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Response of the fruit fly parasitoid *Diachasmimorpha longicaudata* to host and host-habitat volatile cues

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Abstract

Chemical information is crucial to insect parasitoids for successful host location. Here, we evaluated the innate response of *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae), a fruit fly larval parasitoid, to cues from host and host habitat (i.e., fruit infested with host larvae). We first assessed the preference of female parasitoids between oranges infested with *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) and non-infested fruit. Females were highly attracted towards infested oranges on the basis of volatile chemical cues. After this initial experiment, we aimed at revealing the potential sources of volatile cues present in an infested fruit. To this end, we considered five potential sources: (1) punctured fruit; (2) fly feeding, frass, or host-marking pheromone deposited on the orange surface; (3) larval activity inside the fruit; (4) the larvae themselves; and (5) fungi associated with infestation of oranges. Habitat cues associated with host activity and those produced by rotten oranges or oranges colonized by fungi were highly attractive for female wasps, whereas odours associated with the activity of the adults on the surface of the fruit, and those released by the fruit after being damaged (as happens during fruit fly egg-laying) were not used as cues by female parasitoids. Once the female had landed on the fruit, direct cues associated with larval activity became important although some indirect signals (e.g., products derived from larval activity inside the fruit) also increased host searching activity. Our findings indicate that naïve *D. longicaudata* uses chemical cues during host habitat searching and that these cues are produced both by the habitat and by the host larvae.

Introduction

Among the various types of stimuli that provide information to an insect, chemical stimuli, also called semiochemicals (Nordlund & Lewis, 1976) or infochemicals (Dicke & Sabelis, 1988), constitute key cues for a parasitoid when it is searching for its hosts. Chemical compounds that convey information between individuals of different species are termed allelochemicals (to distinguish them from pheromones, i.e., compounds involved in communication between conspecific individuals). Those allelochemicals that benefit the receiver and have a negative impact on the sender are called foraging kairomones and many studies have shown the importance of such infochemicals (Vet & Dicke, 1992). The foraging process is divided into four phases: host habitat location, host location, host acceptance, and host suitability (Vinson, 1976, 1985; van Alphen & Vet, 1986). Kairomones are especially important during the first phase of this process when the parasitoid is searching for host habitats over long distances, whereas at shorter distances other stimuli like visual or acoustic cues can complement chemical cues (or even become more important) (van Alphen & Jervis, 1996).

Volatile infochemicals used by insect parasitoids arise from a variety of sources. There are many examples of parasitoids that respond to odours directly released by their hosts (Jacob & Evans, 2000; Schaffner & Müller, 2001; Conti et al., 2003; Salerno et al., 2006). These include parasitoids of almost every host stage, although rarely pupal parasitoids whose hosts remain motionless and do not feed, keeping odour emission to a minimum (Fischer et al., 2001). However, the release of such cues

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drastically reduces the fitness of the host and therefore natural selection would normally minimize direct cues from the host. In response, some parasitoids follow channels of chemical communication between hosts (e.g., using oviposition deterrent pheromones as kairomones) as indirect cues to locate them (Rice, 1968, 1969; Colazza et al., 1999; Hoffmeister & Gienapp, 1999; Conti et al., 2004; Salerno et al., 2009), a phenomenon termed 'infochemical detour' by Vet & Dicke (1992). Volatiles released by the host's frass are another type of indirect cue (Oatman et al., 1969; Hendry et al., 1973; Auger et al., 1989; Cortesero et al., 1993; Agelopoulos & Keller, 1994; Meiners et al., 2000; Cusumano et al., 2010).

In many tritrophic systems that include a parasitoid, a phytophagous host, and the habitat of the host (a plant), volatile organic compounds coming from the plant attract parasitoids (Godfray, 1994; Jönsson et al., 2005; Wyckhuys & Heimpel, 2007; Belda & Riudavets, 2010). These compounds can be released in the absence of the host, but sometimes plants respond to the damage caused by the phytophagous insect and release volatile compounds that attract the parasitoid. This response has positive effects on both the parasitoid and the plant; hence, the evolution of a communication system between these two actors is favoured. Laboratory and field studies have shown the importance of volatile compounds released by the plant as a response to host feeding (Dicke & Baldwin, 2010 and references therein). In some instances, this system is far more specific and the plant releases parasitoid attracting volatiles only if the damage is caused by the parasitoid host (Turlings et al., 1990, 1991a,b; Mattiacci et al., 1995; Blassiolo Moraes et al., 2005; Fatouros et al., 2005; Tentelier et al., 2005).

Diachasmimorpha longicaudata (Ashmead) (Hymenoptera: Braconidae) is a larval endoparasitoid of several fruit fly species. Females effectively parasitize larvae from the genera *Anastrepha*, *Bactrocera*, *Ceratitis*, *Dacus*, and *Toxotrypana* (all Diptera: Tephritidae), which contain highly polyphagous frugivorous species. *Diachasmimorpha longicaudata* females attack late-second or early-third instars, while larvae are still feeding inside the fruit. Previous studies have indicated that *D. longicaudata* females use volatile (Greany et al., 1977; Eben et al., 2000; Carrasco et al., 2005; Silva et al., 2007) as well as visual (Leyva et al., 1991; Vargas et al., 1991; Messing & Jang, 1992; Segura et al., 2007) stimuli during host habitat (infested fruit) location. Although most studies agree that females discriminate between infested and non-infested fruits, only a few studies have addressed the source of chemical stimuli and generated inconsistent interpretations. For instance, Greany et al. (1977) concluded that females' attraction to infested peaches was not related to the presence of larvae but resulted from fungal fermentation, whereas Carrasco et al. (2005) found that the presence of larvae was essential for females' orientation. More recently, Silva et al. (2007) proposed that *D. longicaudata* females use cues from the fruit even in the absence of host larvae, but that the presence of the larvae enhances the attraction towards a patch. Duan & Messing (2000) proposed that *D. longicaudata* detect chemical compounds derived from larval activity. This has been recently confirmed by Stuhl et al. (2011) who detected a specific compound released by larvae of several Tephritidae species, which enhances host search at short distances (once the female parasitoid is on the fruit). Females would integrate this information with vibrations produced by larvae to fine-tune the location of their hosts (Lawrence, 1981). Nonetheless, the response of the females towards both chemical and vibratory cues has been studied only using artificial substrates, which could distort the relative importance of these stimuli (and also hide other stimuli) on the foraging behaviour of females on a natural substrate (i.e., the surface of a fruit).

