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Differentiation of innate but not learnt responses to host-habitat odours contributes to rapid host finding in a parasitoid genotype

RAQUEL PÉREZ-MALUF[1,2], HALITIANA RAFALIMANANA[1,3], ERICK CAMPAN[1,4], FRÉDERIC FLEURY[5] and LAURE KAISER[1]

[1]Laboratoire de Neurobiologie Comparée des Invertébrés, UR 271/UMR PISC, INRA, Centre de Versailles-Grignon, France, [2]Laboratório de Zoologia, DCN, UESB, Vitoria da Conquista, BA, Brazil, [3]Ecole Supérieure des Sciences Agronomiques, Antananarivo, Madagascar, [4]Laboratoire d'Ecologie Fonctionnelle, UMR CNRS 5245, Université P. Sabatier – Toulouse III, Toulouse Cedex, France and [5]Biométrie et Biologie Evolutive, UMR CNRS 5558, Université Claude Bernard Lyon I, Villeurbanne Cedex, France

Abstract. In parasitoids, host-habitat odour can influence host searching within the habitat. This is the case in *Leptopilina* sp. (Hymenoptera, Figitidae), a *Drosophila* parasitoid searching for larvae by ovipositor probes. This a behaviour can be conditioned to fruit odours. In a previous study, the latency of probing to a fruit odour is reported to have a genetic variability within a laboratory strain. This suggests a link between rapidity of host discovery and fitness. In the present study, this hypothesis is tested by comparing responses to host-habitat odours between two genotypes of *Leptopilina heterotoma* Thomson, from the Mediterranean coast (Antibes) and from Burgundy (Tailly). The two genotypes present contrasting rhythms and levels of locomotor activity linked to contrasting interspecific competition in their area of origin. The high activity observed in the Mediterranean genotype is interpreted as an adaptive response to a limited time-window to win against a competitor species absent in Burgundy. The present study finds differentiation in innate but not learnt responses to host-habitat odours. The more active genotype (Antibes) has a higher probability and a shorter latency of innate probing to the odours than the less active genotype (Tailly); Antibes females also find larvae and complete infestations more rapidly. Learning equalizes the probability and the latency of probing to the odours in both strains, and increases the probing duration. Innate responses to host-habitat odours would allow time-limited insects to increase their reproductive rate, when host predictability is high in the habitat. Selection of faster innate responses to host and habitat cues without evolution of learnt responses indicates that the initial host discovery is more crucial to fitness than subsequent ones.

Key words. Foraging behaviour, genetic variability, learning, parasitic wasp, plant odour.

Introduction

Hymenopteran parasitoids have developed several sensory and behavioural mechanisms to locate and parasitize their specific hosts. Important among these is the cueing on host-habitat odours, which is an essential capacity for host location (Turlings *et al.*, 1993; Smid & Vet, 2006). Learning to prefer the odour of a rewarding host-habitat is particularly well studied (Vet *et al.*, 1995), with the learnt response being the choice for the experienced host-habitat odour. Such a capacity is developed in parasitoids of polyphagous hosts (Geervliet *et al.*, 1998) and is an adaptive mechanism by which females focus their search on plants having a high

Correspondence: Laure Kaiser, Laboratoire Evolution, Génômes et Spéciation, IRD UR 072/CNRS UPR 9034, bât 13, 1 Avenue de la Terrasse, 91198 Gif-sur-Yvette, France. Tel.: +33 169 823 704; fax: +33 169 823 736; e-mail: kaiser@legs.cnrs-gif.fr

probability of being infested. However, memory for a plant odour has other behavioural expressions that have received little attention. It can influence the amount of time spent searching on plants and be involved in both plant species recognition (Kester & Barbosa, 1991) and adjustment of patch time to host resources (Kaiser *et al.*, 2003; Tentelier & Fauvergue, 2007). To obtain a better insight on how plant odour memory contributes to searching efficiency within the host-habitat, it would be useful to document the characteristics of the learnt response, such as latency and duration of exploratory behaviours. The same applies for innate responses to plant odours. Mechanisms enhancing rapidity in host location are particularly expected in pro-ovogenic species that are time-limited, or to maintain coexistence with a better competitor (Hassel & Waage, 1984). However, insect memory is essentially assessed by the probability of responding to the conditioned stimulus.

