

The effect of larval and early adult experience on behavioural plasticity of the aphid parasitoid *Aphidius ervi* (Hymenoptera, Braconidae, Aphidiinae)

Cristian A. Villagra · Francesco Pennacchio ·
Hermann M. Niemeyer

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Abstract The relevance of the integration of preimaginal and eclosion experiences on the subsequent habitat preferences and mate finding by the adult has been rarely tested in holometabolous insects. In this work, the effect of larval and early adult experiences on the behavioural responses of adult males of the aphid parasitoid, *Aphidius ervi*, towards volatiles from the host–plant complex (HPC) and from conspecific females were evaluated. Two experience factors were considered: host diet (normal diet=ND; artificial diet=AD), and eclosion, i.e. extraction or non-extraction of the parasitoid larva from the parasitised aphid (extracted=EX; non-extracted=NE). Thus, four treatments were set up: ND/NE, ND/EX, AD/NE and AD/EX. Glass Y-tube olfactometers were used to investigate the responses of adult *A. ervi* males to the odour sources used. Males from the ND/NE treatment showed a shorter latency to the first choice of olfactometer arms, displayed a marked preference towards the HPC olfactometer arm, and spent more time in the HPC arm than males from the other treatments. Only the interaction of host diet and eclosion experiences proved to be relevant in explaining the differ-

ences in latency to first choice, time spent in olfactometer arms, and behaviours displayed in the olfactometer arms. These results show the importance of the integration of larval and eclosion experiences in the development of stereotyped responses of the adults. This process may involve memory retention from the preimaginal and emergence period, but further research is needed to disentangle the contribution of each stage. The response to conspecific females was much less affected by the treatments in relation to first arm choice and times in olfactometer arms, suggesting a pheromone-mediated behaviour, even though a prompt and more intense wing fanning courtship behaviour was registered in the ND/NE males compared to males from the AD/NE treatment. These results show that sexual behaviours are less affected by early experiences than behaviours related with finding a HPC for foraging or oviposition. Taken together, our results demonstrate for first time that larval and eclosion experiences affect in a differential way the early responses of the adult towards environment-derived cues and mate related cues.

Keywords Innate behaviour · Preimaginal learning · Chemical legacy

C. A. Villagra (✉) · H. M. Niemeyer
Departamento de Ciencias Ecológicas, Facultad de Ciencias,
Universidad de Chile,
Casilla 653,
Santiago, Chile
e-mail: cavillagra@gmail.com

H. M. Niemeyer
e-mail: niemeyer@abulafia.ciencias.uchile.cl

F. Pennacchio
Dipartimento di Entomologia e Zoologia Agraria
“Filippo Silvestri”, Università di Napoli “Federico II”,
Naples, Italy

Introduction

The innate behaviour of insects is not always unequivocally defined, as the role that experience during early stages of development may play in shaping the responses of the adult is still a highly debated topic (Gandolfi et al. 2003). Recent work on holometabolous insects has demonstrated that both larval (e.g. preimaginal conditioning: Tully et al. 1994; Rietdorf and Steidle 2002; Gandolfi et al. 2003) and early adult learning (e.g. chemical legacy: Barron and Corbet

1999; van Emden et al. 2002) may occur. The occurrence of learning at early sensitive stages can be particularly difficult to demonstrate in endoparasitoids because these insects develop inside their hosts and, thus, may be exposed to cryptic experiences derived from the host, the host plant or from other environmental cues affecting host properties (Turlings et al. 1992; Godfray 1994; Vet et al. 1995). These experiences may already be integrated in the first behavioural displays of the newly emerged parasitoid, making the dissection of the contributions of larval and early adult experiences very difficult. Some authors have suggested that in parasitic wasps, early adult experience rather than larval experience plays a key role in determining the behavioural plasticity of the adults (i.e. van Emden et al. 1996; Barron and Corbet 1999; Barron 2001). Thus, a clear definition of “naïve” or “innate” responses by newly emerged parasitoids is possible only if specific experiments are designed to assess the effective role of larval experience, if any, in the determination of the adult behaviour (Vet et al. 1995).

