, these individuals were isolated with a double-sided sticky plastic ring (3MScotch^R; Cergy Pontoise Cedex, France) to trap the crawlers produced by each female following the methodology proposed by Vanaclocha (2012). Sticky plastic rings were placed 21 days after the observation period and replaced weekly for four weeks. The number of crawlers stuck in the rings was then counted under the binocular. To compare the survival and fecundity of the probed females with those of the unattacked females, we

repeated this procedure for two unattacked females on each lemon.

Field observations

We conducted a field assay to determine whether the presence of punctures/scars in field scales was correlated with *Aphytis* and predator activity as well as with climatic variables (mean temperature, maximum temperature, mean wind, maximum gust of wind, accumulated rain and maximum rain in one day), in three commercial citrus groves (Almenara, La Pobla de Vallbona and Betera) located in eastern Spain. Almenara (39°45′02.71″N; 0°12′10.09″W) consisted of 9-year-old clementine (*Citrus reticulata* Blanco) ‘Oronules’ trees (9 years old) grafted on Citrange Carrizo [*Poncirus trifoliata* (L.) Rafinesque-Schmaltz × *Citrus sinensis* (L.) Osbeck] with an extension of 0.2 ha. La Pobla de Vallbona (39°38′05.68″N; 0°30′51.30″W) consisted of 5-year-old clementine ‘Esval’ trees grafted on Citrange Carrizo with an extension of 0.2 ha, and Betera (39°35′10.13″N; 0°24′39.14″W) consisted of clementine ‘Clemenules’ trees (5–10-year-old) grafted on Citrange Carrizo with an extension of 1 ha. Standard agronomic practices for citrus cropping were performed, but insecticides were not sprayed during the assay.

Populations of *A. aurantii* were monitored weekly or every other week from April to November 2007 depending on their phenology (weekly from the beginning of the new generation until the sum of the first- and second-instar hosts represented 60% of the *A. aurantii* population). In each orchard, young shoots infested with *A. aurantii* were collected at random and transferred to the laboratory in plastic bags, and a maximum of ten hosts per shoot were collected to count the number of alive, dead, predated, parasitized or punctured *A. aurantii* of each instar using a stereoscopic microscope. Observations ended when 80 second- and third-instar hosts were counted or when a total of 500 scales were counted per sample. Individuals were considered alive if they were turgid and dead if they were dry and dark (Fig. 1); scales were considered predated when their body had been partially consumed; and scales were considered parasitized if immature parasitoids were found. *Aonidiella aurantii* individuals were noted as being overstung when brown punctures were found on their bodies and they remained alive (turgid) (Fig. 1).

Statistical analysis

We applied generalized linear modeling (GLM) techniques assuming Poisson error variance for the count data (number of behavioral events per patch, number of stings per host) and binomial error variance for the proportional data

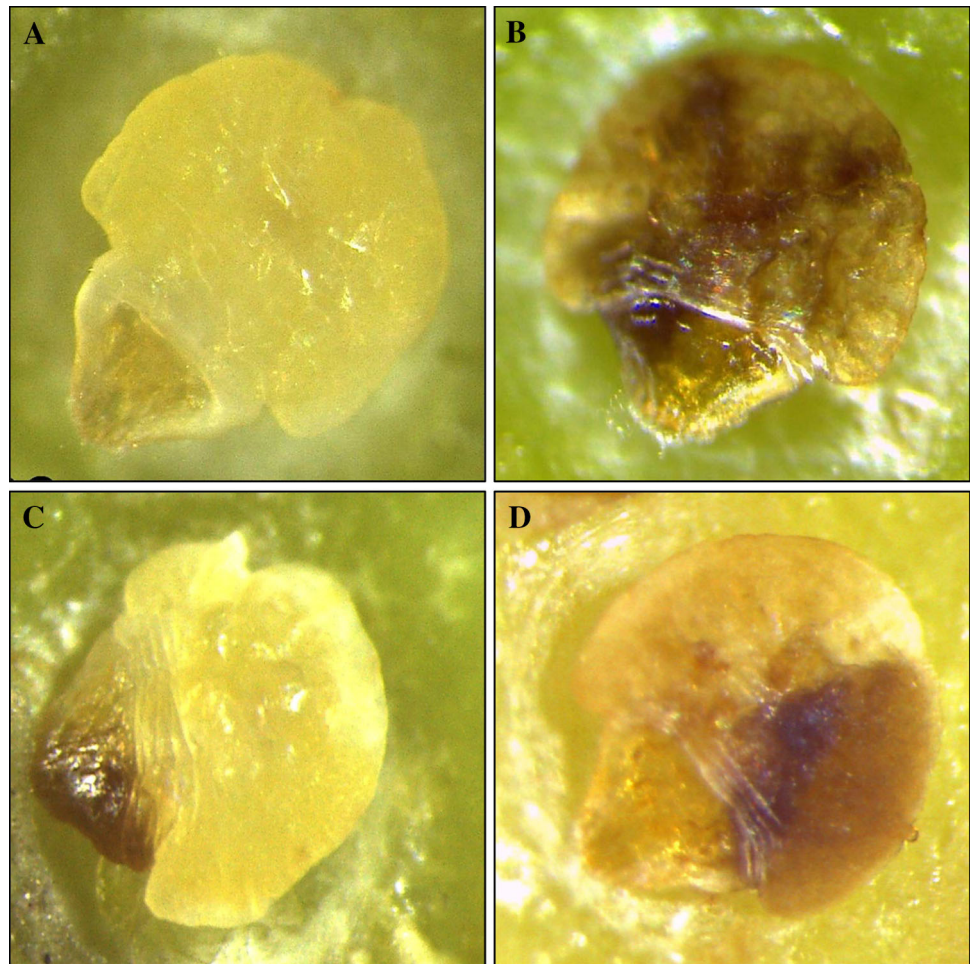
(proportion of scales with punctures in the field, mortality). We assessed significance according to the change in deviance when a variable was removed from the model using a Likelihood Ratio Test with Poisson or binomial errors. Significant values are provided in the text for the minimal model, and the nonsignificant values are those that were obtained before we deleted the variable from the initial model. We assessed the assumed error structures using a heterogeneity factor equal to the residual deviance divided by the residual degrees of freedom. If we detected an over- or underdispersion, we re-evaluated the significance of the explanatory variables using an F test after rescaling the statistical model by a Pearson’s chi square divided by the residual degrees of freedom (Crawley 2007). We present the means of the untransformed proportion and count data (in preference to less intuitive statistics such as the back-transformed means of logit-transformed data). This results in the standard errors being presented as symmetrical, which results in symmetrical standard errors that did not yield impossible values such as a mortality of less than 0. In the multiple logistic regression between the proportion of scales with punctures and the multiple predictor variables, we first created a correlation matrix between all variables: number of parasitoids, number of predated scales, mean temperature, maximum temperature, mean wind, maximum gust of wind, accumulated rain and maximum rain in one day. If two variables were correlated ($P < 0.05$), one of them was removed following biological significance (“Annex 1”). We compared *A. aurantii* fecundity and time using ANOVAs. The normality assumption was assessed using Shapiro’s test, and the homoscedasticity assumption was assessed with Levene’s test. All of the data analyses were performed with the R freeware statistical package (<http://www.R-project.org/>) except the correlation matrix that was performed with Statgraphics.