In the present work, we examined the contribution of host- and fruit-related cues during the first two phases of host searching by the parasitoid *D. longicaudata*: host habitat search and host searching once females arrived to an infested fruit. We first evaluated the ability of *D. longicaudata* females to discriminate between infested and non-infested oranges based only on volatile cues. After this initial experiment, we studied odour sources derived from the host larvae or associated with host activity within the fruit. Finally, we analyzed the importance of close range cues on the oviposition probing behaviour of females foraging on the fruit surface.

Materials and methods

Insects

Parasitoids and fruit fly larvae were obtained from the rearing facility of INTA Castelar, Buenos Aires, Argentina (Viscarret et al., 2006). This colony was initiated with individuals coming from CIRPON, San Miguel de Tucumán, Argentina (Ovruski et al., 2003). *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) was used as host both for rearing and for the experiments. *Ceratitis capitata* larvae were reared on artificial rearing medium (a mixture of carrot, sugar, brewer's yeast, corn flour, and food preservatives, according to Terán, 1977) until they reached the third instar (ca. 7 days) when they were exposed to parasitoid females in small Petri dishes (following Viscarret et al., 2006). Parasitized larvae were transferred to vermiculite for pupariation and placed under controlled environmental conditions (25 ± 1 °C, 65 ± 5% r.h.) until adult emergence, which occurred about 20 days after larval exposure.

From emergence and until the test, female parasitoids were kept in 3-l glass containers and provided with honey and water separately. Containers were placed inside a rearing chamber under controlled conditions (22 ± 1 °C, 65 ± 5% r.h.). Each container hosted 10 females and 10 males. Adult parasitoids had no contact with host larvae, fruit, or artificial media before the tests.

Experimental conditions

Experiments were performed in a screen cage (1 m long, 40 cm high, 40 cm wide) under controlled conditions (25 ± 1 °C, 65 ± 5% r.h.). Screens were made of tulle. Light was provided with daylight fluorescent tubes (ca. 2 500 lux). The cage was symmetrically located in relation to the entrance door, the air-conditioner, and the illumination, to avoid possible biases due to either chemical or visual cues. The room where the experiments were carried out had no windows.

Inside the cage, one 5- to 7-day-old naïve *D. longicaudata* female was offered two options at opposite sides of the cage (90 cm between options). The female was released at the centre of the cage and responded to the options. Two white cardboard sheets (30 × 20 cm) were located as close to the option as possible (about 10 cm from the cage wall) therefore blocking the view of the options from the release point. This forced the female to orientate towards the option based only on chemical cues, because she was able to see the option only after she had moved about 85–90% of the distance from the release point to the option. Only one female was tested at a time. After five females had been tested, the options were discarded and replaced by a new pair, which was placed in the opposite arrangement to avoid any bias in preference due to the location of the option.

The options presented to the females were oranges (*Citrus sinensis* L. Osbeck, Navel variety, ca. 9 cm in diameter) that differed in the treatment received before the choice test. Fruits were obtained from a local market and showed no signs of insect infestation or fungal contamination before they were treated. Oranges were thoroughly washed (only with water) before exposing them to the insects.

To assess females' choice, the cage was divided by two imaginary lines that defined three sectors: one in the centre (20% of the total area) considered as a non-preference area, and two distal sectors towards the sides (40% of the total area each). Each female was observed for 10 min or until the female chose (landed on or started to walk on top of) one option (whichever happened first). During the observation period, we recorded: (1) the first distal sector towards which the female orientated (females had to spend more than 15 s in the sector); (2) the time during which the female remained in each distal sector; (3) the first fruit visited by the female; and (4) the time elapsed between the release and the moment at which the female chose an option (referred to as latency). Eighty females were individually studied in each treatment.

After a female landed on a fruit, any oviposition behaviour was recorded for 10 min or until the parasitoid left the fruit (whichever happened first). We recorded: (1) the time during which the female stayed on the fruit (referred to as residence time); (2) how many oviposition attempts (OAs) were performed; (3) the time elapsed between landing and the first OA (referred to as OA latency); and (4) the duration of each OA. We then calculated: (1) the tendency to perform OAs (= number of females that exhibited at least one OA in a given option/number of females that visited that option); (2) the rate of OAs (= number of OA min^{-1}); (3) the total time spent in OA; (4) mean duration of an OA; and (5) the proportion of time spent in OAs (= time spent in OAs/residence time).

Experiments

1. Orientation towards infested fruit. Females were offered an orange infested with *C. capitata* larvae and a non-infested orange. Infested oranges were obtained by placing one fruit inside a cage containing 60 female flies for 3 h. Fruit was then removed and kept under controlled conditions (25 ± 1 °C, 50 ± 10% r.h.) for 13–14 days until larvae reached the third instar. This treatment induced an infestation level similar to that found in nature (ca. 50 pupae kg^{-1} of orange; Segura et al., 2006). Non-infested oranges from the same lot were kept under the same conditions, but were not exposed to the flies.

2. Odour sources as potential cues for host location. We carried out a series of eight experiments in which several potential volatile cues were analyzed separately (Table 1).

