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Behavioural and genetic approaches to evaluate the effectiveness of deterrent marking by a parasitoid wasp

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Abstract

Some parasitoids deposit chemical signals after oviposition as an indication that the host has already been parasitized. This marking can deter subsequent conspecifics or one's self from laying eggs in previously exploited hosts, thus reducing the risk of superparasitism. We investigated the egg laying behaviour of the parasitoid wasp *Hyposoter horticola*. In a laboratory experiment, we tested whether oviposition, post-oviposition marking, or both together deter subsequent oviposition by conspecifics. We then tested the effectiveness of the deterring mark under natural conditions using maternity assignment based on 14 polymorphic DNA microsatellite markers. The behavioural experiment showed that patch marking deters conspecifics from probing the host eggs, and oviposition deters those that probe from laying eggs in previously parasitized host clusters. These results were confirmed by the maternity assignment showing that under natural conditions, host egg clusters are primarily parasitized by a single *H. horticola* female.

Keywords

DNA microsatellites, foraging behaviour, Hymenoptera, *Hyposoter horticola*, maternity analysis, *Melitaea cinxia*, post-oviposition marking, superparasitism.

1. Introduction

Many insects (including those in the orders Coleoptera, Diptera, Lepidoptera and Hymenoptera) deposit chemical signals after oviposition. This marking enables the individual depositing the mark, and conspecifics, to distinguish

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exploited and unexploited resources, thus reducing the risk of laying eggs in a previously exploited patch (reviewed in Roitberg & Prokopy, 1987; Nufio & Papaj, 2001; Liu et al., 2012), and reducing direct competition among offspring (Prokopy, 1981).

Parasitoids lay their eggs in or on other arthropods. The parasitoid larvae then develop by feeding on host tissue, eventually resulting in the hosts' death (Godfray, 1994). When a host provides a limiting resource, parasitoid larvae can suffer from competition for space or food (Brodeur & Boivin, 2004) with conspecifics (superparasitism, van Alphen & Visser, 1990) or with other species (multiparasitism, Fisher, 1961). For solitary parasitoid species superparasitism is costly in terms of time and fitness because only one individual can complete development inside a host (Harvey & Strand, 2002). Many attempt to avoid superparasitism (Godfray, 1994) but may not always be able to (van Alphen & Visser, 1990; Cronin & Strong, 1993). While some parasitoid species, such as egg parasitoids of gregariously laid host eggs, mark each parasitized host individually (van Baaren et al., 1995), others mark the host patch as a whole. This is especially true for those using concealed hosts. For instance, several species of wasps attacking larvae of fruit flies mark the fruit in which the fly maggot hosts are located rather than marking the maggot (Hoffmeister & Roitberg, 1997; Stelinski et al., 2007). For parasitoids of exposed hosts, it may also be advantageous to avoid previously exploited host patches, even if some hosts remain unparasitized. Under these conditions a wasp may mark an entire patch, rather than individual hosts within it (Hoffmeister & Roitberg, 1997).

Parasitizing a previously exploited host or resource patch can be avoided by responding to general cues, such as those produced by the host as a defence mechanism against predators or parasitoids. For example, the endoparasitoid *Aphidius rhopalosiphi*, rejects hosts based on a secretion released by the host during a previous attack (Outreman et al., 2001). Internal changes in host quality, due to parasitoid development, are also used for discrimination in several systems (Mackauer, 1990; Gauthier & Monge, 1999; Outreman et al., 2001). However, some parasitoids, just as many phytophagous insects, such as the well-studied apple maggot fly (Roitberg & Prokopy, 1987), actively mark the parasitized hosts or host patch with pheromones after oviposition. These markings deter further exploitation by the individual leaving the mark, or by conspecifics (van Lenteren, 1981; Hoffmeister & Roitberg, 1997; Nufio & Papaj, 2001; Stelinski et al., 2007; Liu et al., 2012).

In this paper we investigate the deterrent effect of post-oviposition marking by the solitary wasp *Hyposoter horticola* (Gravenhorst) (Ichneumonidae: Campoplaginae). This egg-larval endoparasitoid is a specialist of the Glanville fritillary butterfly *Melitaea cinxia* (Lepidoptera: Nymphalidae) (van Nouhuys & Ehrnsten, 2004; Shaw et al., 2009). The host butterfly lives as a metapopulation in the Åland Islands, a Finnish archipelago between Sweden and mainland Finland (Hanski, 2011). It lays clusters of around 100 eggs on the underside of leaves of the host plants, *Veronica spicata* and *Plantago lanceolata* (Plantaginaceae) (Kuussaari et al., 2004). Female *H. horticola* forage for these host egg clusters in June, and parasitize them when the caterpillars are close to hatching from the eggs (van Nouhuys & Ehrnsten, 2004). As the parasitized caterpillar develops, the *H. horticola* larva remains inside its host through winter diapause. In the spring the larva consumes the entire caterpillar and pupates within its integument, just before the host itself would have pupated (van Nouhuys & Punju, 2010).