Results

Field observations

Overall, we observed 1933 second- and third-instar *A. aurantii* in the three citrus orchards from April to November. A total of 1079 (55.82%) were alive; 654 (33.83%) were parasitized; and 200 (10.35%) were alive but had brown punctures or scars (Fig. 2). When we distinguished between instars, only 8 out of the 607 (1.32%) second-instar scales were alive with punctures, whereas 192 out of the 1134 (14.48%) third-instar scales were alive with punctures. The percentage of total live scales with punctures per orchard was significantly higher in the third instar than in the second ($F_{1,4} = 1.2$; $P < 0.001$).

Fig. 1 Body of a third-instar *Aonidiella aurantii* when: **a** healthy and alive, **b** dead by host-feeding, and **c** alive and **d** dead 3 days after being overstung by *Aphytis* parasitoids



The proportion of live hosts with punctures or scars was positively correlated with the number of immature *Aphytis* ($\chi^2_1 = 36.81$; $P < 0.001$; Fig. 3) and preyed scales ($\chi^2_1 = 32.6$; $P < 0.001$); negatively correlated with the accumulated rain ($\chi^2_1 = 4.68$; $P = 0.03$) and the maximum gust of wind ($\chi^2_1 = 5.71$; $P = 0.017$); and varied among the three sampled orchards ($\chi^2_2 = 0.0028$; $P = 0.03$). There was also a significant interaction between the number of immature *Aphytis* and predated scales ($\chi^2_1 = 4.43$; $P = 0.035$). However, no relationship was discerned between proportion of live hosts with punctures and maximum temperature ($\chi^2_1 = 0.002$; $P = 0.97$).

Frequency of overstinging

Overall, we observed 28 and 35 *A. melinus* females foraging in patches with either second- or third-instar *A. aurantii*, respectively. These females parasitized 37 second-instar and 79 third-instar scales, host fed on 27 second-instar and 16 third-instar hosts, and overstung (rejected after stinging) 20 second-instar and 42 third-instar hosts. For *A. chrysomphali*, we observed 20 and 24 females

foraging in patches with either second- and third-instar *A. aurantii*, respectively. These females parasitized 19 second-instar and ten third-instar scales, host fed on 18 second-instar and one third-instar hosts; and overstung 22 second-instar and 37 third-instar hosts.

Aphytis melinus and *A. chrysomphali* overstung between 0.8 and 1.6 hosts out of a total of 10 hosts per patch (Fig. 4a, b). The number of hosts overstung per patch depended on host instar (second vs third instar: $F_{1,104} = 5.59$; $P = 0.02$), but it was independent of the parasitoid species (*A. melinus* vs *A. chrysomphali*: $F_{1,104} = 3.58$; $P = 0.062$) (Fig. 4a, b). The interaction between host instar and parasitoid species was not significant ($F_{1,103} = 0.0091$; $P = 0.92$).

