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Host activity and wasp experience affect parasitoid wasp foraging behaviour and oviposition on nematode-infected larvae of the forestry pest *Hylobius abietis*

CHRISTOPHER D. HARVEY and CHRISTINE T. GRIFFIN Department of Biology, National University of Ireland Maynooth, Co. Kildare, Ireland

Abstract. 1. Entomopathogenic nematodes (EPN) are currently being used as introduced biological control agents against the larvae of the native European forestry pest *Hylobius abietis* L. which develop under the bark of stumps and roots of newly dead conifer trees.

2. The potential for resource competition between gregarious ectoparasitoid *Bracon hylobii* Ratz and EPN by recording oviposition and related behaviours of *B. hylobii* females on EPN-infected *H. abietis* larvae was investigated. Wasps did not parasitise EPN-infected host larvae that were dead when presented, but naïve and experienced wasps parasitised live EPN-infected hosts. Naïve wasps parasitised live EPN-infected hosts significantly less frequently than healthy hosts only when the infected larvae were close to death (i.e. died during 24-h trial). Parasitism by experienced wasps was unaffected by host infection.

3. Wasp probing and oviposition were positively associated with the amount of host movement. Preventing *H. abietis* larvae from chewing on bark significantly reduced parasitism by naïve, but not experienced wasps.

4. The number of eggs per clutch was not affected by bark chewing or EPN-infection of *H. abietis* larvae.

5. Naïve and experienced *B. hylobii* parasitised two abnormal hosts (larvae of coleopteran *Rhagium bifasciatum* Fabricius and lepidopteran *Galleria mellonella* L.), both of which moved and chewed on bark during trials.

6. It was concluded that *B. hylobii* can use vibrational cues generated by host movement and feeding to locate hosts at short range and accepts unsuitable (EPN-infected or abnormal) hosts as long as these create such cues. The implications for competition between *B. hylobii* and EPN and possible ways of minimising it when applying EPN are discussed.

Key words. Biological control, clutch size, competition, entomopathogenic nematodes, *Heterorhabditis*, host location, pathogen–host interaction, *Steinernema*.

Introduction

Insects can be host to parasitoid wasps and entomopathogens that occur in their environment naturally or that have been introduced for biological control purposes. In situations where they occur together, competition between parasitoid wasps and entomopathogens may arise either as a result of direct infection of wasps with the pathogen (intraguild predation) or, if the shared host does not sustain wasp offspring when infected with the pathogen, via competition for reproductive resources (Kaya *et al.*, 1978; Rosenheim *et al.*, 1995; Shannag & Capinera, 2000; Sher *et al.*, 2000; Lacey *et al.*, 2003; Mbata & Shapiro-Ilan, 2010). The brood of parasitoids must rely on the mother to find and choose suitable hosts for oviposition. Depending on the progression of infection in the host, some parasitoids seem not to distinguish between healthy

Correspondence: Christopher D. Harvey, Department of Biology, National University of Ireland Maynooth, Co. Kildare, Ireland. E-mail: utopian@gmx.net

and pathogen-infected hosts (Hoch & Schopf, 2001; Lord, 2001; Down *et al*., 2005), although rejection or avoidance of host larvae infected by entomopathogenic nematodes (EPN) has been reported for a number of parasitoids of cryptic hosts (Sher *et al.*, 2000; Head *et al.*, 2003; Lacey *et al.*, 2003). In most of the latter cases it is not clear, however, if wasps located and then did not oviposit on infected hosts or failed to locate them in the first place. The latter may be the case if pathogen infection suppresses or alters the cues the host insect emits, including vibrational cues generated by host movement and feeding and/or volatiles, all of which may facilitate short-range location of cryptic hosts by parasitoids (Meyhöfer *et al.*, 1999; Wang & Yang, 2008). Competition between an entomopathogen and a parasitoid wasp may arise when one or both of these agents are used to control a pest insect (Lacey *et al.*, 2003; van Lenteren *et al.*, 2003; Everard *et al.*, 2009). When predicting the risk of competition between entomopathogens and parasitoids in this and other situations, information on the foraging behaviour of female wasps and any cues they require for host location may therefore be of great benefit. Moreover, as parasitoids are thought to become more efficient foragers (for example via associative learning) once they have successfully parasitised one or more hosts, experience may significantly affect how they respond to pathogen-infected hosts (Du *et al.*, 1997; Vinson, 1998; Meiners *et al.*, 2003).

The infective stage of EPN, the infective juvenile (IJ) enters insects via the cuticle or natural openings and releases insect-pathogenic bacteria (*Xenorhabdus* in *Steinernema* spp. and *Photorhabdus* in *Heterorhabditis* spp.) that frequently kill insects within 48 h of infection by causing toxaemia and/or septicaemia (Kaya & Gaugler, 1993), although it may take several days longer in some insects, including larvae of the large pine weevil, *Hylobius abietis* L. (Ennis, 2009). Insects infected with EPN move less vigorously and may reduce their feeding rate before dying (Alchanatis *et al*., 2000). In laboratory assays, EPN display a wide host range. For example, *Steinernema carpocapsae* Weiser infected insects from 250 species and 75 families across 11 orders in the laboratory (Poinar, 1979). However, in the field the host range of EPN is more limited (Peters, 1996; Gaugler *et al.*, 1997). As a result of their safety for vertebrates, relative ease of production and their potentially wide host range, EPN have become important inundative biological control agents for use against several insect pests (Gaugler *et al*., 1997; Georgis *et al*., 2006; Shapiro-Ilan *et al*., 2006). Entomopathogenic nematodes, like other biological control agents, should be evaluated with respect to the risk they pose to non-target insects (van Lenteren *et al*., 2003). Adverse effects on parasitoids of the targeted pest are particularly undesirable because they reduce the controlling effect these parasitoids already have on the pest population.

We investigated whether *Bracon hylobii* Ratz, a gregarious ectoparasitoid that is one of the main natural enemies of *H. abietis* (Kenis *et al*., 2004; Everard *et al*., 2009) parasitises hosts infected with two distantly related EPN species. *Hylobius abietis* is a major forestry pest native to Northern Europe, with expected annual damages of 140 million Euros if not controlled (Långström & Day, 2004). *Steinernema carpocapsae* is currently being inundatively applied to conifer stumps on clearfell sites in Ireland and the UK to control the immature stages of *H. abietis* (Kenis *et al.*, 2004; Brixey *et al.*, 2006; Dillon *et al.*, 2007). *Heterorhabditis downesi* Stock, Griffin and Burnell, has shown superior potential for this use in field trials (Dillon *et al*., 2006, 2007). Neither of these two EPN species is known to occur in forests in Ireland and the UK, or to cause natural mortality in *H. abietis* (Kenis *et al*., 2004). Larvae of *H. abietis* feed and develop under the bark of conifer stumps for 1–3 years before pupating (Leather *et al.*, 1999). In field trials, EPN IJs applied to lodgepole pine at a rate of 3.5 million IJs per stump infected between 15% and 30% (*S. carpocapsae*) and 50–60% (*H. downesi*) of developing *H. abietis* larvae under the bark within 2 weeks of application (Dillon *et al.*, 2006). When applied on an operational level from a forwarder-mounted tank, *S. carpocapsae* reduced emergence of *H. abietis* by up to 70% compared with untreated stumps (A. Dillon and C. Griffin, unpublished).

Bracon hylobii is found across most of the host distribution range and often exerts considerable natural control of *H. abietis*, with parasitism rates of 20% or more reported (von Waldenfels, 1975; Gerdin, 1977; Henry, 1995; Dillon *et al*., 2008). The only other known host for *B. hylobii* besides *H. abietis* is *Pissodes* spp. (Kenis *et al*., 2004). In the laboratory, a *B. hylobii* female can lay egg clutches on up to 17 hosts. The first clutch usually contains between 20 and 30 eggs and clutch size then tends to decrease with each subsequent host attacked (Henry & Day, 2001; Everard *et al.*, 2009).

Short-range host location by *B. hylobii* has not been investigated in detail, although there is some evidence that females respond to volatiles produced by feeding hosts (Faccoli & Henry, 2003). Once a host is located, the wasp penetrates the bark with its ovipositor. *Bracon hylobii* is an idiobiont parasitoid: the host is injected with paralysing venom before oviposition and generally ceases to move or feed as a consequence, although it does respond with movement when prodded (Henry, 1995; C. Harvey, pers. obs.).