In the Åland Islands about one-third of the hosts in nearly all of the butterfly egg clusters are parasitized by *H. horticola* (van Nouhuys & Hanski, 2002; van Nouhuys & Ehrnsten, 2004). Though the wasps visit the same host cluster several times as the host eggs develop (van Nouhuys & Kaartinen, 2008), they do all their oviposition in one visit during the two days when the hosts are susceptible to parasitism (van Nouhuys & Ehrnsten, 2004; Montovan et al., 2015). Individual host egg clusters are discovered and monitored by multiple females (van Nouhuys & Kaartinen, 2008), and competition among those females is strong (Couchoux & van Nouhuys, 2014). In spite of this competition, a host egg cluster remains only partially exploited. The mechanisms and possible evolutionary explanations for this individual restraint are presented in Montovan et al. (2015). Thus, there is both a risk that multiple females parasitize the same host (superparasitism), and a risk that they parasitize the same host cluster at a rate higher than the optimum, leading to patch overexploitation.

After oviposition *H. horticola* drags its ovipositor on leaves surrounding the host cluster, marking the patch location rather than individual host eggs, or even the cluster of eggs as a whole. *Hyposoter horticola* could mark coarsely because it is adaptive to mark the patch rather than the host (Hoffmeister & Roitberg, 1997), or it may be due to a physical constraint. It is very rare in the family Ichneumonidae to parasitize hosts that have not yet hatched out of the eggs, instead of larger caterpillars or pupae (Shaw et

Figure 1. Female *Hyposoter horticola* parasitizing a *Melitaea cinxia* egg cluster. This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/1568539x.

al., 2009). Thus, in contrast to tiny egg parasitoids such as *Trichogramma* (0.2–1 mm) that are smaller than the eggs they attack, *H. horticola* is large (7 mm) and may not be well adapted to probing and marking individual butterfly eggs (1 mm) (Figure 1).

In this study we tested whether the egg-laying behaviour of *H. horticola*, including both oviposition and patch marking, deterred conspecifics from parasitizing previously used host egg clusters. Using a manipulative behavioural experiment in the laboratory, we first studied the behaviour of a female at a host egg cluster after a previous conspecific had parasitized the cluster and/or marked the patch. We then measured the extent to which multiple wasps may parasitize a single host egg cluster under field conditions. We used 14 DNA microsatellite markers to identify full-sibling offspring in naturally parasitized host egg clusters to determine the number of *H. horticola* females that had parasitized each host cluster. Although maternity analysis has been used to address a variety of questions in insects (Grillenberger et al., 2008; Lepais et al., 2010; Seppä et al., 2012), to our knowledge this is the first time that microsatellite markers and maternity assignment have been used to assess the effectiveness of deterrent marking by insects.

Under natural conditions we would expect only one female to parasitize a host cluster if the marking behaviour were deterrent. If the mark were not

deterrent, the first female to visit the egg cluster should parasitize about one third of the hosts in a cluster, and subsequent females, constrained by optimal foraging strategy should parasitize only a few hosts each (Montovan et al., 2015).

2. Materials and methods

2.1. Behavioural experiment

The parasitoids used for the experiment originated from parasitized *M. cinxia* caterpillars collected in the field in Åland, Finland. The host egg clusters came from laboratory-reared *M. cinxia* butterflies. The laboratory colony is mixed with caterpillars from the field every year to maintain genetic diversity of the colony. Details of rearing the wasps and butterflies are presented in more detail in Couchoux & van Nouhuys (2014). Each leaf bearing a host egg cluster was removed from the plant and kept individually in a Petri dish under controlled temperature and light conditions in an environmental chamber.

To test whether oviposition, post-oviposition marking, or both together deterred parasitism by subsequent females, we studied the wasps' behaviour at host egg clusters under four different conditions: egg clusters that were unparasitized and those that had been previously parasitized, both in the presence or absence of marking.

For each trial we introduced a female *H. horticola* into a mesh cage (40 × 40 × 50 cm) containing a potted host plant (*Veronica spicata*) with an egg cluster on it. The plants were all of the same age, and matched for size. The host egg clusters were used zero to two days before hatching, which is when they are susceptible to parasitism (Montovan et al., 2015). The wasps were at least 2 week old, which is when they are interested in foraging for hosts (Couchoux & van Nouhuys, 2014), fed, not mated, and had been given experience probing a host cluster prior to the experiment.