In a proovogenic parasitoid of *Drosophila*, *Leptopilina boulardi* Barbotin *et al.* (Hymenoptera: Figitidae), it was found that the latency and the duration of a learnt searching response to banana odour depended on the reproductive status (Pérez-Maluf & Kaiser, 1998). The latency also showed significant genetic variability within and between experimental strains (Pérez-Maluf *et al.*, 1998; Campan *et al.*, 2002). In the case of these laboratory strains, such variability was difficult to relate to environmental constraints. In the present study, searching responses to host habitat odour are analysed in a case where genetic variability in activity rhythm is reported as an adaptation to environmental constraints. We compare responses between two strains of a *Drosophila* parasitoid *Leptopilina heterotoma* Thomson originating from a Mediterranean and a northern population. These strains have genetically different circadian rhythms, rates of locomotory activity, rates of oviposition activity (Fleury *et al.*, 1995) and fecundity (Ris, 2003). Higher rates of locomotion and oviposition observed in the Mediterranean strain of *L. heterotoma* are interpreted as a mechanism to coexist with a competitor species *Leptopilina boulardi*, which is absent in northern areas (Allemand *et al.*, 1999). *L. heterotoma* has to oviposit first to 'win' the larval competition when both parasitoids infest a same larva (Fleury *et al.*, 2000), and hence has a limited time-window for successful parasitism.

In *Leptopilina*, naive females are typically attracted by the combined odour of the aggregation pheromone of adult drosophila and fruit odours (Couty *et al.*, 1999), and by fermentation products (Dicke *et al.*, 1984). Once on an infested patch, they search by ovipositor probing for burrowing larvae. This behaviour is triggered innately by larval chemicals and vibrations (Vet & van Alphen, 1985; Vet & Bakker, 1985; Vet *et al.*, 1993). They memorize the fruit odour by associative learning when ovipositing in a larva, and afterwards are attracted by this odour (Vet & Groenewold, 1990; De Jong & Kaiser, 1992). The learnt odour also triggers ovipositor probing, and this conditioned response can occur in the short or long term, dependent upon the number of oviposition experiences (Kaiser *et al.*, 2003). The ability to initiate probing in response to the fruit odour could enlarge the searching area to the entire fruit, and might increase the probability of locating all host patches and even small patches emitting little amount of kairomone. In the present study, a paradigm allowing odour conditioning of the ovipositor searching behaviour (Kaiser *et al.*, 1995) is used to compare naïve and learnt responses of both *L. heterotoma* strains in response to host-habitat odours. *Leptopilina heterotoma* parasitizes mostly frugivorous species, but also fungivorous species (Janssen *et al.*, 1988), which are not parasitized by *L. boulardi* (Carton *et al.*, 1986). Responses to one fruit and one fungus odour are compared.

Materials and methods

Biological material

The strains of *L. heterotoma* (Hymenoptera: Figitidae) originated from populations living respectively in the northern and southern side of the upper limit of *L. boulardi* distribution in France (Allemand *et al.*, 1999). The southern strain came from insects collected along the Mediterranean coast in Antibes (latitude: 43°34′ north; longitude: 7°07′ east). For the northern strain, insects had been collected in the vicinity of Chalon-sur-Soane, in Tailly (latitude: 46°58′ north; longitude: 4°48′ east). For both strains, insects had been collected from fruits and fruit traps in orchards. Both strains were reared on the Rosy 295 mutant of *Drosophila melanogaster* Meigen (for a high parasitization rate; Y. Carton and F. Frey, personal communication), fed on an axenic diet (corn meal, dead pulverulent yeast, sugar and a fungicide). To minimize olfactory experience of parasitoids before the experiments, pupae of parasitized *Drosophila* were collected, washed in 5% bleach (which also prevented fungal infestation), rinsed in water and stored at 25 °C under an LD 12 : 12 h photoperiod in tubes containing agar-agar and honey. After the emergence of parasitoids, tubes were stored at 17 °C (LD 12 : 12 h).