Experiment 2.1: Punctures in oranges: To evaluate whether the damage caused on the fruit peel by *C. capitata* females during oviposition affected the volatile profile of the fruit and acted as a cue to parasitoids, female wasps were offered an orange displaying punctures on its surface (damaged fruit) and a healthy fruit. Punctures were made with a '00' entomological pin, which was pierced through the skin only 1–2 mm. Based on a preliminary test in which we estimated the number of punctures on a fruit exposed to 60 female flies for 3 h (as in experiment 1), each fruit received 15 punctures. Damaged fruit was kept under controlled conditions for 13–14 days (required time to obtain third instars in infested oranges) before testing. Oranges from the same lot kept in the same conditions (but not punctured) were used as the healthy fruit.

Table 1 Options offered to female *Diachasmimorpha longicaudata* parasitoids in a series of experiments carried out to evaluate potential odour sources related to the orientation of females towards oranges infested with *Ceratitis capitata* host larvae. The tested hypotheses are also presented

Exp.	Options	Differences between odour sources	Hypothesis
2.1	Superficially damaged fruit vs. whole fruit	Products released by the fruit after puncturing by fruit fly females	Volatile compounds released by the fruit after fruit fly oviposition are used as cues by female parasitoids
2.2	Fruit whose surface was exposed to adult flies vs. unexposed fruit	Products laid on the fruit surface by adult flies	Volatile compounds derived from the activity of the adult flies on the fruit are used as cues by female parasitoids
2.3	Artificially infested fruit vs. naturally infested fruit	Products deposited inside the fruit by the fruit fly females during oviposition	Volatile compounds released by products that are deposited inside the fruit during fruit fly egg-laying are used as cues by female parasitoids
2.4	Healthy fruit plus a dish containing larvae vs. whole fruit	Products released by host larvae	The host larva releases volatile compounds that are used as cues by parasitoid females
2.5	Infested fruit inside which there are no remaining larvae vs. non-infested fruit	Products associated with host larvae activity inside the fruit	The fruit in which host larvae develop releases volatile compounds associated with larvae activity that are used as cues by female parasitoids
2.6	Fruit contaminated with fungi vs. non-contaminated fruit	Products associated with fungal contamination	The fungus releases volatile compounds that are used as cues by female parasitoids
2.7	Ripe fruit vs. rotten fruit	Products that are released by the fruit when it gets rotten	The rotten fruit releases volatile compounds that are used as cues by female parasitoids
2.8	Used larval diet vs. fresh larval diet	Products associated with host larvae activity inside the substrate	Any substrate in which host larvae develop releases volatile compounds associated with larvae activity that are used as cues by female parasitoids

Experiment 2.2: Odour sources associated to fly activity on the fruit surface: We analyzed the effect of chemical compounds deposited on the fruit surface by adult flies on the orientation of female parasitoids. Such compounds may include host-marking pheromones (Prokopy et al., 1978), faeces, and regurgitated fluids. During the choice test, we offered the female parasitoids one infested orange that contained all these signals and one infested orange with a clean surface. Both fruits were exposed to flies as in experiment 1, but clean fruit had been previously wrapped in Parafilm M (Pechiney Plastic Packaging, Chicago, IL, USA). This thin layer enabled egg-laying by *C. capitata* females but allowed the removal of whatever chemical mark they could have laid on the fruit surface (faeces, host marking pheromones, etc.). After exposure, we removed the Parafilm layer and fruit were kept for 13–14 days until the test. After the test, the number of larvae inside each fruit was assessed and whenever we found substantial differences in the infestation level (>five pupae per fruit) between the two fruits, we removed those data from the analysis (ca. 20% of the cases).

Experiment 2.3: Compounds deposited by flies during egg-laying: During egg-laying, females deposit accessory gland products together with their eggs. This has been under intense research in *C. capitata*, and several compounds have been identified (Marchini et al., 1991, 1993, 1997). To test whether these products act as cues to parasitoids, we compared female parasitoid preference between an infested fruit and a fruit that was artificially punctured and inoculated with *C. capitata* eggs. Fly eggs were collected in artificial devices and rinsed 5× with distilled water. A preliminary study indicated that the mean clutch number was six eggs. Based on these results, 15 punctures were made and each was filled with six eggs. This way, both oranges were punctured and contained fly eggs, but one of them lacked the products that females deposit during egg laying. Artificially and naturally infested oranges were placed under controlled conditions for 24–48 h and then used in the choice experiments. Ideally, we should have tested the fruit after 13–14 days. However, the artificially infested fruit presented a high degree of fungal contamination about 1 week after the experiment, which forced us to run the experiments during the initial phase of the infestation process.

Experiment 2.4: Compounds released by Ceratitis capitata *larvae:* This experiment was designed to evaluate whether *C. capitata* larvae directly release volatile compounds that are used by female parasitoids as cues during host searching. We placed one healthy orange on one side of the cage and one healthy orange + a Petri dish containing 50 third instars that had been rinsed 5× with distilled water on the opposite side. Larvae were obtained from the rearing kept at Instituto de Genética 'Ewald A. Favret', Buenos Aires, Argentina. Oranges were kept under controlled conditions (25 ± 1 °C and 50 ± 10% r.h.) for 13 days before the test.

Experiment 2.5: Compounds derived from larval activity: In order to solely evaluate the importance of larval activity inside the fruit on parasitoid orientation and exclude a potential effect of larval presence, we analyzed parasitoid preference between an artificially damaged orange and an orange that had hosted larvae which had exited the fruit by the time the test was carried out. Infested fruit were obtained as described in experiment 1 and kept under controlled conditions until all larvae exited the fruit (48 h after the first larvae exited the fruit). At the time we performed the choice test, the oranges showed good general conditions (the fruits were ripe and no fungi were evident on the surface). After the experiment, the fruits were dissected to verify that no larvae had remained inside (in which case we removed those observations from the data set). A second lot of oranges was punctured with a '00' entomological pin on the same day on which the first lot was exposed to the flies.