To create the parasitized + marked treatment (P + M), we let a wasp go through the entire process of oviposition and marking the leaves surrounding the cluster. For the parasitized + unmarked treatment (P + UM), we removed the single leaf bearing a parasitized host egg cluster and placed it on a new unmarked plant. Conversely, for the unparasitized + marked treatment (UP + M), we placed an unparasitized cluster at the centre of marked leaves on a plant. Finally, for the unparasitized + unmarked control treatment (UP + UM), the cluster was left unmanipulated. Because of the design of the

experiment, most of the egg clusters were used twice (e.g., first as 'control' and then as 'parasitized'). Due to limited availability of adult parasitoids the wasps were also used several times (three on average) with at least one day between tests. We used 67 host egg clusters and 40 wasps for a total of 142 behavioural observations.

We recorded the wasps' behaviours using The Observer® (version 9; Noldus Information Technology, Wageningen, The Netherlands). The behaviours analysed were: (1) Probing and/or parasitizing: if and for how long a wasp used its ovipositor to probe/parasitize the eggs. These two behaviours were scored together because it is almost impossible to distinguish between probing (poking the eggs with the ovipositor) and parasitizing (actually laying eggs into the hosts). (2) Marking: if and for how long the wasp marked the host patch by dragging its ovipositor on the leaves surrounding the egg cluster. We also measured the parasitism rate of each host egg cluster by dissecting newly hatched caterpillars after each observation and scoring them as parasitized or unparasitized. To assess the effect of a second wasp on the number of parasitized eggs (whether it parasitized or only probed the eggs) and the effect of the mark, we compared parasitism rates of egg clusters parasitized by two wasps (P + UM and P + M treatments) with egg clusters parasitized by a single wasp (UP + UM, UP + M).

All statistical analyses were performed using R (R Core Team, 2012). We used generalized linear mixed models with a binomial distribution and logit link function to analyse the presence of probing and marking, and a Poisson distribution and log link function to analyse durations of probing and marking, and parasitism rate (glmmPQL, package 'MASS'; Venables & Ripley, 2002). For analysis of probing and parasitism rate, the models included egg cluster treatment and cluster size as fixed effects, and their interaction. For the analysis of marking, egg cluster treatment, cluster size, and probing were included as fixed effects. Because we used the egg clusters and wasps several times we included egg cluster identity (ID), wasp ID and the number of times the wasp has been observed before as random effects in all of the models.

2.2. Maternity assignment

We quantified the number of *H. horticola* females that laid eggs in each of 10 naturally parasitized host clusters using maternity assignment based on polymorphic DNA microsatellite markers. The clusters came from six different natural butterfly populations in the main Åland Islands (municipalities of

Strömma (1 locality), Eckerö (1), Hammerland (2) and Saltvik (2)). Each was a large well-established population. After taking full nests of newly hatched caterpillars from the field, we removed all the parasitoids from the host caterpillars, and genotyped each of the 463 parasitoid larvae at 14 microsatellite loci (Hho3 (Kankare et al., 2004) and Hho11, Hho12, Hho13, Hho15, Hho16, Hho17, Hho18, Hho19, Hho21, Hho22, Hho23, Hho24, and Hho25 (Couchoux et al., 2015)). Samples were sexed based on their microsatellite heterozygosity. As a Hymenoptera, male *H. horticola* are haploid. The probability of an individual being a genuine diploid (= female) and a homozygote in all 14 loci at the same time was very low ($p \leqslant 3 \times 10^{-7}$, Couchoux et al., unpublished data). Therefore individuals homozygous in all loci were considered to be haploid males.

Within each parasitized host cluster, we assigned parasitoid larvae to full-sib groups to assess the number of mothers that had parasitized the cluster. This was done using the full likelihood method implemented in Colony 2.0.3.1 (Wang, 2004). For each host cluster, we performed one medium run with error rates of zero for allelic dropout and genotyping errors. Because we did not allow genotyping errors, the number of sib groups in each cluster might be slightly overestimated. The clustering was based on the allelic frequencies of the local habitat patch, and assuming monogamy as the reproductive system (Couchoux et al., unpublished data). We ran the analysis for female (diploid) offspring, which allowed us to identify the full-sib groups in each host cluster, and to deduce the mothers' genotype for each group. The analytical software Colony can only handle diploid offspring so we assigned the male (haploid) offspring to their mothers manually by individually matching their genotypes. The number of mothers per host egg cluster, cluster size and parasitism rate were log transformed and analysed with Pearson correlations in R (R Core Team, 2012).