To what extent competition for host resources occurs between EPN and *B. hylobii* depends in part on whether or not the wasp parasitises infected *H. abietis* hosts (Everard *et al*., 2009), which in turn is determined by wasp foraging behaviour and host acceptance. Everard *et al.* (2009) reported that *B. hylobii* parasitised larvae of *H. abietis* infected with *H. downesi* less frequently than healthy hosts as early as 12 h after infection when given a choice between the two. However, it is unclear if wasps were actively rejecting infected hosts after locating them or if infected hosts were located less frequently than healthy hosts. Moreover, as the wasps used by Everard *et al.* (2009) were naïve, the possible effects of wasp experience on parasitism of infected hosts were not investigated. To address these questions, our three main objectives in the present study were to: (i) test whether *B. hylobii* parasitises infected hosts less frequently than healthy hosts when offered only one host at a time (no choice); (ii) investigate the effect of wasp experience on parasitism of healthy and EPN-infected hosts; and (iii) identify host cues used by foraging *B. hylobii* females to locate and accept hosts

at a short range, with the aim of interpreting altered responses to EPN-infected hosts.

Materials and methods

Source, storage and culture of nematode and insects

Bracon hylobii were collected from stumps of pine on clear-fell sites located in Ireland (site information in Table S1) as cocoons and cultured in the laboratory on *H. abietis* larvae as described previously by Everard *et al.* (2009). To obtain females for experiments, cocoons were transferred to 50-ml plastic tubes with ventilation holes in the lid and incubated at 20 °C. Eclosed female and male wasps were fed on 50 : 50 honey/tap water solution and were kept together for 5 days to allow mating. Naïve wasps were 5 days old when used in trials. Experienced wasps had parasitised a single *H. abietis* larva that they had been offered for 24 h when 5 days old in an arena as described below. They were kept in this arena with food but without further access to males and were transferred to the experimental arena at 7 days of age. Late instar *H. abietis* larvae and larvae of *Rhagium bifasciatum* Fabricius were collected from clearfell sites and stored at 9 °C in 24-well plates (12.7 × 8.6 × 2.7 cm; Costar; Corning Inc.) lined with moist tissue paper. Waxmoth larvae (*Galleria mellonella* L.) were supplied by The Mealworm Company (Sheffield, UK) and stored at 15 °C. Only host larvae with a mass > 150 mg were used in trials. Hosts used in experiments 2 and 3 were incubated at room temperature for 2 h immediately before trials began.

Infective juveniles of *S. carpocapsae* (strain US-S-25) and *H. downesi* (strain K122) were cultured at 20 °C in final instar larvae of *G. mellonella* (Kaya & Stock, 1997). Emerging IJs were collected within 1 week of the onset of emergence and washed three times by allowing them to settle in tap water. Infective juveniles were stored in tap water at 9 °C for 1–4 weeks before they were applied to insects.

Arena used for experiments and culturing

Bracon hylobii females were offered a single host insect in a closed arena (no-choice) similar to that described previously by Everard *et al*. (2009). Arenas were 9 cm in diameter and 2 cm high made from two Petri dish bases taped together. A host larva was placed in a 0.9-cm-diameter chamber in a perspex slide (7.5 cm long by 3 cm wide, 4 mm thick) taped to a standard glass microscopy slide (Menzel GmbH, Braunschweig; Germany). After each use, slides were disassembled and washed thoroughly in 70% ethanol and then tap water.

A 2 × 2 cm patch of Sitka spruce (*Picea sitchensis*) bark approximately 1–2 mm thick was stripped from a log immediately before use (logs stored at 4 °C for no more than 2 months) and was placed over the chamber containing a host and fastened to the slide along all four edges with tape, creating an exposed bark area of approximately 1.5 × 1.5 cm (2.25 cm^2) that represented the host microhabitat available to the wasp for foraging. Wasps therefore had to penetrate the bark to oviposit on a host in the chamber below. A piece of filter paper (1 cm diameter, Whatman No. 1;Whatman, Maidstone, UK) saturated with a 50 : 50 honey/tap water solution was placed in the arena and a wasp was introduced via a 1-cm-diameter hole in the top of the arena. The hole was covered with masking tape and wetted tissue paper was placed on top to maintain humidity in the arena.

Host insects were weighed before being introduced into trials. Trials were carried out in a climate room at 20 °C and under constant illumination as *B. hylobii* females are inactive in the dark (Henry & Day, 2001; Everard *et al*., 2009). Trials were conducted over a 1-year period (June 2009 to June 2010) according to availability of *B. hylobii* females and hosts. Controls (live *H. abietis* larvae) were included in each set of trials conducted at any one time. Trials with naïve and experienced wasps were carried out for all treatments unless otherwise stated. At the end of the trials, the presence and number of *B. hylobii* eggs and whether or not the host had chewed on the underside of the bark patch during the trial was recorded.

Experimental design

Possible host-location cues for *B. hylobii* are: host and substrate volatiles; volatiles released from the substrate by chewing of the bark substrate; vibrations owing to host body movements; and vibrations as a result of bark chewing by the host. The combination of host cues investigated and the hosts presented to wasps in each of the three experiments we conducted are described in Table 1.

Experiment 1: parasitism and insect behaviour in trials with EPN-infected H. abietis *larvae alive at the start of the trial.* This experiment was designed to test whether naïve and/or experienced *B. hylobii* would parasitise EPN-infected *H. abietis* larvae when not offered a healthy host as an alternative and how EPN-infection affected parasitism. Hosts were either healthy (control) or EPN-infected *H. abietis* larvae. Larvae were infected by exposing each one individually to 6000 IJs (*S. carpocapsae* or *H. downesi*) for 48 h before the experiment. Infective juveniles were delivered in 100 µl of tap water in a well of a 24-well plate lined with three layers of filter paper (Whatman No. 1). Hosts were then washed three times in tap water to remove adhering IJs. Controls for this experiment were live *H. abietis* larvae treated the same as infected hosts but exposed to 100 µl of tap water only. Infected hosts were alive when introduced into the trial arena. Mortality of hosts owing to EPN infection was scored at the end of the trials, as evidenced by characteristic cadaver colouration (cream for *S. carpocapsae*, orange to brown for *H. downesi*) and a lack of a response to prodding with forceps. Larvae alive at the end of trials were placed in 24-well plates and incubated at 20 °C for 5 days. Larvae that died of EPN infection during this period were classified as EPN-infected larvae that had survived trials, whereas larvae that were alive after 5 days were excluded from analysis. Thus, data were assigned to five *post facto* treatment groups for analysis: control (healthy *H. abietis* larvae), infected

Table 1. Short-range host location cues (volatile and/or vibrational) investigated in each of the three experiments conducted for the present study.

Host location cues	Host presented	Experiment
Host and bark volatiles, host vibrations	Healthy *H. abietis* larvae	1,2,3
Host vibrations and bark volatiles but putatively abnormal host volatiles/chemistry	*H. abietis* larvae infected with *S.carpocapsae* or *H. downesi*	1
	Hosts not known to be associated with *B. hylobii*: *Rhagium bifasciatum* F. (Coleoptera: Cerambycidae) *Galleria mellonella* L. (Lepidoptera: Pyralidae)	2
Host and bark volatiles only	Healthy *H. abietis* larvae that were immobilized (prepupal, paralyzed or dead)	2
Host and bark volatiles, host vibrations	*H. abietis* larvae that were prevented from chewing on bark, some supplemented with wood shavings as a source of volatiles	3

larvae that died of infection with *S. carpocapsae* or *H. downesi* during trials and larvae that were infected with *S. carpocapsae* or *H. downesi*, but survived trials.

Experiment 2: parasitism and insect behaviour in trials with H. abietis *larvae dead from the beginning of the trial and with abnormal hosts.* This experiment was conducted to test whether naïve and/or experienced *B. hylobii* females would parasitise dead or otherwise abnormal *H. abietis* larvae or hosts other than *H. abietis*. Hosts for this experiment were: *Control:* healthy *H. abietis*. *EPN-killed*: *H. abietis* killed by *S. carpocapsae* or *H. downesi*. These hosts were infected with EPN as described above and had been dead for 24–48 h at 20 °C. *Freeze-killed: H. abietis* larvae frozen at −20 °C (no less than 15 min) and then thawed at room temperature for 2 h before use. Death of freeze-killed larvae was confirmed by prodding them with forceps before and after trials. *Paralysed: H. abietis* larvae that had been parasitised and thus paralysed by a *B. hylobii* female within the 24 h preceding a trial. These hosts were washed in tap water before use to remove eggs and any possible scent or marking associated with the first parasitism. *Prepupae: H. abietis* larvae that were in the transitional stage between the final larval instar and the pupal instar (still in larval cuticle with no distinct formation of pupal morphological structures such as wings or legs). No trials with experienced wasps were conducted for prepupal hosts. *Abnormal hosts:* Trials included two host species not known to be associated with *B. hylobii* (Kenis *et al*., 2004). Larvae of *R. bifasciatum* are saproxylic and commonly found in deadwood on Irish clearfell sites, whereas *G. mellonella*, a lepidopteran, lives in bee hives. We also included trials with an empty host chamber ('empty') for naïve and experienced wasps.