Experiment 2.6: Fungi associated with infested fruit: To evaluate whether fungi associated with fruit could release volatile compounds that act as cues to the female parasitoid, we tested parasitoid preference towards either an orange showing signs of fungal contamination or a healthy one. The green mould *Penicillium digitatum* Saccardo (Eurotiales) grew spontaneously in some damaged fruits. Thus, we punctured oranges with a '00' entomological pin and kept these fruit at room temperature (ca. 25 °C) until the fruit started to exhibit contamination signs (6–10 days). Rotten fruit was not used.

Experiment 2.7: Compounds released by rotten fruit: In experiment 1, infested oranges showed an advanced state of rotting. We considered that fruit was rotten when it started to lose juice, exhibited brownish spots, and it had lost firmness to the point that we had to manage the fruit very gently as to avoid damaging the peel. Here, we aimed at evaluating whether rotten oranges attract female parasitoids, irrespectively of its infestation status. Hence, in this experiment female parasitoids were offered an orange in an advanced decomposing state and a ripe orange. Both fruit belonged to the same batch and showed no signs of fungal contamination (no green mould on the fruit surface).

Experiment 2.8: Compounds derived from larval activity on an artificial substrate: To evaluate whether results of experiment 2.5 were the consequence of a specific interaction between the orange and larval activity on it, this experiment was repeated but using *C. capitata* artificial larval medium instead of oranges. A Petri dish containing artificial medium already used to rear *C. capitata* larvae was placed on one side of the cage, whereas another dish with fresh medium was placed on the opposite side. All the larvae were removed from the infested medium before the test. Both Petri dishes contained the same amount of medium (60 g), which under the rearing methods followed at IGEAF contained ca. 800 larvae. Thus, female parasitoids faced two options inside the cage that differed in compounds derived from larval activity.

Data analysis

The time each female spent in each sector of the cage was compared by a two-tailed paired t-test or a Wilcoxon test if the assumption of normality was not met (Zar, 1996). Preference for one of the two options was evaluated by comparing both the numbers of females that first orientated towards either option and the numbers of females that actually visited them. These variables were analyzed by means of a Yates-corrected G-test of goodness of fit. Latency and residence were compared between fruits by means of a student t-test or a Mann–Whitney test if the heteroscedasticity was mild, or a student t-test preceded by data transformation if the heteroscedasticity was severe (Zar, 1996).

The tendency to oviposit (i.e., OA) on one of the two options was compared by means of a Yates corrected G-test of homogeneity. Latency to the first OA, number of OAs, rate of OAs, mean duration of OAs, and the proportion of time spent in OAs were analyzed by means of a Student t-test or a Mann–Whitney test if the heteroscedasticity was mild, or preceded by data transformation if the heteroscedasticity was severe (Zar, 1996). Tests were performed using STATISTICA for Windows (StatSoft, 2000).

Results

Host searching behaviour

In experiment 1, female parasitoids orientated first towards the sector in which an infested orange was placed significantly more often than expected by chance alone

(G-test of goodness of fit with Yates' correction: Gy = 19.850, P<0.001; Figure 1). In experiments 2.1–4, females showed no preference between the two options (P>0.05; Figure 1). In experiment 2.5, females oriented more frequently towards the sector that contained an infested orange from which all larvae had exited than towards the other sector (Gy = 15.841, P<0.001; Figure 1). In experiment 2.6, females orientated more frequently towards the sector that contained an orange contaminated with the fungus than towards the sector with the non-contaminated orange (Gy = 7.949, P<0.005; Figure 1). In experiment 2.7, females orientated more frequently towards the sector that contained a rotten orange than towards the sector containing a ripe orange (Gy = 14.030, P<0.001; Figure 1). Finally, in experiment 2.8, the females orientated more frequently towards the sector that contained used larval medium than towards the sector containing fresh larval medium (Gy = 38.276, P<0.001; Figure 1).

The pattern of preference as evaluated by female visitation was similar to that found for orientation (Figure 2). We found significant differences in experiment 1 (Gy = 31.790, P<0.001; Figure 2), and in experiments 2.5 (Gy = 15.673, P<0.001; Figure 2), 2.6 (Gy = 7.943, P<0.005; Figure 2), 2.7 (Gy = 15.993, P<0.001; Figure 2), and 2.8 (Gy = 33.255, P<0.001; Figure 2). Again, in experiments 2.1–4, there were no differences in the frequency of visits between options (P>0.05; Figure 2).

The time spent in each sector, latency to visit, and residence times are shown in Table 2. Females spent significantly more time in the distal sector towards which they first orientated preferentially (i.e., the sector containing an infested orange in experiment 1, the sector that contained an orange from which larvae had exited in experiment 2.5, and so on). No differences between sectors were found when times were compared in experiments 2.1–4. Latency and residence times did not differ between options, except in experiments 2.8 and 1, respectively (Table 2). In experiment 2.8, the lack of homoscedasticity and the impossibility to find a proper data transformation impeded the statistical analysis of residence times.

Oviposition behaviour

In experiment 1, a significantly higher proportion of females exhibited at least one OA when they were searching on the surface of infested orange (Figure 3). This proportion was also higher in oranges that were infested but contained no larvae at the time of the experiment (experiment 2.5) and in the artificial rearing medium used by fruit fly larvae that had no remaining larvae in it (experiment 2.8) (Figure 3). In the other experiments, the frequency of OAs was similar between options (Figure 3).