3. Results

3.1. Behavioural experiment

3.1.1. Probing/parasitizing

The wasps probed/parasitized all the host egg clusters in unmarked patches whether or not they had been previously probed/parasitized (P + UM and UP + UM treatments). In contrast, patches that had been marked were probed/parasitized in only 38% of the parasitized (P + M), and

42% of the unparasitized (UP + M) trials (Figure 2a). Thus, the wasps probed/parasitized host eggs in patches that were marked significantly less often than those in patches that were unmarked. Egg cluster size and the interaction of egg treatment and cluster size had no effects (Table 1). Furthermore, duration of the previous marking in P + M and UP + M treatments had no effect on whether the second wasp probed.

The probing/parasitizing of host eggs clusters that had been parasitized and/or marked was very brief. While the wasps spent 11, 21 and 13 min, respectively, on P + M, P + UM and UP + M eggs (Figure 2b), eggs that had not been parasitized and where in unmarked patches (UP + UM) were probed/parasitized for 40 ± 12 min (mean ± SD). Thus, the second wasp spent much less time probing/parasitizing the hosts than the first one, regardless whether the patch had been, marked, parasitized, or both. The wasps in all treatments also spent more time probing/parasitizing as host egg cluster size increased (Table 1).

3.1.2. Marking

After a wasp had probed/parasitized a cluster, it went on to mark the host patch in 66% of the trials (including all treatments $N = 98$), whereas a wasp never marked a patch that it did not also probe in ($N = 44$) (Table 1). Most wasps that probed an egg cluster also marked the patch if the eggs were in the UP + UM (86%) and P + UM (71%) treatments. Fewer of the P + M (19%) and UP + M (39%) patches were marked (Table 1, Figure 3a), though the difference between unmarked treatments and the UP + M treatment is not statistically significant, probably because of the lower effective number for UP + M.

Wasps that marked the patches spent 10 s to 44 min doing it. Because the wasps rarely marked the previously marked patches (parasitized or not), duration of marking was only analysed to compare the UP + UM and P + UM treatments. Egg treatment, duration of probing and cluster size were all related to the amount of time a wasp subsequently spent marking a host patch (Table 1). The wasps marked UP + UM patches (control) for a significantly longer time than P + UM patches (7 ± 4 min and 1.5 ± 1 min (mean ± SD), respectively; Figure 3b). Furthermore, the time spent marking the host patch increased with both the time spent probing the eggs and the size of the cluster (Table 1).