Experiment 3: parasitism of H. abietis *larvae that were prevented from chewing the bark.* This experiment was designed to test the hypothesis that vibrational cues generated by *H. abietis* larvae chewing on bark increase the likelihood of short-range host location and parasitism by *B. hylobii*. Hosts were prevented from chewing by applying a drop of super glue (approximately 20 µl; B&Q, Eastleigh, England) to their mandibles. Therefore, the control for this experiment consisted of *H. abietis* larvae with a similar-sized drop of glue placed on the back of the head capsule. The glue was allowed to set for 15 min and control hosts were checked for the ability to freely articulate the mandibles before trials. Hosts with glued mandibles were offered to wasps in two different contexts: either covered by a bark patch as normal or, to provide putative volatiles released by the chewed bark, in a chamber that contained approximately 25 mg of *Hylobius*-chewed bark shavings and was covered by a bark patch the undersurface of which had been chewed by a *H. abietis* larva previously. Bark shavings and chewed bark patches were produced by allowing a *H. abietis* larva not used in experiments to chew a bark patch for 2–6 h before a trial. At the end of the experiment, control hosts were scored for whether or not they had chewed on the undersurface of the bark patch during the trial, and were grouped accordingly for data analysis. Thus, there were four *post facto* treatment groups: control + chewing, control − chewing, glued + chewed bark and glued − chewed bark.

Measurement variables for wasp and host behaviour

To investigate the effect of host movement as a potential host location cue on the foraging behaviour of naïve and experienced wasps, hosts and wasps in experiment 1 and in some trials of experiment 2 were observed for 2 h. Observations began 5 min after a wasp had been introduced into its arena to give wasps time to adjust to the new surroundings after being transferred. Each wasp was observed for 5 s every 5 min and its behaviour was noted, resulting in 24 records of behaviour for each trial (instantaneous behavioural observation).

Two wasp behaviours were recorded, *probing* and *oviposition*. *Probing* was defined as the wasp touching the tip of its ovipositor to or pushing it through the bark patch that represented the host microhabitat. It was also noted whether the wasp was *probing* directly over the host chamber or elsewhere on the bark patch. If a wasp *probed* in one location over the host for a prolonged period of time (four consecutive records; ≥ 20 min) and it assumed a posture characteristic of *oviposition* (ovipositor usually thrust deep into bark, contracting abdomen that curved toward the bark) and/or if eggs were visible in the host chamber, this was recorded as an instance of *oviposition* commencing during the observation period.

Immediately after the behaviour of a wasp had been recorded, the respective arena was gently lifted and the host was observed through the base of the arena for 5 s. For hosts, it was only recorded whether or not they moved; this included any movement of the head or body, including body contractions, during the 5-s observation period for each record. Lifting the arena had no apparent effect on wasp behaviour (i.e. wasps were never observed to abort *probing* or *oviposition* in response to it). As *B. hylobii* paralyse *H. abietis* larvae when ovipositing, in those trials in which a wasp intiated *oviposition* during the observation period, only records for host movement taken before the beginning of *oviposition* were taken into account for data analysis. For each observed trial, the percentage of records with host movement out of the total number of observational records before wasp *oviposition* was calculated, resulting in a percentage value for each trial. Trials in which wasps had already initiated *oviposition* when observation began were not included in analysis of data with regards to host movement. *R. bifasciatum* and *G. mellonella* larvae were not susceptible to *B. hylobii* venom and all movement data were included in analysis for these hosts.

Statistics analysis

Statistical analysis was carried out with MiniTab Release 15 (MiniTab Solutions, Coventry, UK). To test for differences in parasitism or insect behaviour among treatments in experiments, binomial data for the binary variables of parasitism by wasps and bark chewing by hosts at the end of a trial as well as the incidence of *probing* and *oviposition* by wasps during the 2-h observation period was compared using Pearson's χ^2-test. Likewise, to test for an effect of host movement on host location by wasps, Pearson's χ^2-test was used to compare binary location data for wasp *probing* records (i.e. wasp either *probing* over host or elsewhere on bark patch) as grouped by the percentage of records with host movement in the respective trial (four groups: 0–25%, 26–50%, 51–75%, 76–100%). For 2×2 contingency tables where at least one expected cell count was < 5, Fisher's exact test (FEt) was used. Up to two cells with expected counts < 5 were accepted for χ^2-tests on contingency tables for all five treatments from experiment 1.

To test whether wasp *oviposition* on live *H. abietis* was affected by the amount of host movement in trials, the number of trials in which *oviposition* occurred during observation (out of the total number of trials) was regressed against the mean of the percentage of records per trial with host movement. Treatments from experiment 1 and 2 with a *H. abietis* host alive at the beginning of trials were included in this analysis (i.e. two data sets for controls, four for EPN-infected hosts and one each for prepupal and paralysed hosts). A binary logistic model in the event/trial format with a Gompit link function was used for regression (validity of link function confirmed by Pearson's Goodness-of-Fit test, $P > 0.05$).

Continuous data for host mass were tested for normality (Anderson–Darling method; $\alpha = 0.05$) and for equal variance (Levene's test; $\alpha = 0.05$) and were compared using a one-way ANOVA with Tukey's post-hoc test ($\alpha = 0.05$) to test for differences in host mass among treatments in experiments. Normally distributed data for egg clutch size were analysed using two-way ANOVA with wasp experience and treatment as predictors to test for egg clutch size adjustment by *B. hylobii* in response to different host types. The distribution of residuals was tested for normality (Anderson–Darling method; $\alpha = 0.05$). Non-normal continuous data for host mass and the percentage of records with host movement (to test for differences in movement among host types) were tested for differences among treatments using the Kruskal–Wallis test (KWt) for comparison of multiple treatments and the Mann–Whitney U-test (MWUt) for comparisons of medians between two treatments.

Comparisons between two data sets were only performed where $n \geq 6$ for each. Significance levels of multiple

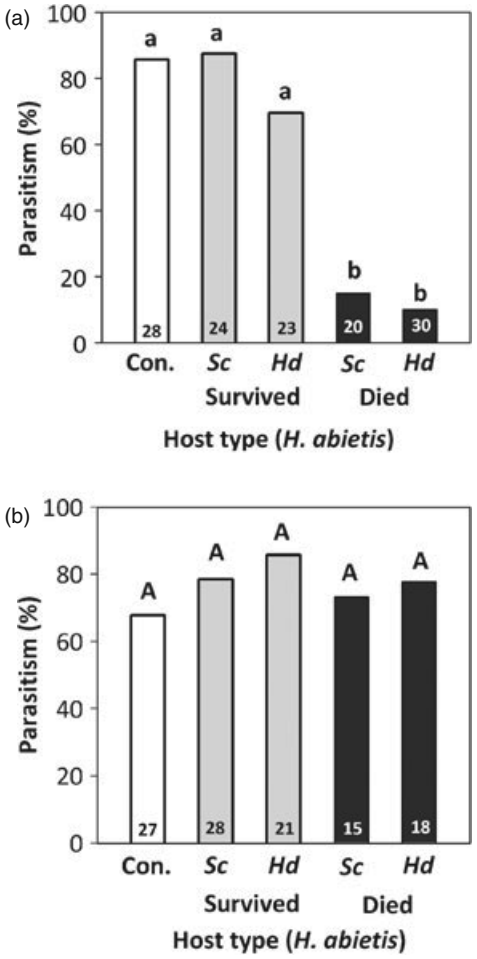

Fig. 1. Parasitism of healthy (Con. = control) or entomopathogenic nematodes (EPN)-infected *Hylobius abietis* larvae (*Sc* = *S. carpocapsae*; *Hd* = *H. downesi*) by naïve (a) and experienced (b) *Bracon hylobii*. Survived = larvae that survived the 24-h trial but died of EPN infection within the 5 days thereafter, died = larvae that died during the trial. Numbers inside bars give n. Within each graph, bars sharing the same letter are not significantly different from each other (χ^2-test or Fisher's exact test; $P < 0.05$; significance level sequentially corrected after Holm–Bonferroni, different case letters used to distinguish tests conducted for each graph).

comparisons performed on parasitism, *probing*, *oviposition* and host movement data were adjusted for type-I family error rate after the Holm–Bonferroni (H-B) sequential P method for the number of comparisons that each data set was included in (Holm, 1979; Rice, 1989). In this method, the significance levels for n comparisons involving the same data set are adjusted to $0.05/n$ for the lowest P-value out of all comparisons, then adjusted to $0.05/(n-1)$ for the second lowest P-value and so on (the highest P-value obtained must therefore be < 0.05 to be considered significant). The adjusted significance level is given in the text where it was decisive for significance of test results.