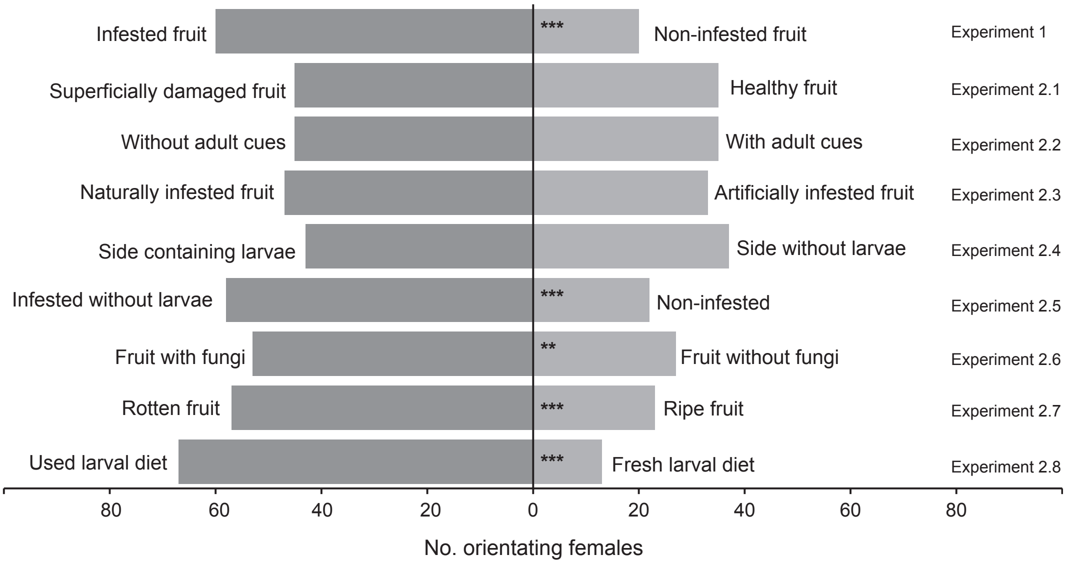

Figure 1 Number of female *Diachasmimorpha longicaudata* that orientated towards either distal sector as first choice. A fruit infested with *Ceratitis capitata* larvae and a non-infested fruit were offered as options in the first experiment. The remaining experiments aimed at evaluating different odour sources as potential volatile cues used by the female parasitoid during host search (see Table 1 for a detailed description of the options offered in each experiment). Preference was assumed when a female spent more than 15 s in one distal sector. Bars indicate the result of the various experiments. Asterisks indicate significant differences between options, evaluated through a G-test of goodness of fit: **0.001<P<0.01; ***P<0.001.

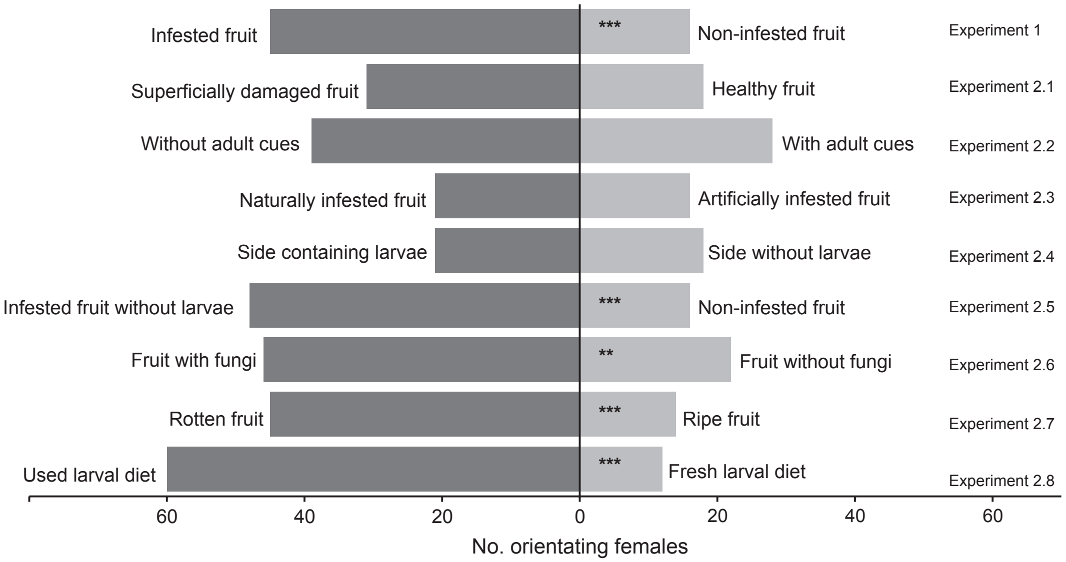

Figure 2 Number of female *Diachasmimorpha longicaudata* that actually visited an option (fruit, Petri dish, etc.) as first choice. A fruit infested with *Ceratitis capitata* larvae and a non-infested fruit were offered as options in the first experiment. The remaining experiments aimed at evaluating different odour sources as potential volatile cues used by the female parasitoid during host search (see Table 1 for a detailed description of the options offered in each experiment). Bars indicate the result of the various experiments. Asterisks indicate significant differences between options, evaluated through a G-test of goodness of fit: *0.01<P<0.05; **0.001<P<0.01; ***P<0.001.