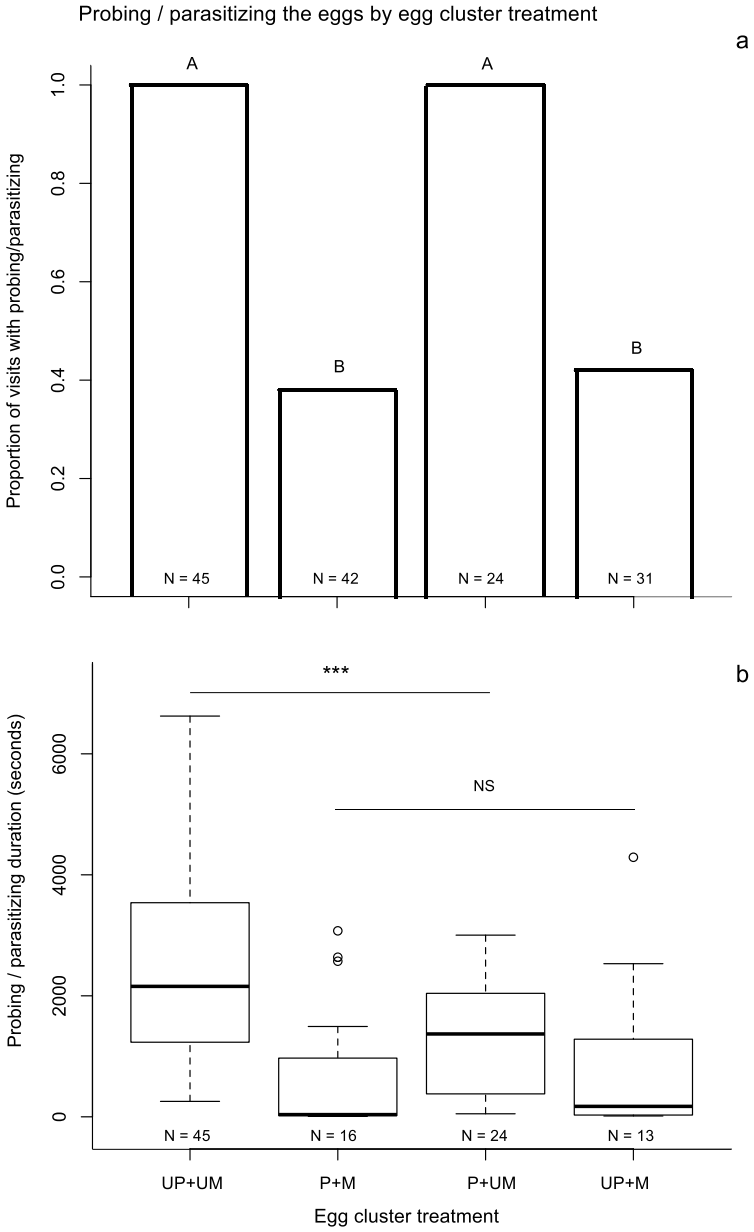

Figure 2. Proportion of visits in which the wasps probed the eggs (a) and duration of the probing (b) for control (UP + UM), parasitized + marked (P + M), parasitized (P + UM), and marked (UP + M) host egg clusters. Different letters indicate a significant difference ($p < 0.001$). *** Significant difference ($p < 0.001$); NS, no significant difference.

Table 1.
Analysis of variance table for the generalized linear mixed models of: probing occurrence, probing duration, marking occurrence, marking duration and parasitism rate.

Response variable	Explanatory variable	df	*F*	*p*
Probing occurrence	Egg treatment[a]	3	168.79	<0.0001*
	Cluster size[b]	1	0.98	0.33
	Egg treatment × cluster size	3	0.85	0.47
Probing duration	Egg treatment	3	23.04	<0.0001*
	Cluster size	1	4.28	0.04*
	Egg treatment × cluster size	3	2.48	0.08
Marking occurrence	Egg treatment	3	101.27	<0.0001*
	Probing occurrence[c]	1	100.90	<0.0001*
	Cluster size	1	0.71	0.40
Marking duration	Egg treatment	3	11.22	0.0005*
	Probing duration[d]	1	8.90	0.01*
	Cluster size	1	5.87	0.02*
Parasitism rate	Egg treatment	3	0.91	0.03*
	Cluster size	1	1.18	0.16
	Egg treatment × cluster size	3	0.48	0.35

Host egg cluster ID, wasp ID and the number of times the wasp has been observed before are included in all the models as random effects.

* Significant value ($p < 0.05$).

[a] Egg treatment is a categorical variable with four groups: control (UP + UM), parasitized (P + UM), parasitized + marked (P + M) and marked (UP + M).

[b] Cluster size is a continuous variable ranging from 31 to 327 eggs.

[c] Probing occurrence is a binary variable with presence or absence of the behaviour.

[d] Probing duration is a continuous variable ranging from 7 s to 110 min.

3.1.3. Parasitism rate

The rates of parasitism (proportion of hosts parasitized in an egg cluster) did not differ between the UP + UM (27%), P + M (27%), and P + UM (32%) treatments, and all were significantly greater than the UP + M treatment (13%). There was no significant effect of egg cluster size on the rate of parasitism in a host egg cluster (Table 1).

3.2. Maternity assignment

All in all, we assigned 96% of the *H. horticola* larvae to mothers. The missing data were due to amplification failing in multiple loci, probably because of poor DNA quality. The host egg clusters contained the progeny of one to eight *H. horticola* females (median = 2, mean = 2) (Figure 4a). On average, 74% (range: 43–100%) of the offspring were full-siblings, showing that only