Results

Experiment 1: parasitism and insect behaviour in trials with EPN-infected H. abietis *larvae alive at the start of the trial*

Both naïve and experienced wasps parasitised EPN-infected *H. abietis* larvae (Fig. 1a,b). For naïve wasps a significant effect of EPN infection on parasitism was detected ($\chi^2_4 = 59.970$, $P < 0.001$; Fig. 1a). This effect was as a result of the low rate of parasitism of infected hosts that died during trials, a rate significantly lower than for infected hosts that survived trials (*S. carpocapsae*: $\chi^2_1 = 23.127$, $P < 0.001$; *H. downesi*: $\chi^2_1 = 20.085$, $P < 0.001$). Infected hosts that survived trials were parasitised at a rate similar to the control (*S. carpocapsae*: FEt: $P = 1$; *H. downesi*: FEt: $P = 0.19$). There was no significant effect of host infection on parasitism by experienced wasps ($\chi^2_4 = 2.577$, $P = 0.631$). Experienced wasps parasitised hosts that died of infection during trials more frequently than naïve wasps did (*S. carpocapsae*: $\chi^2_1 = 12.153$, $P < 0.001$; *H. downesi*: $\chi^2_1 = 22.595$, $P < 0.001$).

Egg clutches laid by naïve wasps contained more eggs than those laid by experienced wasps (two-way ANOVA; $F_{1,149} = 12.97$, $P < 0.001$; Table 2), but treatment did not affect clutch size ($F_{4,149} = 0.96$, $P = 0.43$) and there was no interaction between the two factors ($F_{4,149} = 1.09$, $P = 0.37$). Host mass was similar among treatments (data not shown; KWt,$H^{204}_9 = 8.01$, $P = 0.53$).

Probing incidence was affected by EPN infection for naïve wasps ($\chi^2_4 = 9.557$, $P = 0.049$) and was lowest in trials with infected hosts that died during trials (Table 2). EPN infection had no effect on *probing* by experienced wasps ($\chi^2_4 = 7.508$, $P = 0.11$; Table 2). *Probing* incidence in trials with healthy *H. abietis* larvae (control) was higher for experienced wasps than it was for naïve wasps ($\chi^2_1 = 6.025$, $P = 0.014$) and a similar but usually non-significant trend was seen in all other treatments (χ^2-test, $P > 0.05$ for all treatments except *H. downesi*-infected larvae that died during trials; Table 2).

EPN-infection affected frequency of *oviposition* by naïve wasps ($\chi^2_4 = 15.866$, $P = 0.003$; Table 2); naïve wasps *oviposited* less frequently in trials with hosts that died of infection during trials than they did in trials with infected hosts that survived trials [*S. carpocapsae*: $\chi^2_1 = 4.546$, $P = 0.03$ (H-B adjusted significance level $\alpha = 0.025$); *H. downesi*: $\chi^2_1 = 9.134$, $P = 0.003$]. *Oviposition* by experienced wasps was not affected by EPN infection ($\chi^2_4 = 3.807$, $P = 0.43$; Table 2). Again, a trend was seen in all treatments for experienced wasps to oviposit more frequently than naïve wasps during the observation period (χ^2-test, $P > 0.05$ for all treatments except *H. downesi*-infected larvae that died during trials; Table 2).

In trials with naïve wasps, the movement of infected hosts that survived trials was similar to that of healthy hosts (MWUt; $P > 0.05$; Table 3). However, infected *H. abietis* that died during trials moved less frequently than infected *H. abietis* that survived trials [MWUt; *S. carpocapsae*: W = 398.0, $P = 0.02$ (H-B adjusted significance level $\alpha = 0.017$); *H. downesi*: W = 627.5, $P = 0.003$; Table 3]. Host movement was not affected by EPN infection in trials with experienced wasps (KWt, $H_{4,109} = 2.17$, $P = 0.704$; Table 3). A comparison of median host movement between treatments with naïve and experienced wasps revealed no significant differences, however (MWUt, $P > 0.05$; Table 3 and Table S3).

Table 2. Incidence of *probing* and *oviposition* for naïve and experienced *Bracon hylobii* on healthy and EPN-infected *Hylobius abietis* in experiment 1.

Experiment 1						
	Percentage of trials in which behaviour was observed at least once (n/total)				Mean number of eggs per clutch ± SE (n)	
	Probing		Oviposition			
Host type (*H. abietis*)	Naïve	Experienced	Naïve	Experienced	Naïve	Experienced
Control	50.0 *(14/28)*	**81.5** *(22/27)*	35.7 *(10/28)*	44.4 *(12/27)*	21.9 ± 1.4 (24)	15.2 ± 1.4 (18)
Sc survived	62.5 *(15/24)*	85.7 *(24/28)*	45.5 *(10/22)*	67.9 *(19/28)*	20.1 ± 1.5 (21)	15.6 ± 1.4 (22)
Hd survived	43.5 *(10/23)*	57.1 *(12/21)*	34.8 *(8/23)*	47.6 *(10/21)*	20.6 ± 1.6 (16)	16.5 ± 2.3 (18)
Sc died	35.0 *(7/20)*	60.0 *(9/15)*	15.0 *(3/20)*	46.7 *(7/15)*	17.3 ± 6.7 (3)	17.3 ± 2.8 (11)
Hd died	23.3 *(7/30)*	**66.7** *(12/18)*	3.3 *(1/30)*	**55.6** *(10/18)*	28.7 ± 1.3 (3)	16.5 ± 2.9 (14)

Sc = *S. carpocapsae*; *Hd* = *H. downesi*. Survived = larvae that survived the 24-h trial but died of entomopathogenic nematode infection within the 5 days following, died = larvae that died during trial. Values for experienced wasps in bold were significantly different from those for naïve wasps in the respective treatment (χ^2-test or Fisher's exact test; $P < 0.05$; significance level sequentially corrected after Holm–Bonferroni). Statistical test results in Table S2.

Table 3. Host movement in experiments 1 and 2. Movement of *R. bifasciatum* not recorded in trials with experienced wasps.

		Median of percentage of records per trial with host movement (Mean; *n*)	
	Host type	Naïve	Experienced
Experiment 1 *(H. abietis)*	Control	56.9 (56.7; 28)	37.5 (46.4 ; 27)
	Sc survived	85.4 (70.2; 20)	33.3 (44.9; 24)
	Hd survived	37.5 (45.2; 23)	16.7 (28.8; 18)
	Sc died	22.9 (36.9; 20)	50.0 (52.5; 14)
	Hd died	4.2 (14.4; 30)	10.4 (33.7; 17)
Experiment 2 *(Ha=H. abietis)*	*Ha* control	20.8 (28.0; 55)	20.8 (26.5; 17)
	Ha prepupae	0 (1.1; 30)	NA
	Ha paralysed	0 (0; 14)	0 (0; 13)
	G.mellonella	12.5 (17.1; 38)	20.8 (3.3; 10)
	R. bifasciatum	41.7 (48.8; 30)	NA

NA, not applicable.