Table 2 Average times spent in the vicinity of each one of the options presented inside the experimental arena during the preference test, latency to visit an odour source, and residence time on the visited option (mean duration ± SE; sample sizes in parentheses). The options offered in each experiment are described in detail in Table 1. Statistical analysis: t indicates Student t-test, Z indicates Wilcoxon test, and MW indicates Mann–Whitney test

Exp.	Options offered to female parasitoids	Time in sector		Latency		Residence time	
1	Infested	200.25 ± 21.41 (60)	t = 3.900,	286.58 ± 28.20 (45)	t = 0.048,	522.50 ± 25.92 (45	t = 0.796,
	Non-infested	75.33 ± 16.98 (20)	P<0.001	282.83 ± 35.56 (6)	P = 0.96	307.50 ± 82.37 (6)	P = 0.007
2.1	Damaged	134.64 ± 18.70 (45)	t = 0.500,	240.19 ± 27.31 (31)	t = 1.105,	493.10 ± 31.56 (31)	t = 0.928,
	Healthy	118.38 ± 19.08 (35)	P = 0.62	298.56 ± 47.93 (18)	P = 0.26	443.33 ± 44.74 (18)	P = 0.36
2.2	W/o adult cues	74.15 ± 11.69 (45)	t = 0.283,	212.21 ± 24.00 (39)	t = 0.751,	577.21 ± 14.78 (39)	t = 1.304,
	W/adult cues	79.51 ± 13.76 (35)	P = 0.78	186.04 ± 23.91 (28)	P = 0.46	600.00 ± 0.00 (28)	P = 0.20
2.3	Artificially inf.	154.74 ± 21.42 (33)	t = 0.131,	248.47 ± 35.48 (16)	t = 1.322,	527.44 ± 39.82 (16)	t = 0.764,
	Naturally inf.	159.40 ± 21.42 (47)	P = 0.90	320.07 ± 46.95 (21)	P = 0.20	481.29 ± 43.05 (21)	P = 0.45
2.4	Side w/larvae	142.58 ± 19.27 (37)	t = 1.232,	249.94 ± 24.12 (18)	t = 0.115,	372.50 ± 50.94 (18)	t = 0.014,
	Side w/o larvae	150.03 ± 18.91 (43)	P = 0.91	299. 14 ± 30.67 (21)	P = 0.23	364.71 ± 44.84 (21)	P = 0.91
2.5	Inf. no larvae	144.45 ± 17.15 (58)	Z = 3.801,	267.60 ± 21.26 (48)	t = 1.598,	562.00 ± 15.17 (48)	t = 0.212,
	Non-infested	55.95 ± 13.69 (22)	P<0.001	201.88 ± 31.50 (16)	P = 0.12	568.75 ± 31.25 (16)	P = 0.83
2.6	With fungi	105.03 ± 15.40 (53)	Z = 2.818,	210.87 ± 21.53 (46)	t = 0.676,	373.46 ± 30.86 (46)	t = 0.907,
	Without fungi	49.85 ± 10.82 (27)	P = 0.005	187.00 ± 33.13 (22)	P = 0.50	422.59 ± 44.38 (22)	P = 0.37
2.7	Ripe	58.26 ± 14.66 (23)	Z = 3.702,	239.43 ± 18.22 (14)	t = 0.588,	490.14 ± 47.95 (14)	t = 0.296,
	Rotten	132.18 ± 17.16 (57)	P<0.001	216.89 ± 35.97 (45)	P = 0.56	471.11 ± 32.56 (45)	P = 0.77
2.8	Used diet	130.96 ± 14.34 (67)	Z = 5.701,	248.98 ± 14.72 (60)	MW = 2.213,	599.68 ± 0.32 (60)	–
	Fresh diet	23.75 ± 6.97 (13)	P<0.001	187.25 ± 11.11 (12)	P = 0.027	348.25 ± 77.40 (12)	

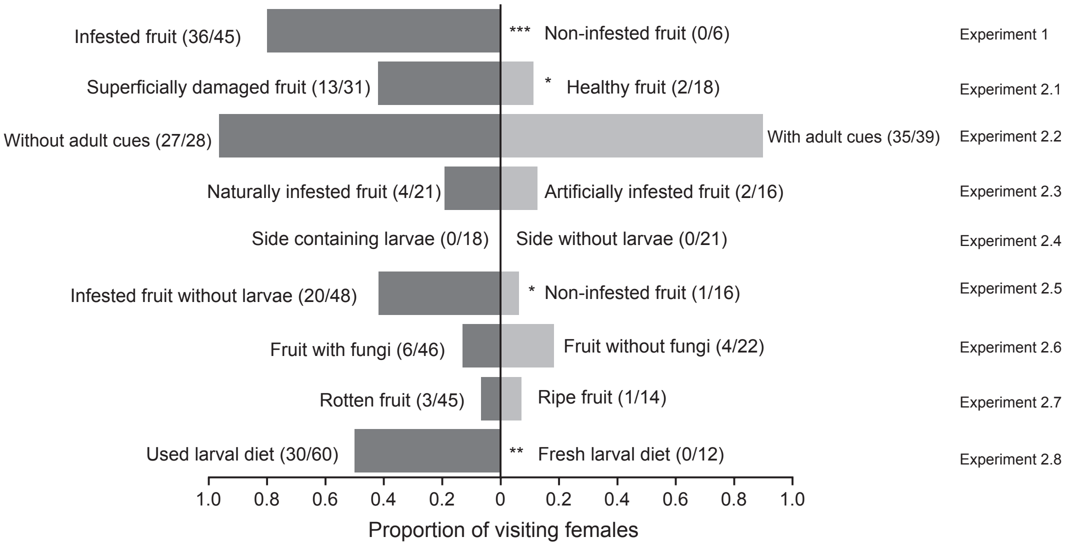

Figure 3 Proportion of *Diachasmimorpha longicaudata* females that performed at least one OA after visiting one of the two options offered inside the experimental arena. The figures of females that attempted to oviposit and females that visited the option are shown next to each column, and is shown as a ratio. A fruit infested with *Ceratitis capitata* larvae and a non-infested fruit were offered as options in the first experiment. The remaining experiments aimed at evaluating different odour sources as potential volatile cues used by the female parasitoid during host search (see Table 1 for a detailed description of the options offered in each experiment). Bars indicate the result of the various experiments. Asterisks indicate significant differences between options, evaluated through a G-test of homogeneity: $*0.01<P<0.05$; $**0.001<P<0.01$; $***P<0.001$.