Moreover, the number of hosts overstung by *A. melinus* and *A. chrysomphali* per patch was similar to those parasitized or host fed when both parasitoids searched patches with second-instar hosts (*A. melinus*: $F_{2,81} = 1.89$; $P = 0.16$; *A. chrysomphali*: $F_{2,57} = 0.31$; $P = 0.74$) (Fig. 4a). Patch use changed when *A. melinus* and *A. chrysomphali* females searched patches with third-instar hosts (Fig. 4b). *Aphytis melinus* females parasitized

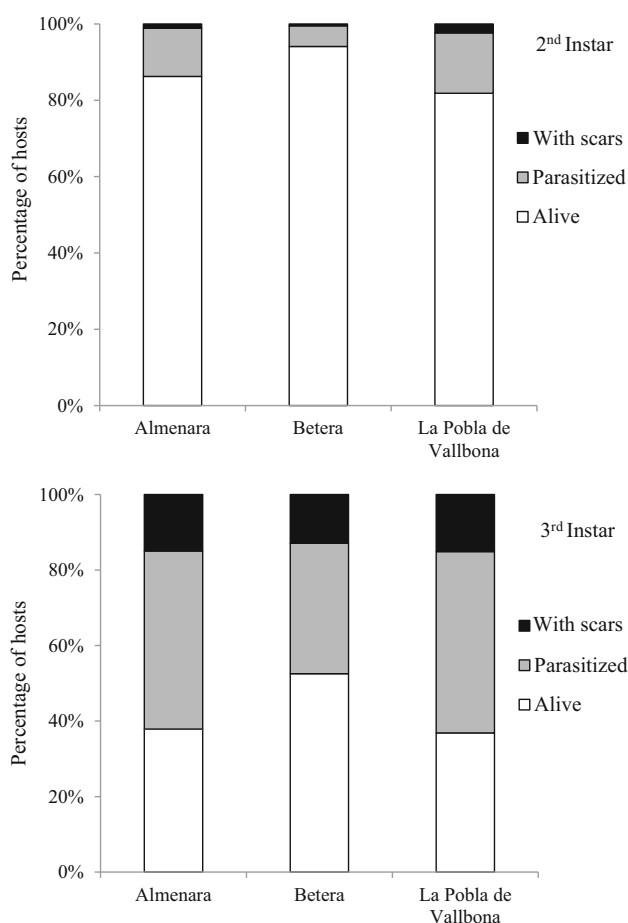


Fig. 2 Percentage of *Aonidiella aurantii* alive, parasitized by *Aphytis*, and likely overstung by *Aphytis* parasitoids in three citrus orchards (Almenara, Bétera and La Pobra de Vallbona) from April to November

significantly more hosts than they overstung or host fed ($F_{2,102} = 23.69$; $P < 0.0001$; uppercase letters in Fig. 4b), whereas *A. chrysomphali* females overstung significantly more hosts than they parasitized or host fed (lowercase letters in Fig. 4b; $F_{2,69} = 28.84$; $P < 0.0001$).

Time spent overstinging and number of stings per host

The mean time spent overstinging per host and patch was independent of host instar (second vs third instar: $F_{1,113} = 0.08$; $P = 0.78$), but it was significantly higher for *A. melinus* than for *A. chrysomphali* ($F_{1,113} = 5.84$; $P = 0.02$) (Table 1). The interaction between host instar and parasitoid species was not significant ($F_{1,112} = 0.002$; $P = 0.96$).

The number of stings per host and patch was independent of host instar (second vs third instar: $F_{1,113} = 0$; $P = 0.99$) and parasitoid species (*A. melinus* vs *A. chrysomphali*: $F_{1,113} = 0.46$; $P = 0.49$) (Table 1). The

interaction between host instar and parasitoid species was not significant ($F_{1,112} = 2.32$; $P = 0.13$).

Effect of overstinging on host fitness

Lethal effect

Respectively, $95 \pm 9\%$ and $91 \pm 6\%$ of the second-instar hosts overstung by *A. melinus* ($n = 20$) and *A. chrysomphali* ($n = 22$) died (Fig. 5), but these figures changed when both parasitoids overstung the third instar. *Aphytis melinus* ($n = 42$) caused $55 \pm 8\%$ mortality in this instar vs $22 \pm 7\%$ caused by *A. chrysomphali* ($n = 37$). Thus, the mortality caused by the overstings depended on the host instar (second vs third instar: $F_{1,70} = 24.92$; $P < 0.001$) and the parasitoid species (*A. melinus* vs *A. chrysomphali*: $F_{1,70} = 5.71$; $P = 0.02$). However, the interaction between host instar and parasitoid species was not significant ($F_{1,69} = 1.48$; $P = 0.23$). As expected, all of the parasitized and host-fed hosts died, whereas all of the unattacked hosts survived.

The probability that the third instar of *A. aurantii* died after being overstung by both parasitoids was positively correlated with the duration of the stings, and it was independent of the sting site, the sequence of visited hosts and number of stings (Table 1, Fig. 6).