Aphidius ervi Haliday (Hymenoptera, Braconidae, Aphidiinae) is an oligophagous endoparasitoid, mainly associated with Macrosiphinae aphids on legumes and cereals (Mackauer and Finlayson 1967; Němec and Stary 1985; Takada and Tada 2000). This species is largely used as an experimental model system, both for behavioural and physiological studies (Powell et al. 1998; Pennacchio and Strand 2006). Learning from experience has been demonstrated for *A. ervi* females in search for host–plant complexes (HPCs), early in ontogeny by chemical legacy (Daza-Bustamante et al. 2002), as well as during adult life, where experiences reinforce the initial HPC preferences through associative learning (Turlings et al. 1992; Powell et al. 1998). Learning in this parasitoid is not limited to host searching. Thus, Villagra et al. (2005) found that young *A. ervi* male parasitoids can learn to respond to an artificial odour cue (vanilla) with a sexual attraction response, if they were trained before to associate the vanilla odour with the encounter with the female. This behavioural flexibility of a trait, which is expected to be rather “innate”, poses a number of intriguing questions about the origin, nature, development and plasticity of behavioural responses towards environmental and mating-related cues in newly eclosed parasitoids.

In this work we address the role of preimaginal and early adult experiences on the behavioural responses of adult males of *A. ervi* toward the odours of HPC and toward the odours of conspecific virgin females. These stimuli correspond to an environmentally derived cue and a mate searching cue, respectively, and are commonly described as fixed and shown to be relatively independent of environmental effects (Ayasse et al. 2001). Larval and early adult (eclosion) experiences were manipulated by rearing

the host aphids on artificial diet and/or by extracting the third instar larvae from the host to let them pupate in an isolated environment (Hérard et al. 1988; Kester and Barbosa 1991).

Materials and methods

Insects Parasitoids were obtained from a laboratory culture maintained on pea aphids (*Acyrtosiphon pisum* Harris) feeding on potted broad bean plants (*Vicia faba* L.). Hosts and parasitoids were maintained in separate environmental chambers, kept at $20\pm 1^\circ\text{C}$, 65% relative humidity, and 14 h L/10 h D photoperiod. For non-manipulative treatments, experimental mummies were detached from the plant surface and isolated in Petri dishes bottom-lined with moistened filter paper. Newly emerged parasitoids had access to water only. To obtain male parasitoids virgin females were used to oviposit the treatment aphids. *Aphidius* parasitoids have haplodiploid sex determination; hence, sex is not determined by the number of chromosome sets, but by heterozygosity at one or several loci. Individuals are male when hemizygous (haploid) or homozygous (diploid) at all sex-determining loci (Godfray 1994; Salin et al. 2004); hence, the use of virgin female ensures that only male offspring will be produced. Virgin females were obtained from laboratory stocks.

Manipulation of parasitoid larval environment The developmental environment of parasitoid larvae was manipulated by rearing the host aphids on artificial diet. Newly born first instar nymphs of pea aphid were reared on artificial diet (AD) treatment, according to Pennacchio et al. (1999), at $20\pm 1^\circ\text{C}$. Third-instar nymphs grown on artificial diet were parasitised by virgin parasitoid females and returned to the liquid diet sachet, under the same environmental conditions indicated above. Aphids maintained on broad bean plants (normal diet [ND] treatment) acted as controls.