In experiment 1, as well as in experiments 2.4, 2.5, 2.7, and 2.8, there was either one or no OA in at least one of the fruit visited. Therefore, the variables that describe the oviposition behaviour could not be compared statistically and are not presented. The results for the remaining experiments are presented in Table 3. The latency to exhibit an OA, the number of OAs, the number of OAs per minute (OA rate), and the time females spent performing OAs, did not differ between options in any of the experiments (Table 3). Duration of the OAs was similar between options in experiment 2.1, 2.2, and 2.3. In experiment 2.6, females showed longer OAs in the fungus-contaminated orange (Table 3). However, the low number of observations could have contributed to the high significance of this comparison (type 1 error).

Discussion

In the present study, we analyzed the use of chemical cues during host searching by naïve *D. longicaudata* females to understand to what extent this parasitoid innately relies on cues derived from the host larvae and cues related to the fruit, at two spatial scales (during host habitat search and during host search within the habitat). We found that female parasitoids were able to discriminate based on chemical cues between patches with host larvae and those that contained no hosts, exhibiting a clear preference for larvae-infested oranges. When different volatile sources were analyzed separately, the odour sources directly associated with adult fruit fly activity were not attractive, whereas those associated with larval infestation attracted parasitoids even in the absence of host larvae. Once the female landed on the fruit, direct cues associated with fruit fly larvae activity became important but some indirect signals also increased host searching activity.

Although the response of *D. longicaudata* to infested fruit is relatively well documented, few studies have explored the sources of volatiles that are responsible for this behaviour. Greany et al. (1977) proposed that fungi that grow in association with infested fruit were the main signal that indicated to the female parasitoids about the presence of hosts, and concluded that infestation itself was not important. Silva et al. (2007) showed that even when rotting guavas attracted females in the absence of hosts, this attraction was increased when the fruit was infested. Carrasco et al. (2005) found that without infestation there is no preference for damaged fruit, but they did not state the fungal contamination and the maturation status of the fruit. Here, we followed a sequential scheme of tests that enabled us to find that females responded to more than one source of volatile compounds. First, we found that females responded to compounds derived from larval

Table 3 Average latency to perform oviposition attempts (OAs), number of OAs, OA rate, mean OA duration, and time spent performing OAs are presented (mean ± SE). The options offered in each experiment are described in detail in Table 1. Statistical analysis: t indicates Student t-test

Exp.	Options offered to female parasitoids	n	Latency to OA (s)		No. OAs		OA rate (OAs min^{-1})		OA duration (s)		Time allocation to OA	
1	Damaged	13	271.15 ± 38.41	t = 0.677,	1.23 ± 0.12	t = 0.721,	0.12 ± 0.01	t = 0.731,	24.02 ± 4.54	t = 0.767,	0.05 ± 0.01	t = 0.771,
	Healthy	2	344.50 ± 49.50	P = 0.51	1.00 ± 0.00	P = 0.48	0.10 ± 0.00	P = 0.48	17.65 ± 6.24	P = 0.46	0.03 ± 0.01	P = 0.45
2	W/o adult cues	35	241.42 ± 25.85	t = 1.669,	2.17 ± 0.15	t = 0.814,	0.24 ± 0.03	t = 0.812,	94.43 ± 7.39	t = 1.202,	0.33 ± 0.03	t = 1.000,
	W/adult cues	27	184.00 ± 24.71	P = 0.10	2.41 ± 0.25	P = 0.42	0.22 ± 0.01	P = 0.42	119.08 ± 20.10	P = 0.23	0.38 ± 0.04	P = 0.32
3	Artificially inf.	2	229.50 ± 202.50	t = 1.193,	1.25 ± 0.25	t = 0.516,	0.15 ± 0.05	t = 0.516,	57.94 ± 14.23	t = 0.511,	0.13 ± 0.01	t = 0.283,
	Naturally inf.	4	74.25 ± 26.27	P = 0.23	1.50 ± 0.50	P = 0.63	0.13 ± 0.03	P = 0.63	77.48 ± 24.79	P = 0.66	0.15 ± 0.03	P = 0.79
6	With fungi	6	216.83 ± 49.54	t = 0.755,	1.00 ± 0.00	t = 2.190,	0.16 ± 0.04	t = 0.695	53.05 ± 8.87	t = 2.547,	0.13 ± 0.02	t = 1.331,
	Without fungi	4	152.50 ± 73.99	P = 0.47	1.50 ± 0.29	P = 0.060	0.21 ± 0.04	P = 0.51	24.65 ± 1.53	P = 0.034	0.09 ± 0.02	P = 0.22

n, sample size.

activity. The fact that used larval medium also elicited a positive response in the females supports the idea that by-products from larval activity are, in fact, an important source of chemical cues, opposite to that postulated by Greany et al. (1977). In contrast, the physical presence of larvae seems unimportant in terms of long-range attractiveness. Stuhl et al. (2011) have recorded an increase in *D. longicaudata* females' host-searching activity in response to a compound that is released by tephritid larvae. In agreement with our results, this compound showed no effect on female parasitoids at long range, as would be expected under natural selection acting to minimize cues that reveal larva location. Attraction to infested plants after removal of hosts has been reported in several parasitoid-host-plant systems (Lo Bue et al., 2004; Voss et al., 2009; Dannon et al., 2010; Peri et al., 2011).