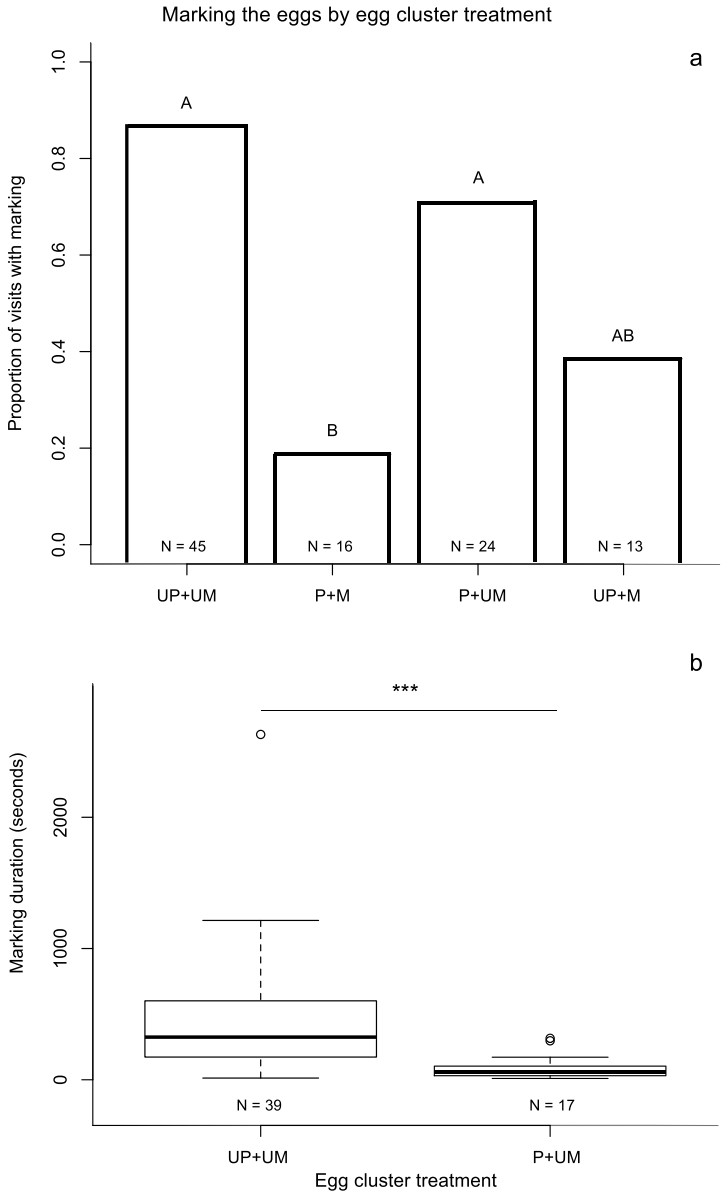

Figure 3. Proportion of visits in which the wasps marked the eggs for control (UP + UM), parasitized + marked (P + M), parasitized (P + UM), and marked (UP + M) host egg clusters (a) and duration of the marking for control (UP + UM) and parasitized (P + UM) host egg clusters (b). Different letters indicate a significant difference ($p < 0.05$). *** Significant difference ($p < 0.001$).

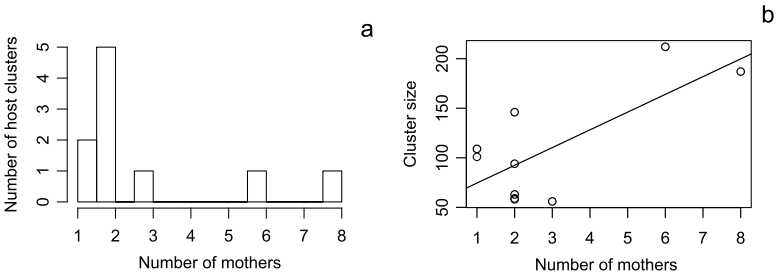

Figure 4. (a) Number of host clusters parasitized by one to eight different *H. horticola* females and (b) number of caterpillars in the host cluster as a function of the number of females that parasitized the host cluster, in ten host clusters naturally parasitized in the field.

one female laid the majority of eggs in each cluster (Figure 5). Parasitism rate of the host clusters varied from 0.18 to 0.86 (average 0.46). The rate of parasitism was not associated with the number of mothers (Pearson's correlation, cor $= 0.19$, $p = 0.60$). The size of the host cluster and the number of females parasitizing were positively associated (Pearson's correlation, cor $=$ 0.73, $p = 0.017$) (Figure 4b). However, when excluding the two unusually large host egg clusters (>180 host individuals, Saastamoinen (2007)), the correlation was marginally non-significant (cor $= 0.63$, $p = 0.067$).

4. Discussion

We examined the egg laying behaviour of the parasitoid wasp *H. horticola* in a competitive foraging environment. We found that *H. horticola* females mark the host patch after oviposition, post-oviposition marking deters conspecifics from probing host eggs, and oviposition deters them from laying eggs in previously parasitized hosts. The deterrent effect that we observed in the laboratory experiment was assessed under natural conditions by determining the number of females parasitizing individual host clusters in the wild. We found that a host cluster is primarily parasitized by one female and, even when several females parasitize the same host egg cluster, the great majority of the offspring are full siblings.