Manipulation of parasitoid eclosion environment Fully grown third-instar larvae of *A. ervi* were extracted (EX treatment) from parasitised aphids 7 days after parasitisation at $20\pm 1^\circ\text{C}$, and maintained until pupation at the same temperature in a Petri dish with a 5-mm-thick agar layer. Meconial pellets were immediately separated from freshly formed pupae. For each host diet treatment described above, synchronous cohorts of parasitised aphids were split into two groups, of which only one was used to extract host larvae (EX treatment), while the other was used as control (NE treatment). Mummies from this latter group were transferred to a Petri dish 24 h before the expected emergence, without performing any further manipulation.

The different combinations of the two manipulation treatments of host larval and eclosion environments led to four possible experimental conditions: normal host diet/non-extracted larva (ND/NE), normal host diet/extracted larva (ND/EX), artificial diet/non-extracted larva (AD/NE), and artificial diet/extracted larva (AD/EX).

Olfactometer bioassays Glass Y-tube olfactometers were used to assess the responses of *A. ervi* males when presented with a choice of odours. Olfactometers utilised (9-cm-long tube and 6-cm-long arms at 60° from each other) allowed parasitoids to explore both arms and to move from one arm to the other through the tube. After each test, the olfactometers were cleaned with ethanol, then soaked in odour-free soap and rinsed with distilled water. Pure air was released into the test chambers of the olfactometer from an ultrapure synthetic air cylinder (Indura®). During tests, the air flow was maintained at 250 ml/min, light intensity at 3,600 lx, and temperature at 20±1°C.

The experimental parasitoids were individually released in the central tube of the olfactometer and their behaviour was observed for 5 min. The following behavioural parameters were scored: latency to first choice of olfactometer arm, first choice of arm inside the olfactometer, proportion of time spent in each olfactometer arm, proportions of time spent running, antennating, grooming the abdomen, grooming the antennae and wing fanning. Latency to first choice represents the time that males spent in the main tube of the olfactometer before choosing one of the arms. Running includes the time that males spent in active exploration. Antennation has been described as part of parasitoid foraging behaviour (see Olson et al. 2003). Grooming behaviours have been associated with insect body cleaning and with homogenizing the chemicals present on the body (Sumana and Starks 2004), a behaviour generally associated with mating and social interaction in wasps and other holometabolous insects (Wuellner et al. 2002). Wing fanning has been shown to be an important part of courtship in many insect species including parasitoids (Mackauer 1969; Hirsch and Tompkins 1994).

To compare the behavioural activity of insects reared under different conditions, the level of activity was defined as the number of behavioural transitions displayed by the wasp plus one. Bioassays were video-recorded and behavioural data were collected utilizing the JWatcher software 0.9® (Blumstein et al. 2000).

With the aim to test the effect on male response to HPC of the manipulations of parasitoid larval and eclosion environments, and of the possible interaction between these experimental factors, male parasitoids from ND/NE, ND/EX, AD/NE and AD/EX treatments were tested in the olfactometer, using a pot with soil in the control arm, and the HPC (3-week-old broad bean plants with ca. 100 mixed instar pea aphids) in the test arm.

To test to what extent sexual responses, such as attraction towards female-derived stimuli and wing fanning, could have been modified by host diet, eclosion environment and their possible interaction, male parasitoids from ND/NE, ND/EX, AD/NE and AD/EX treatments were tested in the olfactometer, using six virgin females (no later than 8 h after their eclosion) in the test arm and air-only in the control arm.

Percentages of individuals first choosing the stimulus or control arms of the olfactometer were compared using the McNemar test for two correlated proportions. This analysis is useful to test two dichotomous responses of each individual from each treatment (McNemar 1947; Kateri et al. 2001). Comparisons between different independent groups were performed with the *G* test.

A preference index was calculated for the analysis of the time spent in each arm of the olfactometer and the time spent performing the behaviours described above. This index collapsed the information from each arm into a single variable that corresponded to the difference between the proportion of time spent in the test arm and in the control arm. Attraction towards the experimental cue was denoted by positive values of the preference index, while negative values indicated the occurrence of attraction towards the control arm. Differences between treatments were analysed with a two-way fixed effects multivariate ANOVA (MANOVA) and *post hoc* least significant difference (LSD) test. Wing fanning responses were ranked and then analysed with two-way fixed effects ANOVA and *post hoc* LSD tests. When values for the times spent in the olfactometer arms were not normal, data were transformed using arcsin square root transformation (Sokal and Rohlf 1998).