Sublethal effects: fecundity of surviving hosts

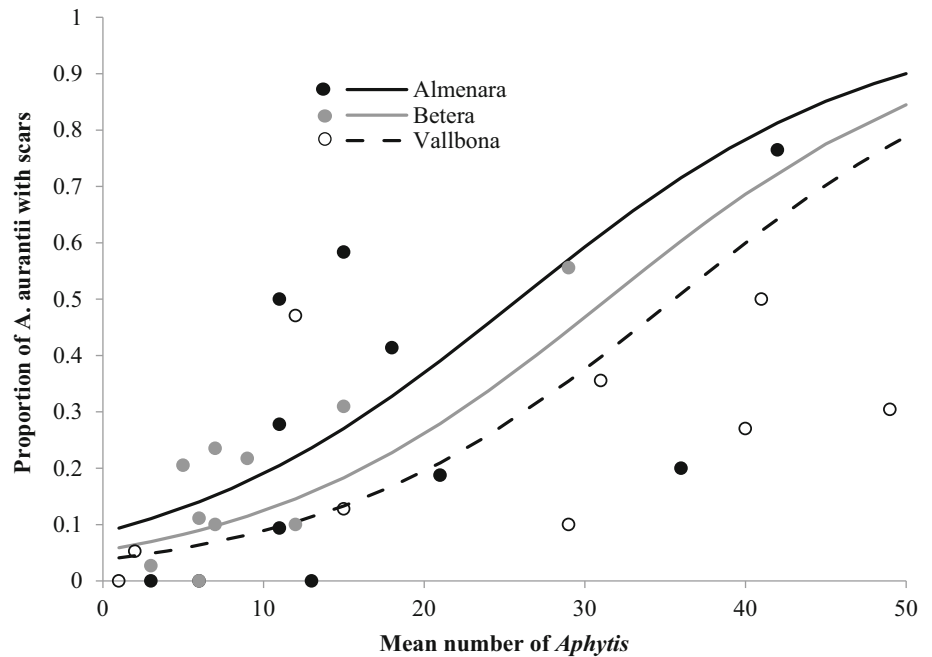
The fecundity (number of crawlers per week) of the surviving hosts that were overstung by *A. melinus* (18.23 ± 3.56) was significantly lower ($\sim 38\%$) than those overstung by *A. chrysomphali* (25.92 ± 1.23) and the unattacked hosts (26.45 ± 0.97) ($F_{2,91} = 5.6$; $P = 0.005$).

Surplus killing

The number of *A. aurantii* killed by *A. melinus* and *A. chrysomphali* without being used for egg laying or host-feeding (surplus killing) depended on the host instar (second vs third instar: $F_{1,118} = 32$; $P < 0.005$) and the parasitoid species (*A. melinus* vs *A. chrysomphali*: $F_{1,118} = 7.34$; $P = 0.008$). The interaction between host instar and parasitoid species was not significant ($F_{1,117} = 1.72$; $P = 0.19$) (Fig. 7).

The number of second-instar *A. aurantii* killed by *A. melinus* without being used for egg laying or host-feeding (surplus killing) was similar to the number of hosts killed for host-feeding, but it was significantly lower than the number of parasitized hosts ($F_{2,78} = 4.68$; $P = 0.012$) (Fig. 7). For *A. chrysomphali*, the number of surplus-killed hosts was similar to the number of hosts killed by host-feeding and parasitism ($F_{2,57} = 0.071$; $P = 0.93$).

Fig. 3 Relationship between the proportion of *Aurantii aurantii* with scars and the number of *Aphytis* observed in three citrus orchards (Almenara, Bétera and La Pobla de Vallbona) from April to November. Each line represents the relation in each orchard when: number of predated scales = 7.19, max gust of wing = 22.5 km/h; accumulated rain = 27.9 mm; interaction between predated host and *Aphytis* = 115.7. Proportion of *A. aurantii* with scars = $1/[1 + (1/\{\exp[(0.07 \times \text{number of } Aphytis) + \text{intercept}]\})]$; $P < 0.0001$; 52.3% deviance explained)



The number of third-instar *A. aurantii* killed by *A. melinus* without being used for egg laying or host-feeding (surplus killing) was similar to the number of hosts killed for host-feeding, but it was significantly lower than the number of parasitized hosts ($F_{2,105} = 26.7$; $P < 0.001$) (Fig. 7). For *A. chrysomphali*, the number of surplus-killed hosts was similar to the number of hosts killed by parasitism but higher than those killed by host-feeding ($F_{2,69} = 4.39$; $P = 0.016$).

Discussion

Overall, our results showed that the rejection of a host after stinging (overstinging) is a common behavior in *Aphytis* parasitoids, and it causes the mortality or mutilation of their common host, *A. aurantii*. The virulence of these stings depended on the host instar being attacked and the parasitoid species. Therefore, this behavior might be an important trait to consider in the selection of parasitoids for biological control programs.

Aphytis females rejected approximately 30% of the *A. aurantii* hosts they encountered after stinging them with their ovipositor, meaning they did not oviposit on the scale or consume its body fluids. This behavior was as common as parasitism or host-feeding when *A. melinus* and *A. chrysomphali* searched in patches with second-instar hosts (low quality) and even more common than host-feeding in patches with third-instar hosts (high quality). In a previous study, Casas et al. (2004) also found that *A. melinus* tended to oversting approximately 12 and 50% of the second and

third instars, respectively, in patches with hosts of different instars. Overstinging seems to also be common in the field, where we recorded many *A. aurantii* scales with punctures that were likely produced by *Aphytis* stings and predators. The number of wounded scales was correlated with parasitoid activity, and most of them were third-instar individuals. This result matches our laboratory observations because the second-instars died after being stung and so could not be recorded in the field samples. Casas et al. (2004) also observed that *Aphytis* tend to sting and reject hosts in the field at even higher rates than in the laboratory when they tracked females for several hours. Therefore, overstinging seems to be a common behavior in the field and not only under experimental laboratory conditions, but its measurement is difficult if the hosts die as occurred with the young *A. aurantii* instars. Although overstinging is commonly observed in parasitoids [see references in Vinson (1976)], its frequency of occurrence and consequences for host physiology have generally been overlooked in the parasitoid literature.