4.1. Deterrent effect

Post-oviposition marking of hosts, as a way to protect offspring from competition by deterring future exploitation, is widespread (reviewed in Roitberg & Prokopy, 1987; Nufio & Papaj, 2001; Liu et al., 2012). As expected,

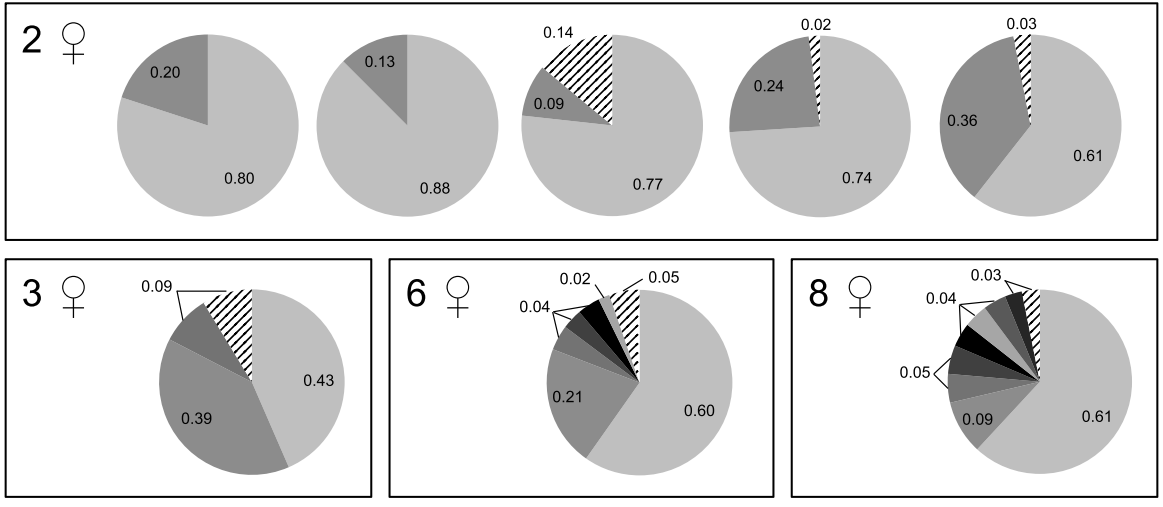

Figure 5. *Melitaea cinxia* caterpillars parasitized by *H. horticola* in eight host clusters (the two clusters parasitized by only one female are not represented here). Different shades of grey represent the proportion of parasitoid offspring mothered by different females in each host cluster. Striped grey represents offspring of unknown mothers (DNA not good enough for genotyping).

marking by *H. horticola* was (1) strongly associated with oviposition as the wasps were only observed marking a host patch in which they had probed/parasitized the eggs, and (2) effective in deterring conspecifics from using an already parasitized host cluster. Behavioural experiments showed that wasps avoided probing marked host patches, whether the eggs had been parasitized or not. Interestingly, the duration of marking did not affect whether or not the second wasp probed the host eggs. That is, longer marking was not more deterrent. As observed in other Hymenoptera (Chow & Mackauer, 1999; Agboka et al., 2002; Stelinski et al., 2007), post-oviposition marking had a deterrent effect but previous oviposition alone had no effect on the wasps' propensity to start to probe the eggs.

Previous oviposition, however, did affect the behaviour of subsequent wasps by decreasing the amount of time spent probing/parasitizing. When the second wasp did probe the parasitized egg cluster, it did so for a short time only, laying few or perhaps no eggs. As a result, parasitism rate when two wasps parasitized the host cluster (P + M and P + UM) was not higher than when only one did (UP + UM). Parasitized but unmarked patches (P + UM) were also not parasitized at a higher rate than marked ones (P + M). Further, parasitism rate of host clusters parasitized in the field did not increase when more than one wasp parasitized the cluster. So, previous oviposition, in contrast to marking, does not deter wasps from probing the eggs. Yet it still prevents subsequent wasps from laying many eggs in previously parasitized host clusters. This maintains the known consistent partial exploitation of each *M. cinxia* egg cluster in the landscape, in spite of competition for hosts (van Nouhuys & Hanski, 2002; van Nouhuys & Ehrnsten, 2004; Montovan et al., 2015).

Our hypothesis is therefore that when the mark is effective only one female parasitizes a host egg cluster, and that when the mark is ineffective or is absent several individuals end up probing the same host cluster. Under these conditions the first female would parasitize most of the available hosts (up to about one third), while the subsequent females would parasitize only few. Genetic analyses conducted on host clusters parasitized in the field showed both situations. Two of the 10 host clusters were parasitized by only one wasp and the others were parasitized by 2–8 different females, with one female mothering the great majority of the offspring. This difference cannot be attributed to different levels of competition between wasps, as each site was

known to have high-densities of wasps. We therefore speculate that host clusters parasitized by only one female were well protected by a deterrent mark while clusters parasitized by several individuals were not. They may have been unmarked, or the mark may have been ineffective, as seen in around 40% of the laboratory trials.