Results

Response to the odours of the HPC Only males from the ND/NE treatment significantly chose the HPC arm over the control arm at the first encounter with the two choices (McNemar test value = 1.96, $p=0.025$). Neither ND/EX (McNemar test value = -1, $p=0.841$), AD/NE (McNemar test value = 0.22, $p=0.413$), nor AD/EX (McNemar test value = 0, $p=0.5$) treatments showed initial marked preferences towards either arm. Moreover, the *G* test showed a global difference between the treatments ($G_h=17.103$, $p<0.001$), ND/NE being different from all other treatments (ND/EX: $G_h=15.152$, $p<0.001$; AD/NE: $G_h=15.152$, $p=0.05$; AD/EX: $G_h=9.721$, $p=0.021$). No other comparisons were significantly different. These results demonstrated that any modification of pre-adult (host diet or eclosion) early experiences may affect the first decision of the juvenile imago towards the cues of a potential HPC. This is also

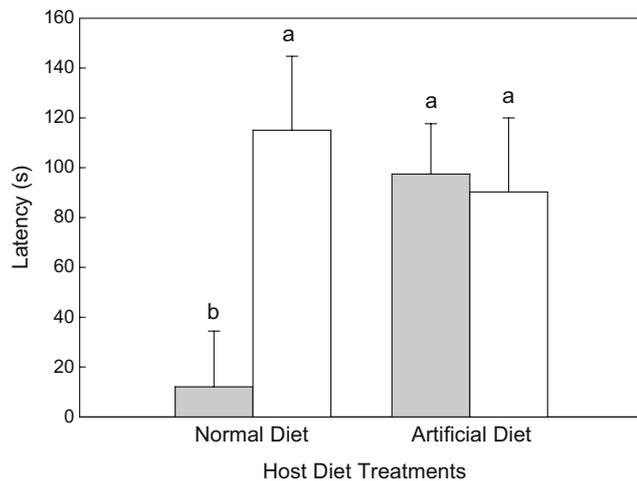


Fig. 1 Latency to first choice, i.e. time (mean with standard error) elapsed until the first preference between Y-tube olfactometer arms by *A. ervi* males offered the choice between odours from the HPC and from soil only (control). Eclosion experience manipulation: *black bars* represent non-extraction of the parasitoid larvae from the host, and *white bars* represent extracted larvae. Different letters represent significant differences ($p < 0.05$) in the *post hoc* test

reflected in latency to choose a side in the olfactometer (Fig. 1, see also Table 1b).

The two-way MANOVA showed no significant global effect of host diet or larval extraction on time spent and behaviours performed in olfactometer arms (Table 1a). However, the interaction of host diet and larval extraction treatments was significant (Table 1a). This result suggests the relevance of the interaction between preimaginal and eclosion experiences in the development of the first responses of the imago.

The MANOVA on the difference of time spent in the stimulus minus the control arms for the different treatments showed the same trend as the global analysis, i.e. only the interaction between host diet and eclosion experience was significant (Table 1b). In the post hoc comparisons, the

ND/NE treatment was significantly different from ND/EX (LSD test, $p = 0.016$), and also from AD/NE (LSD test, $p < 0.001$). ND/NE showed a nonsignificant but biologically relevant difference with respect to AD/EX (LSD test: $p = 0.055$). No other comparisons performed were different enough to be considered (Fig. 2a).