Experiment 2: parasitism and insect behaviour in trials with H. abietis *larvae dead from the beginning of the trial and with abnormal hosts*

Neither naïve nor experienced wasps parasitised dead *H. abietis* larvae (naïve: 11 *S. carpocapsae*-killed, 12 *H. downesi*-killed, 27 freeze-killed; experienced: 12 *S. carpocapsae*-killed, 21 *H. downesi*-killed, 18 freeze-killed). No eggs were laid in empty chambers (naïve: 15 and experienced: 20). Experienced wasps parasitised control hosts more frequently than naïve wasps ($\chi^2_1 = 5.623$, $P = 0.02$; Fig. 2). Naïve wasps parasitised paralysed *H. abietis* larvae in 2 out of 19 trials and prepupal larvae in 5 out of 30 trials, lower parasitism than in the control in both cases ($\chi^2_1 = 32.740$, $P < 0.001$; FEt, $P < 0.001$, respectively). Experienced wasps parasitised 1 out of 25 paralysed hosts, also a rate lower than in the control (FEt, $P < 0.001$). Parasitism of *R. bifasciatum* and *G. mellonella* larvae was lower than in the control for both naïve (*Rb:* $\chi^2_1 = 15.326$, $P < 0.001$, *Gm:* $\chi^2_1 = 49.133$, $P < 0.001$; Fig. 2) and experienced wasps (FEt, *Rb:* $P < 0.001$; *Gm*, $P = 0.008$; Fig. 2). *Bracon hylobii* eggs that were laid on *R. bifasciatum* and *G. mellonella* larvae hatched, but wasp larvae did not develop beyond the first instar on these hosts.

The number of eggs in clutches laid on prepupal and paralysed *H. abietis* larvae and on *G. mellonella* larvae was similar to the number of eggs in clutches laid on *H. abietis* larvae in the control (Table 4). Egg clutches laid by naïve wasps on *R. bifasciatum* larvae contained less eggs than those laid in the control (median of 8 for Rb and 21 for control; MWUt, W = 6216.0, $P = 0.001$). Host mass differed significantly among treatments (data not shown; one-way ANOVA, $F_{13,243} = 2.44$, $P = 0.004$), although Tukey's test detected only one significant difference between the treatments (for naïve wasps, where *R. bifasciatum* hosts were heavier than control hosts, $P < 0.05$).

In control trials with naïve wasps, *probing* incidence was lower than in trials with experienced wasps ($\chi^2_1 = 6.313$, $P = 0.01$, Table 4) and *probing* was also observed in a greater proportion of trials with experienced wasps than naïve wasps for all other treatments, with the exception of paralysed *H. abietis* larvae (χ^2-test or FEt, $P > 0.05$; Table 4). *Probing* occurrence in trials with *R. bifasciatum* and *G. mellonella* was similar to the control for both naïve and experienced wasps (Table 4). Experienced and naïve wasps *probed* the bark patch in trials with dead hosts or an empty chamber (Table 4). Wasps were not once seen *probing* in a location directly over the host chamber in trials with an empty chamber (28 records of *probing* for naïve and experienced wasps combined), a freeze-killed host (6 records) or a host killed by *H. downesi* (34 records). One experienced wasp *probed* directly over a *H. abietis* larva killed by *S. carpocapsae*, representing 3 out of 9 *probing* records overall for this host type.

Oviposition occurred less frequently in control trials with naïve wasps than it did in control trials with experienced wasps ($\chi^2_1 = 6.313$, $P = 0.01$; Table 4). No oviposition was recorded in any of the treatments with dead *H. abietis* larvae. Naïve wasps oviposited on 1 *G. mellonella* and 1 *R. bifasciatum* larva.

No movement was recorded for paralysed *H. abietis* and prepupae moved on only 8 out of 720 records for that host type in total (Table 3). In trials with naïve wasps, *R. bifasciatum* larvae moved more frequently than *H. abietis* larvae in the control (MWUt, W = 1841.5, $P = 0.002$; Table 3). Movement in trials with *G. mellonella* was similar to the control (MWUt, W = 2529.0, $P = 0.18$; Table 3). *Galleria mellonella* and *R. bifasciatum* larvae showed no signs of paralysis when parasitised.

Experiment 3: parasitism of H. abietis *larvae that were prevented from chewing bark*

Naïve wasps parasitised a higher proportion of control hosts that chewed on the bark patch (control + chewing) than hosts that were prevented from chewing, but were supplemented with *Hylobius*-chewed bark (glued + chewed bark) ($\chi^2_1 = 4.787$,

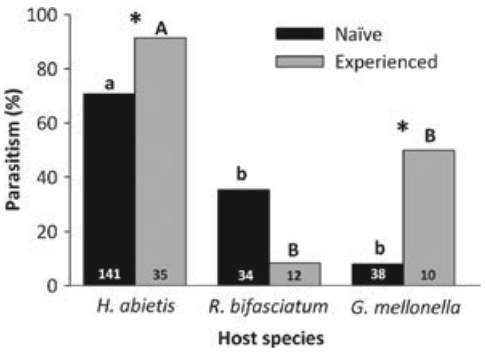

Fig. 2. Parasitism of healthy *Hylobius abietis*, *Rhagium bifasciatum* larvae or *Galleria mellonella* larvae by naïve and experienced *B. hylobii* in experiment 2. Numbers inside bars give *n*. Bars sharing the same letter are not significantly different from each other (χ^2-test or Fisher's exact test, $P < 0.05$, lowercase letters for naïve wasps, capital letters for experienced wasps). Pairs of bars marked with an asterisk are significantly different from each other (χ^2-test ; $P < 0.05$). Significance level sequentially corrected after Holm–Bonferroni.

Table 4. Incidence of *probing* and *oviposition* (observational data recorded during the 2-h observation period at beginning of the 24-h trial) and mean egg clutch size (recorded at the end of the 24-h trial) for naïve and experienced wasps in experiment 2.

Experiment 2						
	Percentage of trials in which behaviour was recorded at least once *(n*/total*)*				Mean number of eggs per clutch ± SE (*n*)	
	Probing		*Oviposition*			
Host type (*Ha* = *H. abietis*)	Naïve	Experienced	Naïve	Experienced	Naïve	Experienced
Ha control	35.7 *(20/56)*	**65.4 *(17/26)***	23.2 *(13/56)*	**57.7 *(15/26)***	20.3 ± 0.6 (102)	13.1 ± 1.1 (32)
Empty chamber	6.7 *(1/15)*	33.3 *(4/12)*	0 *(0/15)*	0 *(0/12)*	NA	NA
Ha killed by *Sc*	9.1 *(1/11)*	14.3 *(1/7)*	0 *(0/11)*	0 *(0/7)*	NA	NA
Ha killed by *Hd*	42.0 *(5/12)*	50.0 *(5/10)*	0 *(0/12)*	0 *(0/10)*	NA	NA
Ha freeze-killed	0 *(0/9)*	66.7 *(5/15)*	0 *(0/9)*	0 *(0/15)*	NA	NA
Ha prepupae	16.7 *(5/30)*	NA	0 *(0/30)*	N/A	26.2 ± 4.0 (5)	NA
Ha paralysed	10.5 *(2/19)*	6.7 *(1/15)*	0 *(0/19)*	0 *(0/15)*	26 (1)	20.0 ± 2.0 (2)
G.mellonella	31.6 *(12/38)*	60.0 *(6/10)*	2.6 *(1/38)*	0 *(0/10)*	14.7 ± 2.9 (3)	10.8 ± 2.5 (5)
R. bifasciatum	46.7 *(14/30)*	66.7 *(4/6)*	3.3 *(1/30)*	0 *(0/6)*	9.3 ± 2.0 (12)	2.0 (1)

In columns for *probing* and *oviposition*, values for experienced wasps in bold were significantly different from those for naïve wasps within the respective treatment (χ^2-test, $P < 0.05$). Statistical test results in Table S4.
Ha, *H. abietis*; *Sc*, *S. carpocapsae*; *Hd, H. downesi*; NA, not applicable.

$P = 0.03$; Fig. 3a), whereas parasitism of control hosts that did not chew on the bark was similar to parasitism of hosts prevented from chewing ($\chi^2_1 = 0.798$, $P = 0.37$; Fig. 3a). Chewing of bark had no significant effect on parasitism by experienced wasps (comparison of most disparate treatments: control + chewing vs. glued - chewed bark; FEt, $P = 0.66$; Fig. 3b). However, experienced wasps parasitised hosts that did not chew on bark during trials more frequently than naïve wasps did, both in the control (control - chewing; $\chi^2_1 = 5.808$, $P = 0.016$) and in trials with hosts prevented from chewing bark (glued + chewed bark: $\chi^2_1 = 11.768$, $P < 0.001$; glued - chewed bark: $\chi^2_1 = 8.705$, $P = 0.003$; Fig. 3a,b).