Apparatus

The conditioning protocol was developed for *L. boulardi* and described in Kaiser *et al.* (1995) and Pérez-Maluf & Kaiser (1998). Briefly, this protocol allows conditioning of *Leptopilina* females to probe agar in response to an olfactory stimulus. Conditioning is achieved by delivering the odour during an oviposition, and this association is repeated *n* times. The conditioned responses are subsequently tested by delivering the odour in the absence of host larvae.

To achieve this, a small plastic cap (internal diameter 11 mm, height 3 mm) with a 4-mm hole at its centre, covered with gauze, was used. A ring of agar-agar (internal diameter 6 mm) was placed in the cap, on the gauze. For conditioning, 20 second-instar larvae were placed on the ring of agar. For testing female behaviour after conditioning, a ring of agar without host larvae was used to record probing responses to the odour. For conditioning as well as for testing, a single

female *L. heterotoma* was introduced in the centre of the ring of agar, which was later covered with a piece of overhead projection film, perforated with pin holes. The cap was placed on a support slightly above the outlet of an airflow tube. A pump regulated at 520 mL min^{-1} was used to generate the main airflow. To deliver the odour stimulation to the female, half the flow was sent through a glass vial containing the odour source and connected to the main flow via a Pasteur pipette just under the caplet.

Odour sources

The used food flavourings were made from banana and from mushroom instead of whole fruit or mushroom to avoid uncontrolled variations of odour sources. The flavourings were made from natural extracts and supplied by Haarmann and Reimer (Holzminder, Germany; banana ref. 211202, mushroom ref. FR 4887). Two glass capillaries (length 1 cm, internal diameter 1.56 mm) with one extremity sealed with wax were filled with the same flavour and placed in the glass vial described above.

Conditioning and test protocols

Females from each strain were allocated to four groups. The first two groups were subjected to the conditioning phase: one with banana odour, the second with mushroom odour. The last two groups were used as control to provide naïve females tested either to banana or to mushroom odour without oviposition experience or exposure to the odour.

The conditioning phase consisted of five ovipositions each associated with the odour delivery. Once in the ring of agar, a female typically walked and preened, then started to probe the agar when walking or not (ovipositor searching). When the larva was touched, the female inserted the ovipositor and remained immobile (ovipositing) for some time. The oviposition ended when the female retrieved the ovipositor and resumed search until the second larva was found, and so on. Insect spent most of the time between two ovipositions probing the agar, with eventual bouts of preening or walking without probing. The female was removed from the arena as soon as it finished the fifth oviposition. Four parameters were recorded: (i) the latency to initiate ovipositor searching, from the introduction of the female into the caplet; (ii) the cumulative duration of the five ovipositions; (iii) the total time to parasitize the five larvae (from introduction to removal of the female); and (iv) the time spent to find the five larvae [i.e. the accumulated duration of the time elapsed between the end of an oviposition and the beginning of the next one, estimated by (iii) – (ii) – (i)].

The testing phase (the same for naive or conditioned females) was undertaken 24 h after the conditioning (if any). A female was placed into a ring of clean agar. For the first minute, the airflow was not odourized so that the insect could become acclimatized with the device. The odour was then delivered for 30 s, representing the mean duration of an oviposition. The occurrence of a probing response in the minute after the initiation of the odour delivery was noted. If the female did not probe the agar within this time, it was counted as not responding to the odour (a preliminary study showed that only 1.5% females started probing after 1 min). When probing, the female was observed until it stopped for at least 1 min (females were never observed resuming probing after this delay), or up to 5 min if it did not stop. Two parameters were recorded: the latency of the response from the onset of the odour delivery and the cumulative probing duration.

