

Discrimination of the age of conspecific eggs by an ovipositing ectoparasitic wasp

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Abstract

The recognition and avoidance of already parasitized hosts is a major issue in parasitoid behavioural ecology. A key factor affecting the fitness reward expected from superparasitism is the probability that the second or subsequent egg laid on a host will win the contest with the first-laid egg. The present study investigated the ability of females of the solitary ecto parasitoid *Anisopteromalus calandrae* Howard (Hymenoptera: Pteromalidae) to (i) discriminate between unparasitized *Callosobruchus maculatus* (Fabricius) (Coleoptera: Bruchidae) hosts and those parasitized by a conspecific, and (ii) discriminate between a host parasitized by an egg just laid (2 h) and one parasitized by an egg about to hatch (28 h). However, they did not adjust their offspring sex ratio on already parasitized hosts compared to unparasitized ones. Our results show that *A. calandrae* females can discriminate between parasitized and unparasitized hosts, as they lay more eggs on the latter. The probability of the second or subsequent egg laid on a host (superparasitism) winning the contest with a conspecific increases as the time between the two ovipositions decreases. Consequently, parasitoid females should lay more eggs on recently parasitized hosts than on those that have been parasitized for a long time (i.e., when the first eggs are about to hatch), and that is indeed what they were found to do. To increase their fitness in spite of the presence of already parasitized hosts, *A. calandrae* females have developed highly discriminative capacities regarding the parasitism status of hosts.

Introduction

Parasitoid females are expected to adapt their oviposition strategies in different ways in order to maximize their reproductive success. For example, they could adjust their offspring sex ratio to environmental factors (Godfray, 1994). One such factor that has been studied in depth is the level of intra-specific competition. According to the local mate competition theory (LMC; Hamilton, 1967), females foraging alone on a patch produce more daughters than sons, while females competing on a given patch adjust their oviposition strategies to an unbiased sex ratio. By depositing more sons, a female increases the chance that her sons will inseminate most of the females in the reproductive patch, including her own daughters and those of other females (King, 2002). While the LMC theory was primarily developed for gregarious species, it can also be applied to

solitary species that reproduce on an aggregate of hosts and are thus considered as semi-gregarious (Godfray, 1994). The LMC model can also be applied to indirect competition, that is, when an isolated female is confronted with already parasitized hosts (Werren, 1980; Shuker et al., 2006; Darrouzet et al., 2008). In this situation, females adapt their sex ratio as if they are in a direct competition situation, even if they never encounter a conspecific female.

When a female encounters a parasitized host, she can either reject it and continue to search for unparasitized hosts, or accept it and superparasitize that host (i.e., lay an egg on an already parasitized host). Gregarious as well as solitary species can superparasitize hosts, but in solitary parasitoids, the presence of supernumerary juveniles results in a contest competition and the death of all but one immature. This competition has been defined as lethal larval combats (Ueno, 1997), physiological suppression (Vinson & Hegazi, 1998), or scramble competition (Mayhew & Hardy, 1998). While the expected fitness gain per host

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is lower when females superparasitize, superparasitism by solitary species can be adaptive under certain conditions (van Alphen & Nell, 1982), for example, when the number of unparasitized hosts is small or when travel time between patches is long (Waage, 1986; van Alphen & Visser, 1990). In fact, this behaviour is adaptive when the second egg laid on an already parasitized host can win the competition with the first immature (van Baaren & Nénon, 1996). In some species, females perform ovicide (i.e., killing the first egg) before ovipositing. In this situation, females restore host quality and the host is once again acceptable for oviposition (Godfray, 1994).

In solitary parasitoids, as females have to optimize their investment in eggs on a given host, they need to be able to assess the quality of that host and the probability of survival of their progeny. Female parasitoids have been shown to discriminate between unparasitized and parasitized hosts in several species (Gauthier et al., 1996; Weber et al., 1996; Santolamazza et al., 2004; Darrouzet et al., 2007). However, additional information could also help the female to adjust her progeny allocation on already parasitized hosts, such as the sex of the juvenile on the host (van Baaren et al., 1999), its age (Visser et al., 1992), or its species (Gauthier et al., 1999). Age, that is, the development stage of the first juvenile, is an important factor, as it could strongly influence the survival of an egg laid on an already parasitized host. The probability of survival of the second egg generally decreases as the time between the first and second oviposition increases (van Baaren & Nénon, 1996). This is due to the previous hatching of the first egg: the first instars actively seek eggs and larvae on the hosts, and try to eliminate these competitors by attacking them with their mandibles (van Alebeek et al., 1993). If the survival of the second juvenile depends on the development stage of the first, we could expect a strong evolutionary pressure on the female to be able to assess this development stage and to adjust her oviposition strategy accordingly. While some studies have shown the influence of the time interval between ovipositions on a female's acceptance of a parasitized host (Yamada & Ikawa, 2005), or on the survival of the second egg laid (Visser et al., 1992; van Baaren & Nénon, 1996), these studies have not generally investigated these two aspects in combination. However, Goubault et al. (2003) have shown that females of the solitary pteromalid *Pachycrepoideus vindemniae* Rondani adapt their oviposition strategies according to the development stage of the first juvenile, from egg to pupal stages. Furthermore, the period of egg maturation on the host could be particularly important. Because of the capacity of the first immature to kill eggs, the survival of a second egg should strongly decrease after the first egg hatches. Therefore, the oviposition strategies of superparasitizing females during this