The effects of host diet and eclosion experience on differences in behaviours performed in the HPC and control arms were not significant for antennation, running, grooming the abdomen, grooming the antennae or latency to first choice; however, the interaction of host diet and eclosion experience was significant in the case of antennation (Fig. 3a), running (Fig. 3b), and latency to choice (Fig. 1; statistical results shown in Table 1). As antennation behaviour is related with orientation and searching in parasitoids (see Olson et al. 2003), grooming behaviours are related with interspecific recognition in many hymenopterans (Wuellner et al. 2002), and walking parasitoids display an active searching behaviour by wiping the surface with their antennae (C.A. Villagra, personal observation), the difference found in the times assigned to these behaviours by the males while deciding between HPC and control odours suggests that the host diet and eclosion experience can integrally modify performances related to foraging in parasitoid wasps.

The level of activity did not differ between treatments neither in the factors diet ($F_{1, 73} = 3.370$, $p = 0.070$) nor extraction ($F_{1, 73} = 2.459$, $p = 0.121$), nor in the interaction between both factors ($F_{1, 73} = 2.14 \text{ E-}06$, $p = 0.999$; two-way fixed-effects ANOVA). This result confirms that the different manipulations did not affect the capability of the insects to perform different behaviours.

Response to the odours of virgin females All parasitoids displayed a marked first preference towards the olfactometer arm that is releasing the virgin female volatiles, independent of treatment: ND/NE (McNemar test value = 3.46, $p < 0.001$),

Table 1 Two-way fixed effects MANOVA for behavioural responses exhibited by *A. ervi* males exposed to host plant complex (test arm) and potted soil (control arm) in a Y-tube olfactometer

Effect	Wilk's lambda	Numerator DF1	Denominator DF2	F ratio	P
A. Global effects					
Host diet (D)	0.856	9	65	1.21	0.303
Extraction (E)	0.892	9	65	0.87	0.555
D*E	0.648	9	65	3.93	0.001*
B. Effect of Host diet X Eclosion experience interaction on behavioural responses					
Time in arm	1.555	1	73	5.17	0.037*
Latency to first choice	0.578	1	73	4.53	0.026*
Antennation	0.073	1	73	6.78	0.011*
Running	0.680	1	73	4.82	0.031*
Grooming abdomen	0.000	1	73	0.01	0.917
Grooming antennae	0.011	1	73	3.69	0.058

ND/EX (McNemar test value=2.12, $p=0.016$), AD/NE (McNemar test value=3.32, $p<0.001$), and AD/EX (McNemar test value=2.11, $p=0.017$). This suggests a strong pheromone-guided behaviour not subject to modification by early experiences.

No significant effect of host diet (two-way ANOVA, $F_{1, 42}=2.392$, $p=0.129$), eclosion experience (two-way ANOVA, $F_{1, 42}=0.003$, $p=0.954$) or their interaction was found on time spent in the stimulus minus the control arms (two-way ANOVA, $F_{1, 42}=0.831$, $p=0.367$; Fig. 2b). The differences between proportions of time devoted to wing fanning behaviour showed a significant effect of the interaction between host diet and eclosion experience (two-way ANOVA, $F_{1, 42}=4.56$, $p=0.038$), but no significant effect of diet (two-way ANOVA, $F_{1, 42}=1.16$, $p=0.287$) nor extraction (two-way ANOVA, $F_{1, 42}=0.035$, $p=0.852$) by themselves. Parasitoids from the ND/NE treatment displayed