Overstinging affected host survival and fecundity, and its virulence depended on the host instar being attacked. Most immature instars (second-instar hosts) died when overstung by *Aphytis*, whereas ~50% of the adults survived being attacked. Our results supported our initial hypothesis that young hosts are likely more vulnerable to this parasitoid because their immune defences are possibly weaker. Through the insertion of the ovipositor, parasitoids can inject biochemical compounds as well as cause mechanical injury to host tissues that can lead to increased premature mortality of young hosts (Vinson 1976; Strand

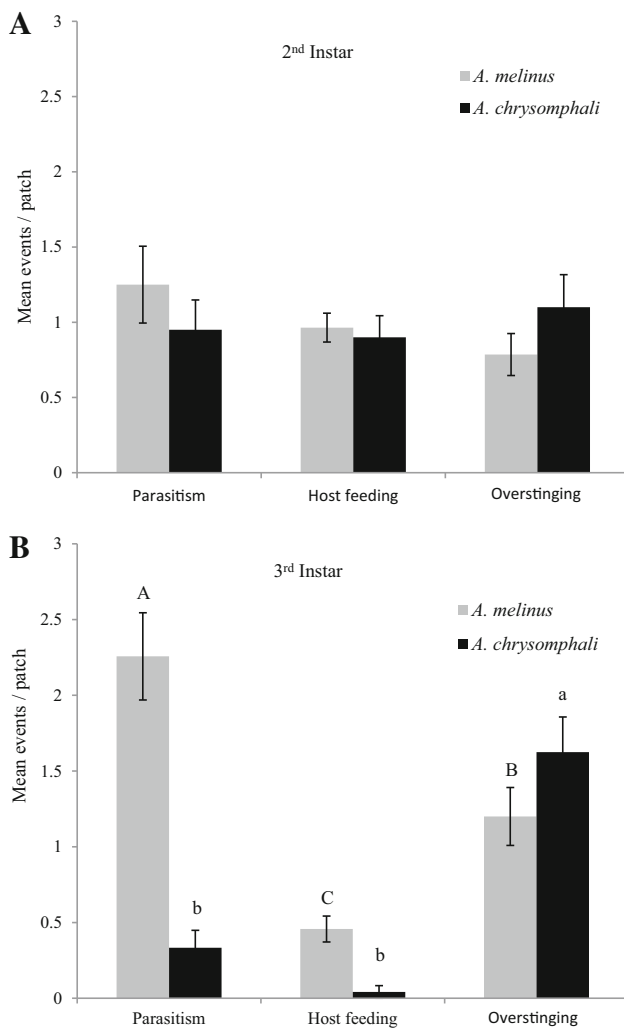


Fig. 4 Behavioral events (mean \pm SE) of the parasitoids *Aphytis melinus* and *A. chrysomphali* in patches with ten second- (a) and third-instar (b) *Aonidiella aurantii*. Different uppercase letters above the columns denote significant differences between the occurrence of the different behaviors for *A. melinus*, and lowercase letters denote differences for *A. chrysomphali*

1986; Van Driesche et al. 1987; Beckage 2008). In two different systems, the parasitoids of mealybugs (Hemiptera: Pseudococcidae) and leaf miners (Lepidoptera: Gracillariidae) also cause higher mortality rates in younger instars when they reject the host after stinging (Neuenschwander et al. 1986; Van Driesche et al. 1987; Barrett and Brunner 1990).

Parasitoid species also affected the virulence of the overstinging when *A. melinus* and *A. chrysomphali* attacked the third instar. The former parasitoid killed more adult hosts than *A. chrysomphali* and, moreover, reduced the fecundity of the surviving females, which demonstrates the superiority of *A. melinus* as a biological control agent of *A. aurantii* compared with *A. chrysomphali* (DeBach and Sisojevic 1960; Rosen and DeBach 1979; Pekas et al.

Table 1 Influence of several parasitoid behaviors on *Aonidiella aurantii* mortality when the scale was rejected by either *Aphytis melinus* or *A. chrysomphali* after stinging (overstinging)

| Variable | Parameter estimate | F | P |
|--|--------------------|--------|--------------|
| Host overstung by <i>A. melinus</i> | | | |
| Intercept | −0.92 | −1.96 | 0.058 |
| Sting duration | 0.0081 | 2.21 | 0.034 |
| Sting site | 1.99 | 1.9 | 0.07 |
| Order | −0.62 | −1.064 | 0.3 |
| Number of stings | −0.66 | −0.71 | 0.49 |
| Host overstung by <i>A. chrysomphali</i> | | | |
| Intercept | −2.89 | −3.47 | 0.0015 |
| Sting duration | 0.016 | 2.2 | 0.035 |
| Sting site | 0.24 | 0.14 | 0.89 |
| Order | 0.33 | 0.58 | 0.56 |
| Number of stings | 0.43 | 1.06 | 0.3 |