period (before hatching) are crucial to their fitness. To our knowledge, the capacity of parasitoid females to assess egg development on a host prior to superparasitism has never been demonstrated.

The main purpose of this study was to investigate whether isolated females of a solitary parasitoid wasp can assess the developmental stage of a previously deposited egg and if they adjust their reproductive strategy accordingly. *Anisopteromalus calandrae* Howard (Hymenoptera: Pteromalidae) is a solitary generalist ectoparasitoid that attacks many coleopteran pest larvae and pupae (Ahmed, 1996) and is used throughout the world for biological control of pests in seed stock. We investigated (1) the host-discrimination capacity (ability to discriminate between unparasitized and parasitized hosts) of ovipositing females, and (2) the survival probability of an experimentally deposited egg under superparasitism conditions depending on the time interval after the first egg was laid. We then analyzed whether females could adapt their oviposition strategies, that is, superparasitism, ovicide, and offspring sex ratio, when confronted with a host parasitized by an egg just laid and a host parasitized by an egg about to hatch.

Materials and methods

Rearing conditions

Anisopteromalus calandrae were reared in the laboratory on larvae and pupae of one of their natural hosts from the tropical zones of West Africa, *Callosobruchus maculatus* (Fabricius) (Coleoptera: Bruchidae). Both *C. maculatus* and *A. calandrae* originated from Ivory Coast (collected in 2000) and were reared in the laboratory under conditions close to that of their area of origin: 12 h light at 30 °C, 12 h dark at 22 °C, and 71% r.h. Two homozygous strains of *A. calandrae* were isolated in 2003, one red-eyed mutant (R) and one black-eyed wild type (B), to allow the offspring to be monitored. As previous studies found no differences in oviposition behaviours between the two strains (Do Thi Khanh et al., 2005; E Darrouzet, personal observations), the oviposition strategy of only one strain was tested in the following experiments.

General methods

To obtain parasitoid eggs, five gelatine capsules each containing one *C. maculatus* L4 larva were offered to a group of 10 virgin *A. calandrae* females of a single strain confined to an arena (2.5 cm high × 8 cm in diameter). Hymenoptera parasitoids reproduce by arrhenotokous parthenogenesis, whereby fertilized (diploid) eggs develop into females and unfertilized (haploid) eggs into males. By using virgin females, we were thus able to obtain male eggs only, thereby preventing a bias due to the sex of eggs

presented to tested females. The bruchid L4 larvae were placed inside the capsules after removal from seeds by dissection and selection by size (9.12 ± 0.20 mg). The gelatine capsules mimic the bruchid pupal chamber in the seed and are accepted for oviposition by females (Darrouzet et al., 2003). Oviposition activities occurred at L12 (30 °C):D12 (22 °C) and 71% r.h. At the end of the exposure period, eggs on parasitized hosts were located under a dissecting microscope and manipulated as described below.

Mated females were obtained by placing a 2-h-old virgin female with a 24-h-old virgin male of the same strain in a Petri dish. Mating was confirmed by direct observation.

Host discrimination capacity of an ovipositing female

We wished to investigate whether females are able to discriminate between unparasitized and parasitized hosts. Groups of 10 virgin females (strain B) were exposed to five hosts for 8 h. Parasitized capsules were isolated and stored at 4 °C overnight. Capsules with parasitized hosts (one egg per host) were then used to prepare reproductive patches that were offered to isolated mated females of strain R. Each patch contained a circle of six equidistant gelatine capsules each containing one host. To perform aggregates of hosts with different qualities, two areas were prepared (Darrouzet et al., 2007): three adjacent capsules contained one unparasitized host, and three contained a host parasitized 24 h earlier by a virgin female of strain B. For 5 days, individual R-mated females ($n = 11$) could lay eggs in patches containing unparasitized and parasitized capsules (two 4-h exposure periods per day).