It is possible that some individuals do not respect the deterrent mark and lay a few eggs in many host clusters as an alternative strategy (Gross, 1996; Taborsky, 1998), or that strong competition among foraging *H. horticola* females (Couchoux & van Nouhuys, 2014) could increase females' motivation to oviposit, leading them to override the deterrent signal. Indeed, in other research systems, competition for hosts has led to several parasitoids using the same host patch and an increase of superparasitism (van Alphen & Visser, 1990). On average, the dominant female *H. horticola* has at least four times more offspring in a host cluster than the other females. Thus, to achieve the same reproductive success, a female following the alternative strategy would have to lay eggs in at least four times as many host clusters.

We also found that the number of females parasitizing a host cluster increased with cluster size, but only when the clusters were exceptionally large. It is possible that there is a size threshold above which clusters attract more foraging females. Indeed, large host clusters are of high value, both because of the number of hosts available for parasitism, and because the gregarious host caterpillars benefit from large group size (van Nouhuys et al., 2003; Kuussaari et al., 2004).

4.2. Why mark?

We found that oviposition, even in the absence of a mark, deters wasps from laying eggs in previously parasitized host clusters. Furthermore, *H. horticola* successfully avoids superparasitism of individual eggs while parasitizing (Montovan et al., 2015). Therefore, because marking has physiological costs and takes time, we can ponder why the wasps do it.

For the mark to have evolved, it should benefit the marking female. It might mark to avoid wasting effort (time or energy) revisiting and defending clusters it has already parasitized. Avoiding revisitation also may insure the physical integrity of the parasitized host egg cluster, which is beneficial to the wasp. *Hyposoter horticola* is much larger than the host eggs it attacks and, when parasitizing, often causes some eggs to fall on the ground, where the newly hatched caterpillars die. Furthermore, probing the eggs could be

harmful to the caterpillars as it might increase vulnerability to disease and infection.

For the mark to be deterrent to subsequently visiting conspecifics it also has to be beneficial to them. Montovan et al. (2015) showed that when about one third of the hosts in a cluster are parasitized the wasp encounters many already parasitized hosts and, according to optimal foraging theory (including a high cost for superparasitism and later hyperparasitism), it becomes beneficial to leave the cluster and forage for another one. Therefore, a mark indicating a host cluster is already parasitized would be beneficial to all individuals, not only the one marking.

A topic not considered in this study is discrimination between self and non-self mark and consequently, between self-superparasitism and superparasitism by conspecifics. Indeed, superparasitism (self- or conspecific) can be adaptive under certain circumstances, and wasps have evolved to recognize hosts that they themselves have recently parasitized (Bernstein & Driessen, 1996; Field & Keller, 1999; McKay & Broce, 2004). For example, conspecific superparasitism can be adaptive when there is some probability of eliminating non-sibling competitors (Nufio & Papaj, 2001), and self-superparasitism can be adaptive when increasing the density of juvenile stages saturates the defences of a host (van Alphen & Visser, 1990). In *H. horticola* superparasitism is not adaptive as only one individual can develop inside a host, and, in case of competition, the oldest larva is the one that is likely to survive. However, under extreme circumstances, an *H. horticola* might resort to superparasitism, in which case it would be better to superparasitize hosts in clusters parasitized by conspecifics, rather than clusters it has parasitized itself (van Alphen & Visser, 1990).

4.3. Conclusion

Intraspecific communication is central to the lives of many animals such as territorial birds (Mougeot et al., 2003), and of course social insects (d'Ettorre, 2008). However, this behaviour, which has costs to both the signaller and the recipient, has not been well studied in solitary insects. Here we show that a solitary parasitoid wasp defends its offspring by depositing a deterrent chemical mark after ovipositing into host egg clusters. Under laboratory conditions we show that, while the wasp is known to avoid superparasitism (Montovan et al., 2015), patch marking deters subsequent wasps from probing the host eggs, and oviposition deters those that probe from laying eggs

in previously parasitized host clusters. We go on to show, using maternity assignment, that this behaviour generally carries over to wasps in the natural environment. While there are several studies of host marking by parasitoid wasps (Nufio & Papaj, 2001; Stelinski et al., 2007), this is to our knowledge the first study addressing how the deterrent marking by a parasitoid functions in a complex natural setting as well.

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