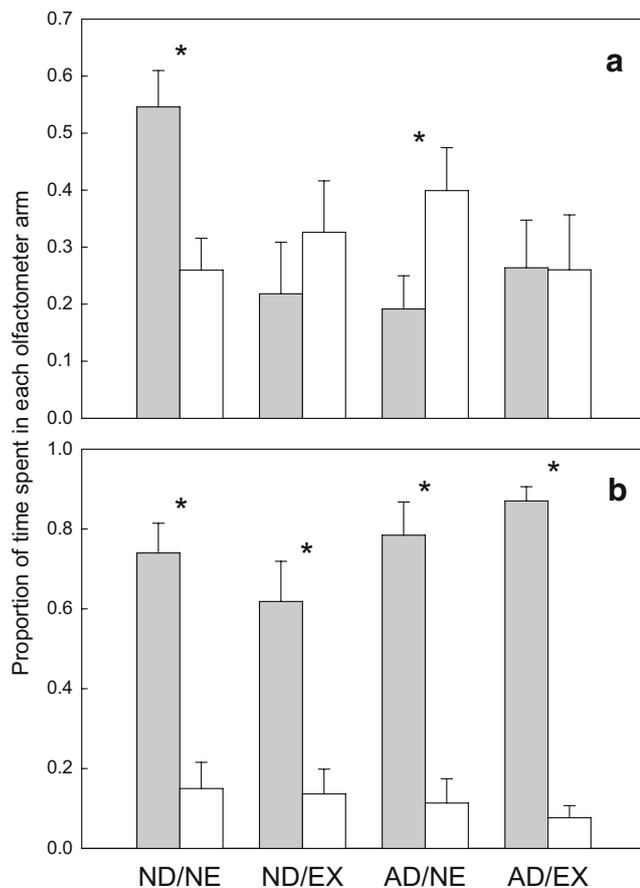


Fig. 2 Proportion of time (mean with standard error) spent in each arm of the Y-tube olfactometer by *A. ervi* male parasitoids offered: **a** the choice between host plant complex and soil-only control odours; and **b** the choice between the odours from six virgin females and air-only control. Treatments are as follows: ND=normal diet; AD=artificial diet; NE=non-extracted larvae; EX=extracted larvae. Grey bars represent proportion of time in the stimulus arm, and white bars represent proportion of time in the control arm. Asterisks represent significant differences ($p<0.05$) in the *post hoc* test