Female parasitoids preferred visiting rotten over ripe oranges, suggesting that parasitoids are attracted to compounds released by the fruit even in total absence of host larvae. Silva et al. (2007) found that *D. longicaudata* females preferred visiting rotting over unripe guavas. This shows, again, that *D. longicaudata* uses volatile compounds indirectly associated with *C. capitata* larvae. Infested fruit become rotten more rapidly than non-infested fruit, and hence volatiles released by rotten fruit will indicate infestation with a higher probability than ripe fruit. At large distances, compounds released by rotten fruit may guide parasitoids to habitats with higher probability of host encounters and then, at a closer range, parasitoids could use less conspicuous compounds related to larval activity. In agreement with our results, Henneman et al. (2002) found that *Diachasmimorpha juglandis* (Muesebeck) innately responded to non-infested fruit. However, as this species is a specialist parasitoid of *Rhagoletis juglandis* (Cresson) larvae, which in turn are specialised on walnuts, an innate response to its host habitat is expected (Vet & Dicke, 1992). In the case of *D. longicaudata*, however, the host habitat includes many different plant families and we would therefore expect use of general host-habitat cues such as those produced by microorganisms likely to be associated with the host. This led us to think that the repertoire of host-habitat cues that *D. longicaudata* females can detect is much wider and does not necessarily need to be reinforced by learning in order to trigger a response. Evidence on the use of cues from the host habitat in the absence of hosts has also been found in other generalist parasitoids (Jönsson et al., 2005; Wyckhuys & Heimpel, 2007; Belda & Riudavets, 2010).

Mouldy oranges elicited a positive response in female parasitoids. Greany et al. (1977) found attraction of *D. longicaudata* females towards methanolic extracts of the fungus *Monolinia fruticola* (Wint) isolated from peaches and also towards extracts of *P. digitatum* isolated from grapefruits. In our study, *P. digitatum* was evaluated in association with oranges. This response is puzzling when the effect of the fungus on the survival of the host larvae and the behaviour of the female parasitoids after landing on a mouldy fruit are taken into account. First, the fungus affects larvae and normally no pupae are recovered from contaminated fruit (DF Segura, personal observation), probably because of the mycotoxins released by the fungus (Castillo et al., 2000). Therefore, cues emitted specifically by *P. digitatum* would likely indicate a poor habitat in terms of host quality. In addition, when females encountered a mouldy area in an orange, they first stopped antennal activity and then stopped walking, turned, and walked in the opposite direction or even flew away from the fruit. This suggests that the presence of the fungus limits the searchable area in a fruit. The apparent contradiction between females' preference and females' expected reward could rely on the fact that the plant releases (actively or not) volatile compounds after the fruit is attacked, irrespective of the agent causing the damage (fruit fly or fungus). These compounds would then be used by parasitoids to locate potentially infested fruit. A possible alternative explanation is that fruit infested by larvae or infected by fungi were colonized by bacteria, and that the parasitoids were attracted to cues from the bacteria. Although females' attraction towards fruit colonized by fungi may well reflect a 'contamination' of the chemical signals used by parasitoids, we cannot rule out an adaptive role because, at long distances, volatiles related to fungal contamination might aid in the search of mature fruit (potentially infested by fruit fly larvae).

In the present work, artificial fruit damage, fruit fly eggs, and host marking pheromones were not cues for *D. longicaudata* females. These odour sources are related to the initial phases of the infestation and thus would only be useful to find eggs or, at most, intermediate instars (Hoffmeister & Gienapp, 1999). In fact, studies on fruit fly egg parasitoids have shown that host marking pheromones are cues during host search (Prokopy & Webster, 1978; Roitberg & Lalonde, 1991). However, the marked preference of *D. longicaudata* females for late instars (Cancino Díaz & Yoc, 1994) makes these compounds useless as cues.

After landing on an infested orange, females readily initiated oviposition even when larvae had already exited the fruit. The response was also observed when they foraged on the artificial larval diet. Duan & Messing (2000) reported that *D. longicaudata* increases its oviposition activity when foraging in artificial larval medium that had hosted larvae, and suggested that this behaviour is due to chemical signals excreted by host larvae. The concordance between our results using host *C. capitata* in oranges and those obtained by Duan & Messing (2000) using *Anastrepha ludens* Loew could indicate that these cues are similar among host larvae from different species. In fact, Stuhl et al. (2011) have recently identified a compound that is released by larvae from several tephritid species which enhances host searching behaviour in *D. longicaudata* females. Lawrence (1981) found that during host searching *D. longicaudata* used vibratory signals produced by larvae. In our case, when larvae were present in the fruit (as in experiment 1), females that attempted to oviposit increased up to 80%, suggesting that females combine various types of cues.

We found that punctures on the surface of the fruit elicited ovipositor searching in *D. longicaudata*, pointing out that females might use visual and/or mechanical cues during host searching on the fruit. However, even when females exhibited an increased tendency to oviposit in punctured non-infested oranges, these attempts had a short duration (similar to that recorded in whole fruit). The fact that female parasitoids found no other stimuli (larvae or chemical cues associated with larvae, as suggested by Stuhl et al., 2011) after they bored with the ovipositor on those holes, may explain why they stopped ovipositor searching. The usefulness of ovipositor searching in these holes is not clear, but females could respond to them because holes indicate that hosts are near (third instars are located close to the surface of the fruit) or because they represent areas in which the ovipositor drilling is easier.

Understanding the biology, ecology, and behaviour of a parasitoid and its target pest species is essential to produce high-quality natural enemies that ensure the success of a biological control program (Glenister & Hoffmann, 1998). Our results allow a better understanding of the cues used by *D. longicaudata* females during host searching. Further research should contribute to the identification of the chemical compounds involved in the attraction found in the present study. Chemical identification of these cues could determine whether the attractiveness is based on a single volatile compound or a blend and whether different fruit species release common compounds. Furthermore, the identification of the chemical basis of this behaviour will provide valuable information to design specific attractants for this species that enable to follow the populations in time and space after a release as a part of a biological control program against fruit flies.

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