Statistical analysis

Proportions of females probing in response to the odours were compared using the chi-square test. A two-way factorial analysis of variance (ANOVA) was used to test both effects of strain and odour (crossed factors) on the parameters of the naive probing response to the odour during the test. The same analysis was run on the parameters of oviposition behaviour during the conditioning phase. A one-way ANOVA was used to analyse the treatment effect between the conditioned groups. A Wilcoxon–Mann–Whitney test under the one-sided hypothesis was used to compare the responses of naive and conditioned groups. The numbers of observed females are reported in Table 1. For the analysis of latency and duration of the probing response, sample sizes corresponded to the number of responding females.

Results

Behaviour during conditioning

In the presence of host larvae, the latency to start probing the substrate depended on the strain, with more rapid responses in Antibes females ($F_{1,126} = 28.42$, $P < 0.001$; Fig. 1a). Latency was not influenced by the odour ($F_{1,126} = 2.64$, $P = 0.11$). The time spent to find five larvae depended on both strain and odour ($F_{1,126} = 33.94$, $P < 0.001$ and $F_{1,126} = 9.54$, $P = 0.002$, respectively). It was again shorter in Antibes than in Tailly females and shorter in banana than in mushroom odour (Fig. 1b).

Cumulative duration of the five ovipositions depended on the strain ($F_{1,126} = 11.11$, $P = 0.001$; Fig. 1c), with the duration of Antibes females being longer. This parameter was not influenced by the odour ($F_{1,126} = 1.21$, $P = 0.27$). The total time to

Table 1. Number of observed females in the different testing situation.

	Banana	Mushroom
Antibes	39/26	41/37
Tailly	51/28	26/28

Naïve and experienced groups are shown to the left and right of solidus, respectively.