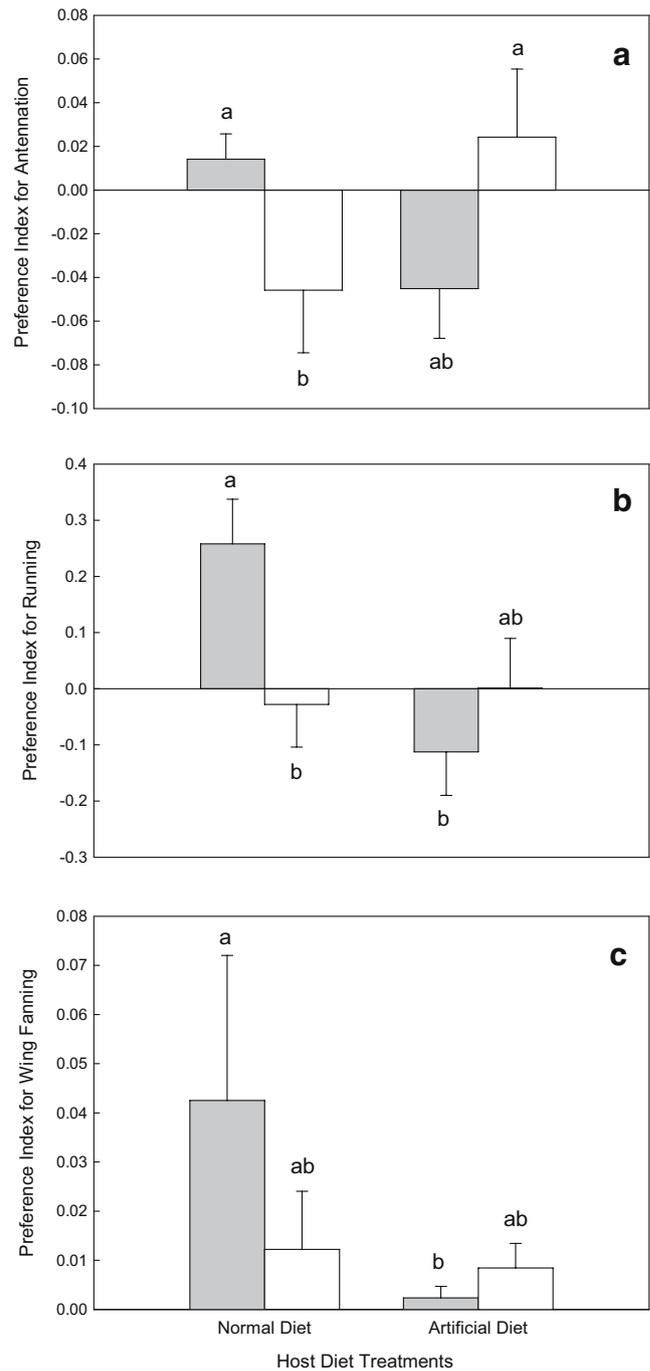


Fig. 3 Differences (mean with standard error) between the proportions of time spent in each arm of the Y-tube olfactometer performing antennation (**a**), running (**b**), and wing fanning (**c**) behaviours by *A. ervi* male parasitoids offered. **a** and **b**: the choice between host plant complex and soil-only odours; and **c**: the choice between the odours from six virgin females and air-only control. Eclosion experience manipulation: grey bars represent non-extraction of the parasitoid larvae from the host, and white bars represent extracted larvae. Different letters in each graph represent significant differences ($p<0.05$) in the *post hoc* test

significantly more wing fanning inside the olfactometer arm containing the conspecific females than those from the AD/NE treatment (post hoc LSD test, $p=0.03$). Extraction did not significantly affect this behaviour (Fig. 3c). Thus, even though attraction towards the female parasitoid pheromone (reflected in time spent in arms) is not affected by diet and extraction treatments, the specific courtship behaviour (wing fanning) is more sensitive to such early experience manipulations.

Discussion

Males from the non-manipulated treatment (ND/NE) displayed the shortest latency to the first choice between odours from the rearing environment (HPC) and control odours, and showed a marked preference towards the odours from the HPC. Male behavioural responses towards the rearing host plant complex were modified by the interplay of host diet environment and eclosion experience (ND/NE compared to ND/EX, AD/NE and AD/EX treatments), as shown by the significant interaction of these two factors in the global effects MANOVA (Table 1), and the significant effects found in latency to first choice, time in arm, antennation and running behaviours (Figs. 1, 2 and 3). These results suggest a role for the integration of experiences faced during preimaginal and emergence periods in the behaviour of an adult holometabolous insect, as has been documented by other authors (i.e. Gandolfi et al. 2003).

Our results also show that neither extraction of the larva nor change in the host diet alone generated relevant changes in the time spent by males in the olfactometer arm with virgin female odours (Fig. 2b), suggesting the existence of a female volatile pheromone that strongly attracts males, as has been suggested by McNeil and Brodeur (1995) for *Aphidius nigripes* and by Battaglia et al. (2002) for *A. ervi*. However, the wing fanning courtship behaviour was more promptly and intensely exhibited by parasitoids from the ND/NE treatment than by those from the AD/NE treatment (Fig. 3c). This latter result suggests the relevance of rearing environment on the triggering of courtship behaviour of males towards receptive females. However, it is not possible to exclude the possibility that the reduced quality of the host reared on artificial diet may have affected the parasitoid vigour and its capacity to react more promptly to sexual cues, as has been demonstrated for other insect species. For example, in the oriental fruit moth, *Grapholita molesta* (Lepidoptera: Tortricidae), when males were reared on artificial diets or were starved as adults the concentration of the most active male pheromone component was altered and female courtship behaviour was negatively affected (Löfstedt et al. 1989). In synthesis, our experiments show that larval and eclosion experiences affect in a differential

way the early responses of the adult towards environment-derived cues and mate-related cues.