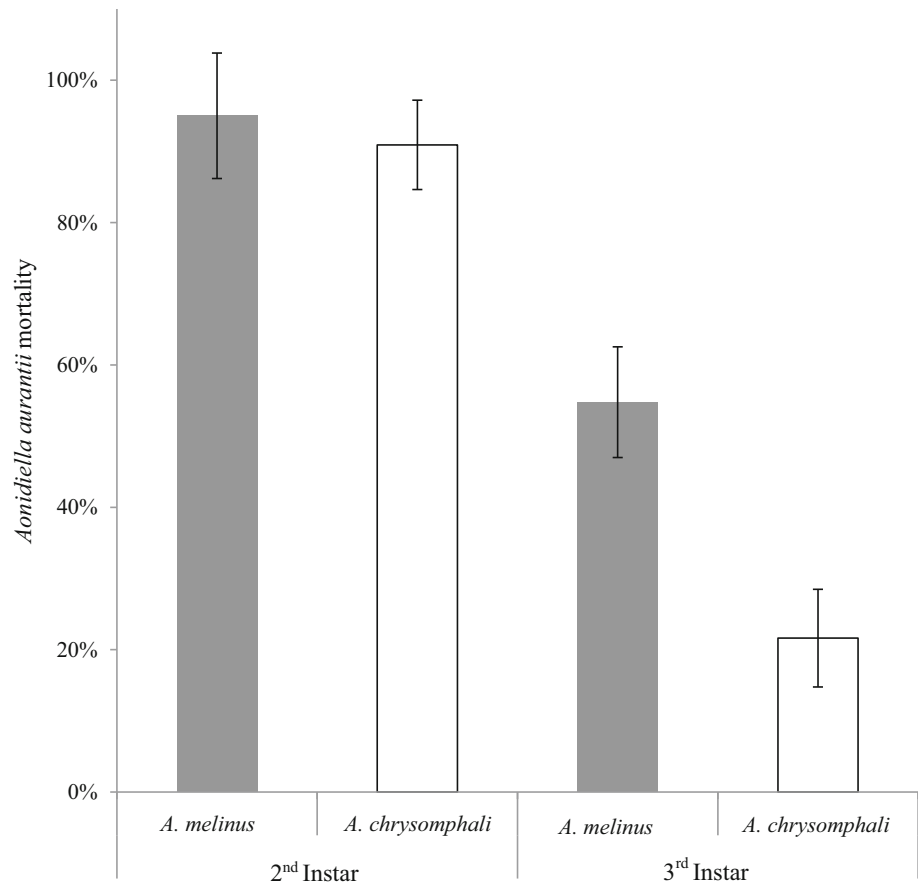
Host mortality was analyzed with a GLM based on quasi-binomial distribution with sting duration, sting site, host encounter sequence (order) and number of stings as factors

Significant *P*-values are presented in bold

2010, 2016; Boyero et al. 2014; Cebolla et al. 2017). The mortality caused by *A. melinus* was fourfold greater than that caused by *A. chrysomphali* when considering the three behaviors measured in this assay (parasitism, host-feeding and overstinging). Van Driesche et al. (1987) also compared the mortality caused by two parasitoids of the mealybug *Phenacoccus herreni* Cox and Williams (Hemiptera: Pseudococcidae) and observed that the mortality caused by *Epidinocarsis diversicornis* (Howard) was almost twice that of *Acerophagus coccois* Smith (Hymenoptera: Encyrtidae) when both reject their common host after stinging. Both results confirm the importance of measuring the frequency of occurrence and the consequences of overstinging on host physiology.

The mortality caused by *Aphytis* parasitoids depended on the duration of the stings when third-instar hosts were encountered, and it is likely that the mechanical damage as well as the potential amount of venom proteins (Asgari and Rivers 2011) and polynadivirus (Beckage 2008) injected by the parasitoids increased with the time spent stinging. In fact, more than the 80% of the hosts died when the ovipositor was inside for more than 240 s. Keinan et al. (2012) studied the fitness implications of multiple stinging events and found that all of the hosts died after 4–5 stings, but this study included mortality induced by parasitoid oviposition. In our study, we did not find a correlation between mortality and the number of stings. Regardless, *A. melinus* spent more time than *A. chrysomphali* overstinging its host, which might partially explain the differences in the virulence of both parasitoids.

Fig. 5 *Aonidiella aurantii* mortality caused by the parasitoids *Aphytis melinus* and *A. chrysomphali* when they probed and rejected different scale instars



Overstinging by *Aphytis melinus* also reduced the fecundity of the surviving host, whereas this effect was not observed with *A. chrysomphali*. Previous studies have reported detrimental fitness costs, such as the suppression of gonad development in the host after being stung (Reed-larsen and Brown 1990; Brown and Kainoh 1992; Münster-Swendsen 1994; Tagashira and Tanaka 1998; Digilio et al. 2000; Barratt and Johnstone 2001). These studies are based on hymenopteran parasitoids attacking lepidopteran hosts in the egg or larval stages, but the authors could not determine whether the female parasitoid laid an egg or just stung its host. Therefore, the damage could be caused by the sting or the immature parasitoid. Generally, these attacks end with the castration of the young instars (Baudoin 1975). Adult host castration is uncommon and rarely complete, and fecundity is generally only slightly reduced (Spencer 1926; Beard 1940; Schlinger and Hall 1960) as occurred when *A. melinus* attacked adult *A. aurantii*. This is because gonadal tissues are generally well formed by the time the host reaches the adult instar (Reed-larsen and Brown 1990).