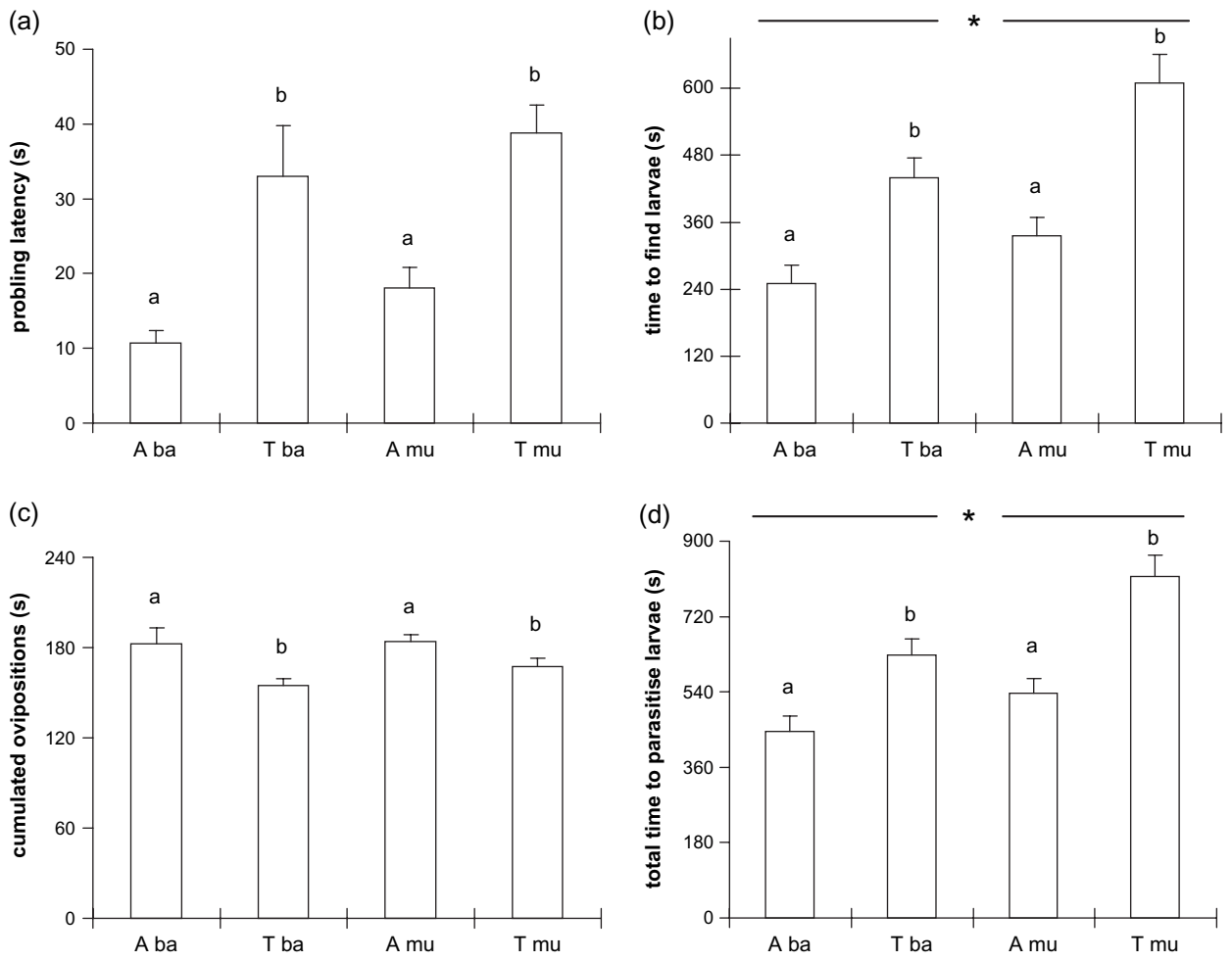

Fig. 1. Behaviour during conditioning. The observation was stopped when five larvae had been parasitized. Bars represent the mean ± SE of the parameters: (a) latency of initiating probing after introduction in the caplet; (b) time to find five larvae; (c) accumulated duration of five ovipositions; and (d) total time to parasitize five larvae. A ba, Antibes strain responding to banana odour; T ba, Tailly strain responding to banana odour; A mu, Antibes strain responding to mushroom odour; T mu, Tailly strain responding to mushroom odour. A two-way analysis of variance demonstrated a significant effect of strain ('a' versus 'b') and/or odour (star between groups tested with different odours) at $P < 0.05$.

parasitize five larvae varied with the strain ($F_{1,126} = 32.13$, $P < 0.001$; Fig. 1d) and the odour ($F_{1,126} = 11.01$, $P = 0.001$). It was shorter in Antibes females and with banana odour (Fig. 1d). This result came from the observed differences of searching time, which represented most time of the behavioural sequence.

Probing responses to the olfactory stimuli

Percentage of females probing in response to the odour. There was evidence of variation between strains of naïve females in probing response to banana odour (Fig. 2). A higher percentage response to banana odour was observed in Antibes strain ($\chi^2 = 18.36$, d.f. = 1, $P < 0.001$). However, there was no evidence of variation between the strains in response to mushroom odour ($\chi^2 = 2.02$, d.f. = 1, $P > 0.05$). In the conditioned groups, the proportion of females responding to the odour was significantly higher than in the corresponding naïve groups for each combination of strain and odour ($\chi^2 > 5.25$, d.f. = 1, $P < 0.01$), and most conditioned females of both strains responded to both odours, without any significant variation between the groups ($\chi^2 = 7.43$, d.f. = 2, $P > 0.05$).

Parameters of the probing response. When naive females probed in response to banana or mushroom odour, the probing latency depended significantly on strain ($F_{1,48} = 6.22$, $P = 0.016$; Fig. 3a), with shorter latency in Antibes females. The latency was not influenced by the odour ($F_{1,48} = 0.15$, $P = 0.70$). Compared with naïve females, the latency of conditioned Tailly females was reduced, significantly in the case of banana odour ($W = 177.5$, d.f. = 1, $P = 0.04$), whereas the latency of Antibes