Experienced wasps laid fewer eggs per clutch than naïve wasps did (data not shown; two-way ANOVA; $F_{1,232} = 164.11$, $P < 0.001$), but treatment had no effect on egg clutch size ($F_{1,232} = 0.76$, $P = 0.38$) and there was no interaction between wasp experience and treatment ($F_{1,232} = 0.27$, $P = 0.60$). Host mass among treatments was similar (data not shown; One-way ANOVA, $F_{7,380} = 0.91$, $P = 0.50$).

Occurrence and effect of bark chewing on parasitism in experiments 1 and 2

In trials of experiment 1 with naïve wasps, *H. abietis* larvae infected with *S. carpocapsae* and *H. downesi* that survived trials chewed bark in 75.0% and 65.2% of trials, respectively, a rate similar to the control (79.2%; for *n* see Table 2). Infected hosts that died during the trial chewed the bark less frequently (*Sc:* 20.0% of trials, Hd: 10.0% of trials, for n see Table 2) and EPN infection affected chewing of bark ($\chi^2_4 = 42.649$, $P < 0.001$). Differences between treatments were less pronounced for experienced wasps (control: 44.4%, *S. carpocapsae* survived: 17.9%, died: 33.3%; *H. downesi* survived: 57.1%, died: 16.7%; $\chi^2_4 = 11.999$, $P = 0.017$; for n see Table 2).

Combining data on bark chewing for controls of experiments 1 and 2 revealed that naïve wasps were more likely to parasitise a healthy *H. abietis* larva that had chewed bark during a trial than one that had not chewed bark [94 hosts parasitised in 111 trials with chewing (94/111) vs. 24 hosts parasitized in 48 trials without chewing (24/48); $\chi^2_1 = 19.740$, $P < 0.001$]. Bark chewing had no significant effect on parasitism by experienced wasps, however (35/44 vs. 10/18; $\chi^2_1 = 0.626$, $P = 0.429$).

Paralysed *H. abietis* larvae chewed on bark in two trials with experienced wasps (one of these larvae was parasitised) and in one trial with naïve wasps. Only one prepupal larva chewed bark and it was parasitised. The larvae of both *R. bifasciatum* and *G. mellonella* chewed the bark in some trials (naïve wasps: *R. bifasciatum:* 24/30, *Gm:* 4/38; experienced wasps: *R. bifasciatum:* 1/6 and *G. mellonella:* 4/10), but bark chewing did not affect parasitism of these hosts (data not shown; FEt, $P > 0.05$).

Wasp behaviour related to H. abietis *host movement in experiments 1 and 2*

There was a significant positive relationship between the amount of movement by *H. abietis* hosts in treatments and the frequency of *oviposition* by wasps during observation in those treatments (logistic regression; naïve: $n = 8$, Coef = 3.7, Z = 4.85, $P < 0.001$, Fig. 4a; experienced: $n = 7$, Coef = 2.96, Z = 2.64, $P = 0.008$, Fig. 4b). For naïve and experienced wasps, the likelihood of wasps *probing* over a host rather than elsewhere on the bark patch increased with the amount of *H. abietis* host movement during the observation period (naïve: $\chi^2_3 = 212.674$, $P < 0.001$; experienced: $\chi^2_3 = 104.348$, $P < 0.001$ Fig. 5).

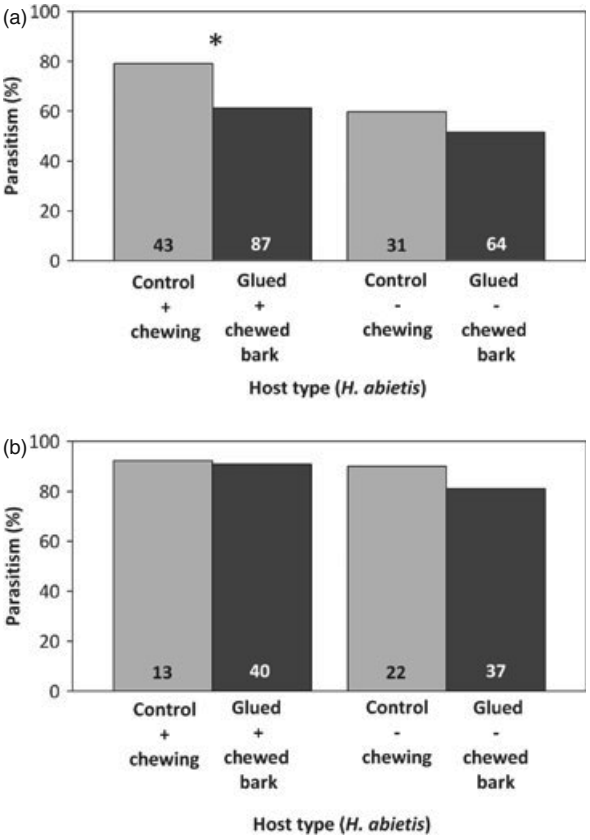

Fig. 3. Parasitism of bark chewing and non-bark-chewing *Hylobius abietis* larvae by naïve (a) and experienced (b) *Bracon hylobii*. Some of the larvae had been prevented from chewing bark by gluing their mandibles ('glued') and were either supplemented with a *Hylobius*-chewed bark patch and bark shavings (+ chewed bark) or not (− chewed bark). Control hosts are grouped by whether they chewed the bark patch during trials (+ chewing) or not (− chewing). Numbers inside bars give n. Pairs of bars in graph A marked with an asterisk are significantly different from each other (χ^2-test; $P < 0.05$). Significance level sequentially corrected after Holm–Bonferroni.

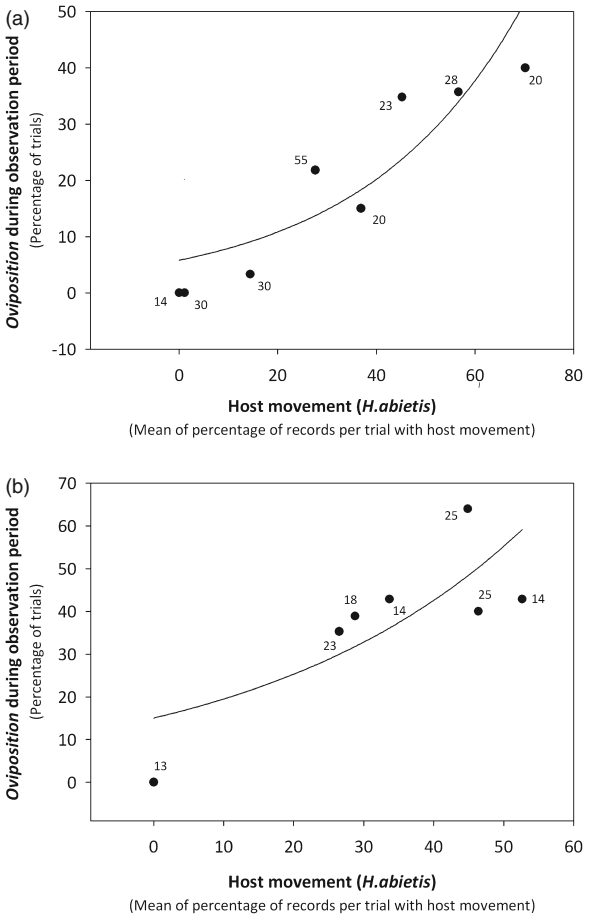

Fig. 4. Scatter plot relating *oviposition* incidence in treatments with *Hylobius abietis* hosts [healthy controls, entomopathogenic nematodes (EPN)-infected, prepupal (naïve only) and paralyzed] to the amount of host movement in those treatments during 2 h of observation. (a) naïve wasps; (b) experienced wasps. Each datum point is labelled with n (number of trials per treatment from which data were calculated). The fitted regression line in each graph represents event probabilities for the respective binary logistic model used for analysis.