From a biological control point of view, our result supports the idea that overstinging should be considered when evaluating the efficacy of parasitoids as biological

control agents, as has also been recently suggested for other cases of parasitoid-induced mortality (Abram et al. 2016). In this sense, it is important to highlight the differences between parasitoids and predators. In the literature considering natural predators, surplus killing or overkilling is generally taken into consideration when describing predator behavior and the potential for use as a biological control agent (Pekár 2005; Monzó et al. 2009; Pérez-Hedo and Urbaneja 2015). The importance of overstinging and its consequences for the host (mortality and mutilation) is far from being a phenomenon isolated to this system as this behavior has been widely described in numerous parasitoids (Vinson 1976). One of the best-known cases of overstinging and its consequences on the host was described by Münster-Swendsen (1994, 2002). He demonstrated that the parasitoid *Apanteles tedellae* Nix. (Hymenoptera: Braconidae) caused the sterilization of its host *Epinotia tedella* (Cl.) (Lepidoptera: Tortricidae) when parasitoids are disturbed before depositing an egg. This effect was later included in several models to analyze the dynamics of the host and detect the causes of population cycles (Münster-Swendsen 2002; Münster-Swendsen and Berryman 2005). These authors demonstrated that is the total combined impact of parasitism on mortality and fecundity that

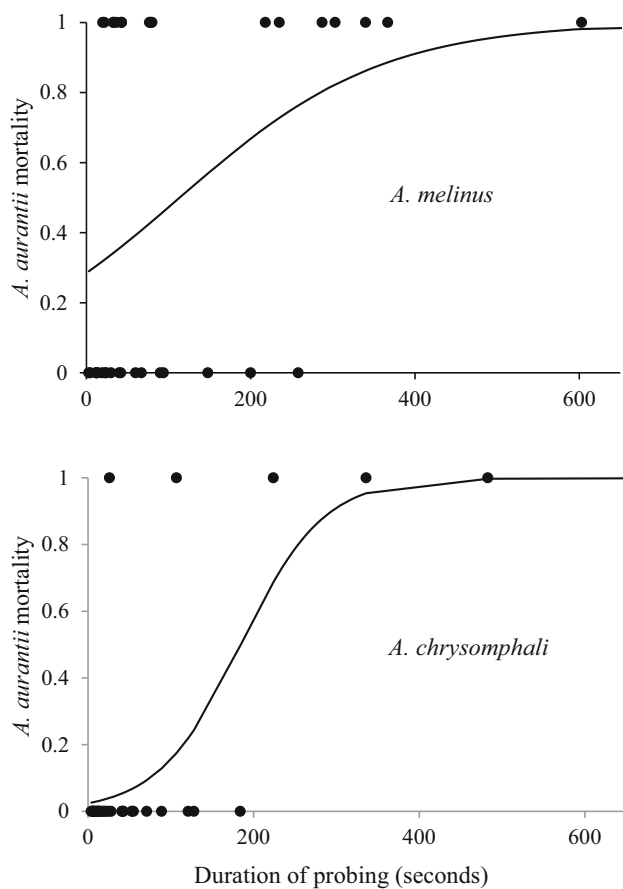


Fig. 6 Effect of oversteering duration (in seconds) by *Aphytis melinus* and *A. chrysomphali* on the mortality of *Aonidiella aurantii* (statistics in Table 1). The curve represents the fitted values from the logistic regression model of the proportion of dying hosts. Points at the *bottom* and *top* of the figure represent the actual data from alive and dead hosts

apparently provides the strong negative feedback needed to drive population cycles in all species of this community. The population dynamics of *A. aurantii*-*Aphytis* has been also analyzed (Murdoch et al. 1995, 1996, 2005), but the frequency and consequences of oversteering have not been included. Further research should consider them and, likely, also parasitoid state because the frequency of oversteering might depend on parasitoid state (i.e., number of mature eggs, age or nutritional state), which also affects stability in insect host–parasitoid population models (Shea et al. 1996; Murdoch et al. 1997”).

Annex 1 Correlation matrix between all variables to select them for the multiple correlation analysis

| | Parasitoids | Predators | Mean T ^a | Max. T ^a | Mean wind | Max wind | Accumulated rain | Max rain |
|-------------|----------------|-----------|---------------------|---------------------|-----------|----------|------------------|----------|
| Parasitoids | R ² | −0.04 | 0.11 | −0.06 | 0.02 | 0.31 | 0.26 | 0.20 |
| | P | 0.82 | 0.54 | 0.77 | 0.90 | 0.09 | 0.16 | 0.27 |

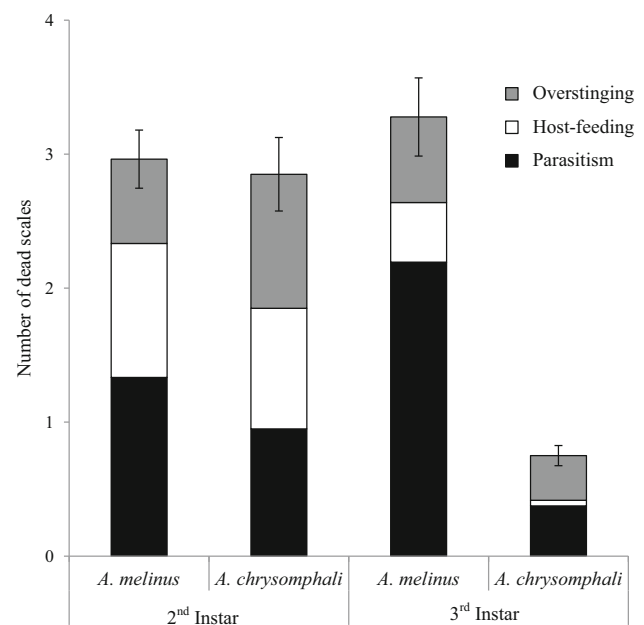


Fig. 7 Mean number of *Aonidiella aurantii* (\pm SE) dead by parasitism, host-feeding and oversteering when the parasitoids *Aphytis melinus* and *A. chrysomphali* found second- and third-instar scales

Author contribution statement

AU and AT conceived and RC, AU, AT designed research. RC and PV conducted experiments. RC and AT analyzed data. All authors wrote, read and approved the manuscript.