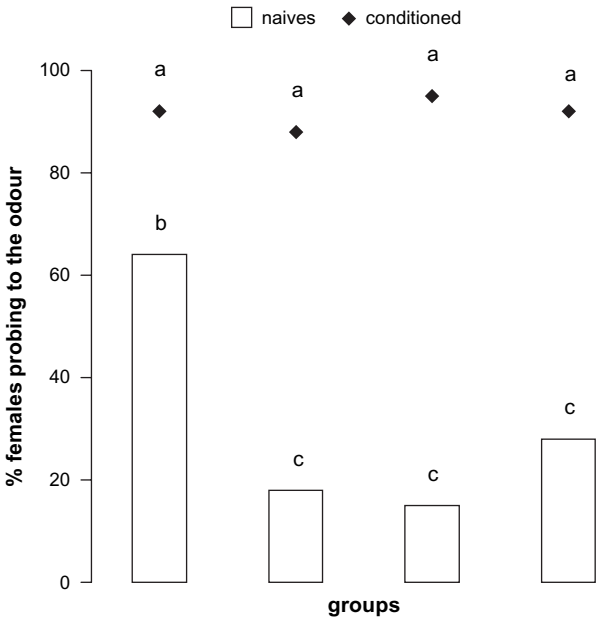

Fig. 2. Percentage of females probing in response to the olfactory stimuli. Bars give the value of naive groups, and points of experienced groups. Letters indicate differences at $P < 0.05$ by a chi-square test (see text).

females remained unchanged. Both conditioned strains showed a low latency to both odours without significant difference ($F_{3,110} = 0.675$, $P = 0.57$).

The duration of the probing response was highly variable in naive females, and was not significantly influenced by the strain or the odour ($F_{1,48} \leq 1.79$, $P \geq 0.19$; Fig. 3b), although it tended to be longer in the Antibes strain. Conditioning uniformly increased the probing duration of the four groups ($W \geq 79.5$, $P \leq 0.04$), without variation between the groups ($F_{3,110} = 1.53$, $P = 0.21$).

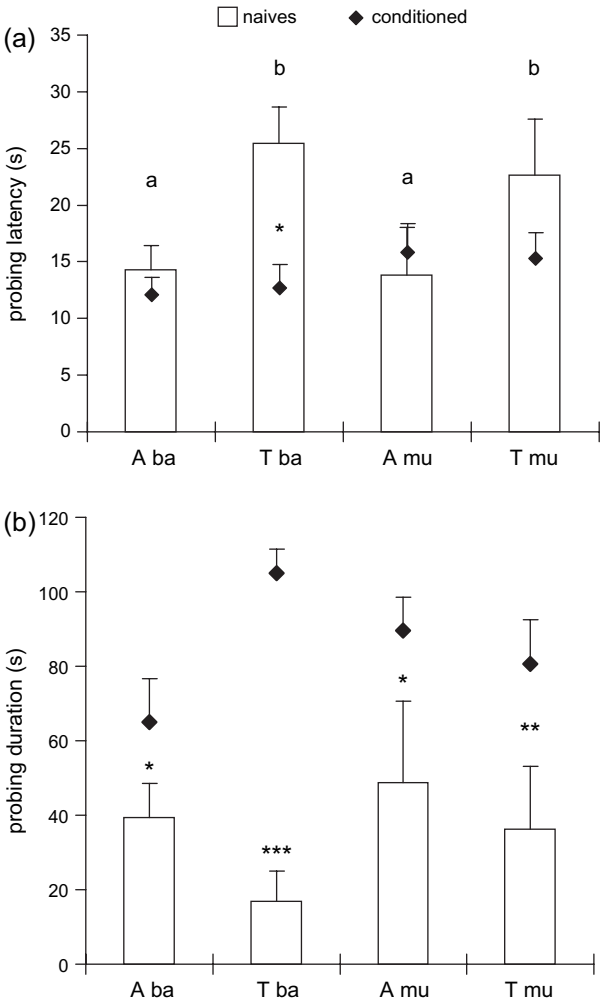

Fig. 3. Parameters (mean ± SE) of the probing response to the odours: (a) mean latency and (b) mean duration. Bars give the value of naive groups, and points, of experienced groups. A ba, Antibes strain responding to banana odour; T ba, Tailly strain responding to banana odour; A mu, Antibes strain responding to mushroom odour; T mu, Tailly strain responding to mushroom odour. A two-way analysis of variance demonstrated a significant effect of strain ('a' versus 'b') but not odour at $P < 0.05$. Stars indicate significant difference between naive and experienced females (Wilcoxon–Mann–Whitney: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$). Neither the probing latency, nor the probing duration differed significantly between experienced groups (see text).