The relevance of early sensitive stages in parasitoid learning not only is related to the acquisition of the capability for attraction towards olfactive cues derived from the HPC rearing environment (Kester and Barbosa 1991), but also towards an array of environmental cues presented during parasitoid adult life. Studies of male courtship behaviour by Villagra et al. (2005) further reinforce this idea, by demonstrating that adult males are capable of learning a novel cue (vanilla) by associating it with copulation as an anchoring experience. Based on these results, it seems that anchoring is produced by a given sequence of experiences during larval and adult stages. Thus, not all behaviours of parasitoids are derived from learning, but rather the responses are generated by an ontogenetic process of behavioural development (Lehrman 1970), with emergent traits being built into pre-existing cognitive conditions (Schneirla 1956; Bonner 1988; Wcislo 1989). It would be desirable to design finer-grain experiments with the aim to define more precisely when experiences of the larva are able to influence adult behavioural responses to HPC and conspecifics.

Innate behaviour is usually defined as early responses under “natural conditions”. However, the level of “innateness” that could be attributed to a given response also depends on the nature of the stimulus presented (compare Fig. 3a and b with c). In our system, the response towards the HPC showed more plasticity than that towards conspecific females, supporting the idea of the existence of different degrees of “innateness” for initial responses (Papaj and Lewis 1993). Hence, the general attraction of males to female volatiles (Fig. 2b) seems to be highly canalized (sensu Tierney 1986). However, it is worth noting that male courtship behaviour, i.e. wing fanning, seems to be influenced by early life environment, as males displayed more wing fanning in the ND/NE treatment than in the other treatments, although this tendency was significant only with respect to AD/NE males most likely due to the high variability of the data (Fig. 3c).

Notwithstanding the relevant role of genetics in insect behavioural development, the term “innate” does not seem fully informative for denoting behavioural responses, which may develop before emergence of an adult holometabolous insect. This is supported by the growing literature that demonstrates the existence of different degrees of conservation of memory through metamorphosis (Borsellino et al. 1970; Carlin and Schwartz 1989; Tully et al. 1994; Rietdorf and Steidle 2002; Gandolfi et al. 2003), and the clear evidences of early adult learning of surrounding environmental cues at emergence (Caubet and Jaisson 1991; Barron and Corbet 2000; van Emden et al. 2002). It would be more appropriate to recognise that many insect behav-

joural traits may be the result of continuous interactions between genes and environment, which may determine the necessary functional adjustments at any developmental stage. In other words, behaviour develops, and the degree of its plasticity varies with the type of response, and in relation to the predictability/stability of the external cues to which the animal has to react. On these premises, the term “initial response” seems more informative than “innate response”. Furthermore, it is evident that our knowledge of the neurophysiological bases of behavioural plasticity in insects is very limited and the research activities in this field are still at their infancy.

The study of parasitoid initial responses as affected by larval and early adult experiences may help to explain some characteristics of the parasitic life style. The environment where the parasitoid larva develops is strictly determined by the preferences of the ovipositing adult female. Thus, if larval and early adult experiences can affect behavioural preferences of adults, this will imply a transgenerational reiterated effect of environmental cues (Corbet 1985; West and King 1987; Bateson 1988), which may lead to the inheritance of environmental preferences (Rossiter 1996; Maturana and Mpodozis 2000) as a consequence of the functional link between early learning and parental behaviour (Bernardo 1996; Wcislo 1989). This possibility was already suggested to explain in part how some specific populations of parasitoids can specialize and strictly link their life cycle to hosts, which represent a predictable resource over space and time (i.e. a perennial plant infested by a monophagous species), which may provide a stable ecological niche promoting natural population splitting and speciation (Pennacchio and Tremblay 1989; Pennacchio et al. 1994). However, if and how behavioural changes during ontogeny are related to the maintenance of parasitoid host range and specificity largely remains to be experimentally substantiated.

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