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Compliance with ethical standards

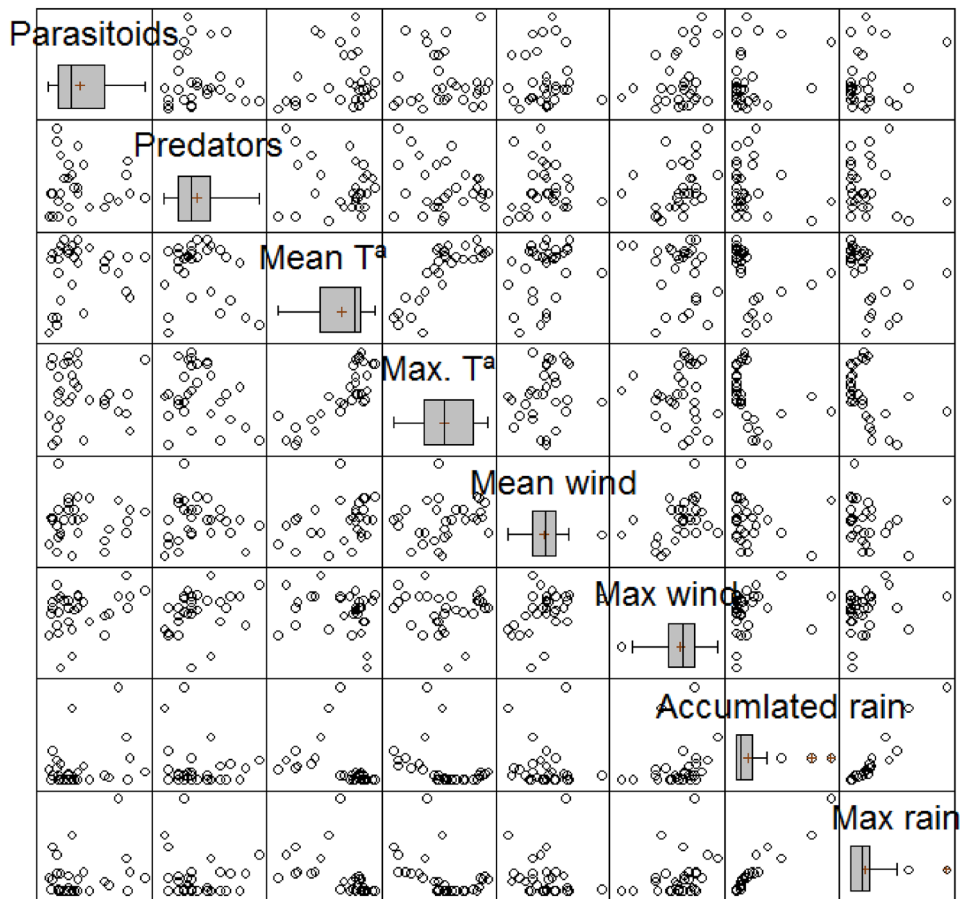
Conflict of interest The authors declare that they have no conflict of interest.

Annex

Annex 1 continued

| | | Parasitoids | Predators | Mean T ^a | Max. T ^a | Mean wind | Max wind | Accumulated rain | Max rain |
|---------------------|----------------|-------------|-----------|---------------------|---------------------|-------------|----------|------------------|----------|
| Predators | R ² | -0.04 | | -0.22 | -0.22 | -0.03 | 0.30 | -0.16 | -0.12 |
| | P | 0.82 | | 0.23 | 0.23 | 0.85 | 0.10 | 0.38 | 0.54 |
| Mean T ^a | R ² | 0.11 | -0.22 | 0.82 | 0.32 | -0.20 | -0.38 | -0.49 | |
| | P | 0.54 | 0.23 | 0.00 | 0.08 | 0.28 | 0.03 | 0.01 | |
| Max. T ^a | R ² | -0.06 | -0.22 | 0.82 | 0.23 | -0.06 | -0.37 | -0.44 | |
| | P | 0.77 | 0.23 | 0.00 | 0.22 | 0.76 | 0.04 | 0.01 | |
| Mean wind | R ² | 0.02 | -0.03 | 0.32 | 0.23 | 0.40 | -0.14 | -0.13 | |
| | P | 0.90 | 0.85 | 0.08 | 0.22 | 0.02 | 0.47 | 0.49 | |
| Max wind | R ² | 0.31 | 0.30 | -0.20 | -0.06 | 0.40 | 0.22 | 0.26 | |
| | P | 0.09 | 0.10 | 0.28 | 0.76 | 0.02 | 0.23 | 0.16 | |
| Accumulated rain | R ² | 0.26 | -0.16 | -0.38 | -0.37 | -0.14 | 0.22 | 0.96 | |
| | P | 0.16 | 0.38 | 0.03 | 0.04 | 0.47 | 0.23 | 0.00 | |
| Max rain | R ² | 0.20 | -0.12 | -0.49 | -0.44 | -0.13 | 0.26 | 0.96 | |
| | P | 0.27 | 0.54 | 0.01 | 0.01 | 0.49 | 0.16 | 0.00 | |

Significant P-values are presented in bold



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