Discussion

In Everard *et al.*'s (2009) choice trials, naïve *B. hylobii* did not parasitise *H. abietis* larvae killed by *H. downesi*, and infected hosts were less likely to be parasitised than healthy hosts as early as 12 h after infection. In the present study, it has been shown that even without a healthy larva as an alternative host, naïve wasps still do not parasitise larvae killed by *H. downesi* or the distantly related EPN, *S. carpocapsae*. Both naïve and experienced wasps accepted live nematode-infected hosts for oviposition, however, even although their progeny would have no chance of survival. This is not unusual: parasitoids frequently do not reject hosts in advanced stages of infection by entomopathogens including fungi, bacteria, and microsporidia (Chilcutt & Tabashnik, 1999; Lord, 2001; Down *et al.*, 2005). Rejection of pathogen-infected hosts has been reported in some instances, however (Sher *et al.*, 2000; Head *et al.*, 2003; Lacey *et al.*, 2003). For example, Lacey *et al.* (2003) showed that, in choice trials, infection of cocooned codling moth larvae with *S. carpocapsae* only 12 h before they were offered to the parasitoids *Mastrus ridibundus* Gravenhorst and *Liotryphon caudatus* Ratz significantly reduced parasitism compared with healthy hosts. The authors suggested that, when probing hosts, wasps detected changes in their quality brought on by the activity of the pathogenic symbiotic bacteria that EPN release in the host (Forst *et al.*, 1997; Dowds & Peters, 2002; Lacey *et al.*, 2003) and consequently rejected these unsuitable hosts. Rejection of hosts on which the brood will suffer competition or fail to develop appears to be an adaptive behaviour in parasitoids (Vinson & Iwantsch, 1980; Charnov & Skinner, 1984). Our further investigations indicate, however, that where it occurs, 'rejection' of EPN-infected hosts by *B. hylobii* is not a specially evolved behaviour, but rather a fortuitous by-product of the way in which this species locates hosts at short range, as explained below.

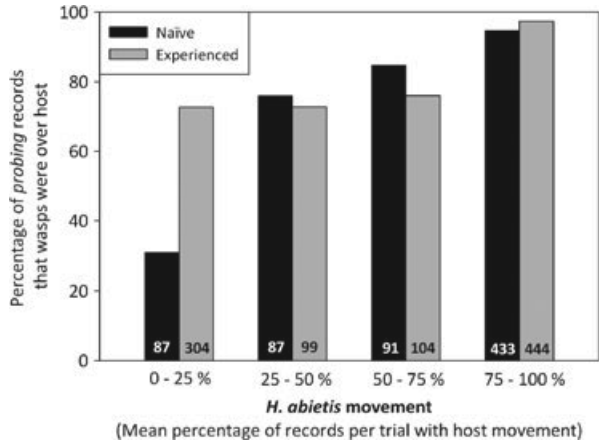

Fig. 5. Percentage of *probing* records during 2-h observation of naïve and experienced wasps where *probing* occurred over the host chamber rather than elsewhere on the bark patch. Data combined for treatments with *H. abietis* hosts [healthy controls, entomopathogenic nematodes (EPN)-infected, prepupal (naïve only) and paralysed] and grouped according to the mean percentage of records per trial that hosts moved. Numbers inside bars give *n*.

Especially for parasitoids that target cryptic hosts such as *H. abietis*, volatiles released by the host or the substrate containing it and vibrations caused by host movement and feeding are important cues for short-range host location (Meyhöfer *et al*., 1999; Wang & Yang, 2008). Host volatiles produced by the cryptic bark beetle *Ips typographus* L. attract parasitoid wasps to infested trees and induce and sustain probing, even after beetles have been removed from the bark (Mills *et al*., 1991; Pettersson & Boland, 2003). In olfactometer assays conducted by Faccoli and Henry (2003), female *B. hylobii*, especially when experienced, responded to volatiles released by feeding *H. abietis*. Laboratory assays necessarily exclude a large part of the complex set of volatiles wasps may encounter when foraging in nature and may therefore lead to an overestimation of the importance of vibrational cues (Duan & Messing, 2000). Also, the bark used in our experiments was cut from recently felled spruce logs (stored for up to 2 months after felling) and was probably fresher than the bark that *B. hylobii* usually forages on in its natural habitat, as *H. abietis* larvae in spruce tree stumps reach a size suitable for *B. hylobii* 2 months after felling at the very earliest (Leather *et al*., 1999; Henry & Day, 2001). However, parasitism in our controls was high (70–90%), indicating that whatever amount and composition of volatile cues *B. hylobii* may require for short-range location and parasitism of its standard host were adequately represented in our trials.

As *B. hylobii* females *probed* the bark in trials with empty chambers, it seems that on their own, bark substrate cues (e.g. volatiles, contact chemicals, and texture) suffice to trigger foraging behaviour in *B. hylobii*. However, even with the addition of a dead host as a source of host volatiles, *probing* occurrence was lower than in trials with a live host (*H. abietis*, *R. bifasciatum* and *G. mellonella*) capable of producing vibrational cues by moving and chewing bark. Moreover, *B. hylobii* almost never *probed* directly over a paralysed or dead larva, so volatile cues from the bark substrate and/or host did not seem to allow precise location of the host, even within the small host microhabitat in our experiments (bark patch area: 2.25 cm^2). Wasp venom (in paralysed hosts), EPN infection or freezing may have changed the chemistry of immobile hosts in our experiments. However, such host-associated cues are probably not as informative as vibrational cues to wasps in assessing host quality (Ulyshen *et al*., 2011). Moreover, a wasp would most likely only be able to detect changes to chemical host cues if it probed the host (Vinson, 1998). If wasps were rejecting immobile larvae because of chemical changes within them, we therefore would not expect to see the reduction in probing incidence and accuracy that we observed in our trials with immobilized hosts compared with the control.

Our finding that *oviposition* frequency and the frequency of wasps *probing* over a host rather than elsewhere on the bark patch increased significantly with increasing host movement suggests that wasps were using vibrational cues to locate hosts at short range. As both naïve and experienced wasps readily parasitised *H. abietis* larvae that did not chew on bark during trials, host movement (with host and substrate volatiles present) seems a sufficient source of vibrations for *B. hylobii* to locate hosts in our experimental set-up. However, host feeding can be an important additional source of vibrational and volatile cues (Wang & Yang, 2008; Wang *et al*., 2010). Naïve *B. hylobii* were less likely to parasitise *H. abietis* larvae that did not chew on bark than larvae that did chew, even when larvae were supplemented with *Hylobius*-chewed bark and bark shavings as a source of putative feeding volatiles. This indicates that it was the additional vibrational cues, rather than volatile cues that were responsible for the increased probability of *B. hylobii* females locating and then parasitising chewing hosts at short range. In summary, we conclude that vibrational cues generated by moving and/or feeding hosts are of significant importance in short-range host location and possibly also host acceptance by *B. hylobii*, as speculated by Henry & Day (2001) and Everard *et al.* (2009). Although feeding-associated volatiles released from chewed bark appeared to have little importance for short-range host location in our trials, the bark in our trials was chewed on for 24 h at most, so we cannot rule out the possibility that volatiles emitted from a substrate that has been chewed or fed on for longer and under natural conditions may contribute to *B. hylobii* host-finding to a greater extent.

The use of vibrational cues by foraging *B. hylobii* may explain the pattern of parasitism of EPN-infected hosts we recorded. In our experiments, parasitism of EPN-infected *H. abietis* by naïve wasps was only reduced compared with healthy hosts if the infected larvae died during the 24-h trials. Everard *et al.* (2009) found that EPN-infected *H. abietis* that were not parasitised by *B. hylobii* during trials (also 24 h) died sooner than those that were parasitised. They proposed that the lack of parasitism was as a result of host lethargy (reduced larval movement and feeding) brought on by EPN infection, thereby reducing the likelihood of such hosts being detected by foraging wasps. Our data support this hypothesis. Even within the first 2 h of or our trials, *H. abietis* larvae that died of EPN-infection during trials moved less frequently than larvae that

survived trials and larvae that died were also less likely to chew on bark during trials. As insect death because of *Photorhabdus temperata* K122 (the symbiont of *H.downesi* K122) occurs when the bacteria reach the stationary phase (Clarke & Dowds, 1995), the bacterial pathogen load of infected *H. abietis* larvae that died during trials was almost certainly greater than that of larvae that survived trials. Whether this also reflects different numbers of nematodes invading and/or different rates of progression of the infection in different hosts is uncertain. However, differing pathogen loads between insects that died during or after a trial is unlikely to directly account for the differences in parasitism among EPN-infected hosts that we observed, as *B. hylobii* females parasitise *H. downesi*-killed hosts even 24–48 h after host death if these hosts are supplemented with artificially created vibrations (C. Harvey and C. Griffin, unpubl. data), showing that infected hosts with bacteria at stationary phase are not rejected by *B. hylobii* if they can be located.