Discussion

The present study reveals differing responses to host-habitat odours between strains previously known to differ in activity rhythm. The more active Antibes strain is more inclined and shows a more rapid response to a fruit odour and to host larvae when naive. Differences are only visible, however, when females are naïve because learning equalizes their performances. A novel finding of the present study is the differentiated naïve response to host-habitat odour in the two strains. Previous studies on the species *L. heterotoma* established that responses to host-habitat odours including fruits are the result of associative learning (Vet & Schoonman, 1988; Vet & Groenewold, 1990). These previous studies were conducted on strains from northern populations, having a low activity rhythm (Fleury *et al.*, 1995). The response of naive Antibes females is innate because wasps are not exposed to fruit components in their rearing environment. It is not restricted to banana odour because Antibes females are reported to show a high percentage of naïve probing responses to pear odour, which is not observed in a northern population (Pérez-Maluf, 1998). Such naïve response to fruit odours would facilitate the discovery of a first host, depending on its predictability on the fruit. Antibes females are also more rapid compared with Tailly females in finding and parasitizing the host larvae. This strain gains time in finding the larva, but not at the level of the egg deposition behaviour.

It is assumed that the observed behavioural differences between the strains have a genetic basis. First, an effect of

the rearing environment is excluded because both strains are reared under the same condition. Second, both strains differ genetically for other behavioural parameters, as noted in the Introduction. The Mediterranean population would have evolved this rapid genotype in response to environmental constraints because high activity has a high physiological cost. Rapidity would have been selected in Antibes, possibly as an adaptive response to a limited time-window to win the competition with *L. boulardi*, found on banana but not fungi. However, a shorter latency and more rapid location of larvae are observed with both banana and mushroom odours. This may be also favoured by other environmental factors, such as shorter periods of host or fruit availability due to higher temperature range. Experiments in more realistic conditions are required to address the cost of being less rapid in host location, especially when two species are competing.

Selection of faster innate responses to host and host-habitat cues without evolution of learnt responses indicates that the first host discovery is more crucial to fitness than the subsequent discoveries. This is coherent with the structure of the host population and the life cycle in the tri-trophic association. When parasitoids emerge after approximately 3 weeks of development, the old fruit is too decayed and dry to be infested by young *Drosophila* larvae and the subsequent emerging wasps have to search for new host patches. Because host larvae are gregarious, once a first larva has been discovered, further host discoveries are guaranteed.

The present study also shows that the parameters of the searching behaviour have different sources of variation. The latency is determined genetically and by experience, as seen in the Tailly strain, the duration of the probing response depends mostly on experience. This matches the lack of genetic variability of this parameter between isofemale lines from one population of *L. boulardi* (Pérez-Maluf *et al.*, 1998). The searching behaviour of *L. heterotoma* also depends on the odour. Both strains are more rapid in the presence of the fruit odour compared with the mushroom odour. In the present study, the individuals came from insects trapped with banana baits, and thus better laboratory performances could result from preferential parasitism of frugivorous *Drosophila* by the founders.

Finally, the present study provides original data on the role of the host-habitat odour at the level of host finding within the habitat. In a general model, wasps find a potential host-habitat by oriented locomotion to plant volatiles, which depends on learning, whereas exploratory behaviours are triggered by the innate recognition of host cues. In some specialist tri-trophic systems, plant volatiles can condition host acceptance (Kester & Barbosa, 1991; Kaiser & Cardé, 1992) and host handling, and hence reproductive rate (Mattiacci *et al.*, 2000). In some more generalist systems, learning plant odour is involved in host patch exploration and exploitation (Vet & Schoonman, 1988; Kaiser *et al.*, 2003; Tentelier & Fauvergue, 2007). The present study documents an unusual situation where a population of a generalist parasitoid has developed the innate use of host-habitat odour in its exploratory behaviour. Because learning is time consuming, such physiological capacity can be seen as a strategy that allows time-limited insects to increase their reproductive rate, implying high host predictability in the host-habitat.

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