Unlike naïve wasps, experienced wasps parasitised hosts that died of EPN-infection during trials as frequently as healthy hosts. Preventing hosts from chewing on bark also had no significant effect on parasitism by experienced wasps. Parasitoid wasps can become more sensitive to innate host cues or learn novel host-associated cues of the host habitat when encountering a host (Turlings *et al.*, 1993; Vinson, 1998; Meiners *et al.*, 2003). Learning is thought to increase the fitness of wasps by enhancing their ability to locate and thus parasitise subsequent hosts (Vet *et al*., 1990; Vinson, 1998; Meiners *et al*., 2003). For instance, Papaj and Vet (1990) report that not only were experienced females of the parasitoid *Leptopilina heterotoma* Thomson more likely to locate microhabitats containing their host than naïve females were, they also did so more quickly. Faccoli and Henry (2003) found that experienced *B. hylobii* females were more responsive than naïve females to volatile cues emitted by a feeding *H. abietis* larva. In our trials, experienced *B. hylobii* females were more likely than naïve females to initiate *probing* and *oviposition* within the first 2 h of trials, indicating that they either were quicker to locate the host microhabitat (i.e. the bark patch) in our experiments, and/or required less time to initiate *probing* and *oviposition* once the microhabitat had been found. Parasitism by experienced wasps therefore pre-empted the onset of significant reductions in host movement as a result of EPN infection, as is illustrated by the lack of significant differences in host movement between treatments in the first 2 h of trials with experienced wasps (only host movement data recorded prior to oviposition were included). Thus, experienced wasps overall had a greater chance than naïve wasps of locating and parasitising moribund EPN-infected *H. abietis* larvae before these grew too lethargic to allow precise location via vibrational cues.

To optimally exploit available resources and thus maximise fitness, parasitoids should adjust clutch size to host quality (Charnov & Skinner, 1984; Godfray *et al*., 1991). Host quality may be judged based on a variety of cues, including volatiles (Vinson & Iwantsch, 1980; Vinson, 1998), but possibly also vibrational cues (Henry & Day, 2001; Ulyshen *et al*., 2011). However, in agreement with results reported by Everard *et al*. (2009), we found no indication of *B. hylobii* females adjusting egg clutch size in response to EPN-infection (experiment 1), host species (experiment 2) or host activity as represented by bark chewing (experiment 3). The small clutch size we recorded on *R. bifasciatum* larvae is most probably as a result of the insects moving while wasps were *ovipositing* (*R. bifasciatum* larvae are not susceptible to wasp venom), thus causing wasps to interrupt *oviposition* to protect their ovipositor (Gross *et al*., 1993). Our results as well as those of Everard *et al*. (2009) indicate that once a stimulus threshold is surpassed, *B. hylobii* females commit to laying a full clutch of eggs on a host, even when it is infected with EPN *(H. abietis)* or unlike the normal host (e.g. *G. mellonella*).

Bracon hylobii appears to be a specialist parasitoid with a very limited host range (Kenis *et al*., 2004), yet females parasitised two hosts outside of the reported host range (*R. bifasciatum* and *G. mellonella*). There are no previous reports of *B. hylobii* parasitising *R. bifasciatum* larvae, even although they have a saproxylic lifestyle similar to that of *H. abietis* and both species frequently occur on the same coniferous clearfell sites (C. Harvey, pers. obs.). In their natural environment, female *B. hylobii* probably follow specific volatile and textural cues associated with wood, bark, and soil not replicated in our experiments in order to locate tree stumps harbouring *H. abietis* (Vinson & Iwantsch, 1980; Vinson, 1998), thus avoiding deadwood containing the unsuitable host *R. bifasciatum* (Duffy, 1953; Twinn & Harding, 1999). At short range, however, *B. hylobii* females appear to use cues generic enough to allow oviposition on a host as phylogenetically removed from its target host as the waxmoth larvae *G. mellonella*.

As shown above, *B. hylobii* females do not seem to actively reject hosts infected by entomopathogenic nematodes, but, as a consequence of the role that vibrational cues play in their foraging strategy, are simply less likely to locate infected hosts that are close to death and therefore lethargic. It follows that, if entomopathogens are abundant in the host population, *B. hylobii* fitness may suffer as unsuitable pathogen-infected hosts are accepted for oviposition when they are still moving (Charnov & Skinner, 1984; Godfray *et al*., 1991). While natural EPN infection of *H. abietis* has been reported (Kenis *et al*., 2004) there is no evidence that this is widespread and natural infections have not been recorded in Ireland to date (Dillon *et al*., 2006, 2008), so there probably is little selective pressure on wild *B. hylobii* populations to evolve the capacity for EPN detection. The entomopathogenic fungus *Beauveria spp*., however, is widespread among *H. abietis* populations on coniferous clearfell sites (Glare *et al*., 2008; C. Harvey, pers. obs.). It would be interesting to investigate whether *B. hylobii* females actively reject *H. abietis* larvae infected with this pathogen that they may encounter more frequently. As *B. hylobii* is an idiobiont parasitoid that paralyses hosts, one advantage of reliance on vibrational cues for host location may be the avoidance of superparasitism, which can reduce overall reproductive success (Potting *et al*., 1997; Dorn & Beckage, 2007; Wang *et al*., 2010). In our trials, paralysed hosts did not move during observation, chewed bark infrequently and, presumably as a consequence of this, were only rarely parasitised.

As both naïve and experienced wasps parasitised EPN-infected hosts, our results imply that there is a risk of competition between EPN and resident *B. hylobii* populations on clearfell sites when EPN are applied against the immature stages of *H. abietis* for biological control. However, as healthy, active host larvae may be more attractive than moribund, lethargic hosts infected with EPN (Everard *et al*., 2009), results of no-choice trials will probably overestimate the risk of competition. Field data collected by Dillon *et al*. (2008) indicate that *B. hylobii* parasitism on clearfell sites was not adversely affected 1 month after application of *S. carpocapsae* or *H. downesi* to tree stumps, although the long-term effects and developmental success of *B. hylobii* were not investigated. Applying nematodes just after most adult wasps have eclosed in late spring, but parasitism of *H. abietis* in tree stumps is still relatively low (Henry, 1995), may help to minimise the direct impact of EPN on *B. hylobii* larvae and adults, which are highly susceptible to EPN infection (Everard *et al*., 2009). Numbers of eclosed *B. hylobii* adults should then be sufficient to maintain the wasp population over the period immediately after EPN application, when EPN-infection of *H. abietis* larvae is expected to be highest (Dillon *et al*., 2006) and competition between EPN and *B. hylobii* brood for *H. abietis* resources is therefore likely to peak. The resident *B. hylobii* population may complement large pine weevil control efforts not only in the short term, by parasitising *H. abietis* larvae that are not killed by the nematodes in the summer of EPN application, but also in the long term over subsequent seasons when weevils often remain abundant in tree stumps (Leather *et al.*, 1999).

As a parasitoid of cryptic hosts, *B. hylobii* appears to utilise vibrational cues for short-range host location. As we found no evidence that the species has evolved the capacity to actively discriminate between hosts different from its standard host, including pathogen-infected hosts, we conclude that the abundance of unsuitable insect hosts that may act as potential 'decoys' under the bark of tree stumps is too low to create selective pressure for a more stringent host selection mechanism in *B. hylobii*. The reduction in parasitism by naïve *B. hylobii* of hosts in advanced stages of pathogen-infection is most likely because of wasps locating such hosts less frequently, rather than any active rejection or avoidance of such hosts. The same may be true of other parasitoids that appear to 'reject' pathogen-infected hosts. Our results also indicate that parasitoid wasp experience can significantly influence how frequently pathogen-infected hosts are parasitised, particularly if wasps lack the ability to actively detect and reject such hosts. This should be taken into account when assessing the compatibility of entomopathogens and parasitoids for biological control.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/j.1365-2311.2012.01362.x

Table S1. Information on clearfell sites from which insects were collected.

Table S2. Test results for pairwise comparisons of probing and oviposition between naïve and experienced wasps for each treatment in experiment 1 (see Table 2). χ^2 – test or Fisher's exact test.

Table S3. Test results for pairwise comparisons by treatment between trials with naïve and experienced wasps for host movement in experiment 1 (MWUt, see Table 2).

Table S4. Test results for pairwise comparisons of probing and oviposition between naïve and experienced wasps for each treatment in experiment 2 (see Table 4). χ^2 – test or Fisher's exact test.

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