At the end of each 4-h exposure period, the positions of all eggs laid on unparasitized and parasitized hosts were noted and each egg laid on a host was transferred onto a different unparasitized host and deposited in a separate cell in a Plexiglas sheet closed by a Plexiglas cover-slide until emergence of the parasitoid adults (Darrouzet et al., 2003, 2007). At emergence, the maternal origin of each individual was identified by its eye colour (strain R or B). The sex ratio (proportion of males) was noted at emergence for the individuals (secondary sex ratio) in each series of experiments (with or without indirect competition). At the start of each 4-h period, entire patches were replaced (Darrouzet et al., 2003, 2007).

The sex ratio of females without competition was controlled in another experiment in which R-mated females were confronted with patches composed of six unparasitized hosts. The experiment was performed as previously described.

Survival of supernumerary eggs

We wished to investigate whether the development stage of an egg on a host could influence the survival of a second egg added to that host. Virgin females of both R and B

strains were offered hosts for a 1-h period and eggs were collected as previously described. Eggs of a given strain (R or B) were deposited individually on a host and stored in a climatic chamber. After different time intervals covering the entire development of the first egg, that is, 0 ($n = 79$), 2 ($n = 76$), 5 ($n = 82$), 6 ($n = 80$), 22 ($n = 50$), 24 ($n = 61$), and 30 h ($n = 77$), a second freshly laid egg from the other strain was added to the parasitized host. Under our experimental conditions, hatching took place after about 31 h. Oviposition intervals of 8–16 h were not considered, because that would imply that oviposition took place during the night, when *A. calandreae* females do not oviposit (S Lebreton, personal observation). The replicates were balanced so that the first eggs deposited came almost equally from one strain or the other. At emergence, the phenotype (eye colour) of the surviving individual was noted for each oviposition interval.

Discrimination of the age of eggs

Are females able to discriminate between a host parasitized by an egg just laid (2 h) and one parasitized by an egg about to hatch (28 h)? These two extremes were used to analyse the oviposition behaviour of females confronted with hosts of different quality. Strain B virgin females were allowed to oviposit during 2-h intervals on unparasitized hosts. After this period and/or after 28 h, gelatine capsules with parasitized hosts were collected. One capsule of each category (2 or 28 h, only one egg per capsule) was placed in a Petri dish in a climatic chamber and presented to an R-mated female for a choice test ($n = 61$). The oviposition sequence was noted: first capsule visited, capsule selected (i.e., the capsule on which the female settled after examining the patch), time elapsed before oviposition, and oviposited capsules. After oviposition, the female was removed and each egg was transferred onto a different host and deposited in a separate cell in a Plexiglas sheet until emergence of parasitoid adults. After emergence, the sex ratio of the second egg laid (identified by the adult's eye colour) and the mortality rate of the first egg laid (to determine whether the action of the second female would interfere with the development of the first egg) were determined for each kind of selected capsule (2 and 28 h).

Statistical analysis

To analyse host discrimination capacity, a non-parametric paired design (Wilcoxon test) was used to compare the number of eggs laid on the parasitized and unparasitized hosts and the number of hosts parasitized. A χ^2 -test was performed to analyse sex ratios.

The proportion of surviving eggs deposited under superparasitism conditions was analysed in relation both to oviposition intervals and to each B-R and R-B combination

using a general linear model (GLM) with a logit link and a binomial error distribution. Factor effects were analysed using a χ^2 -test on a complete model containing the following effects: oviposition intervals, combination of strains, and the interval–combination interaction.

To analyse whether females could discriminate the age of the egg, a χ^2 -test was used to analyse the choice test results and the sex ratio. A Fisher's exact test was used to compare sex ratios with a small sample. A non-parametric Mann–Whitney U-test was used to analyse the laying time. Analyses were carried out with R software (R 2.1.1, R Development Core Team, Free Software Foundation Boston, MA, USA). For all tests, a threshold level of $P < 0.05$ was used.

Results

Host discrimination capacity

In an indirect competition context, significantly more unparasitized hosts were attacked ($Z = 2.93$, $P = 0.0033$) and more eggs were laid on them than on parasitized hosts ($Z = 2.93$, $P = 0.0033$; Figure 1). The overall sex ratio in the entire patch was female biased (0.42; $\chi^2 = 7.1$, d.f. = 1, $P = 0.007$) and not different from those laid by females without competition (0.37; $\chi^2 = 1.84$, d.f. = 1, $P = 0.17$). The sex ratio deposited on unparasitized hosts (0.42) was similar to that on parasitized hosts (0.44; $\chi^2 = 0.18$, d.f. = 1, $P = 0.67$). Under our conditions, 16% of eggs failed to develop.

Survival of supernumerary eggs

The proportion of surviving eggs deposited under superparasitism conditions was similar for the two strains

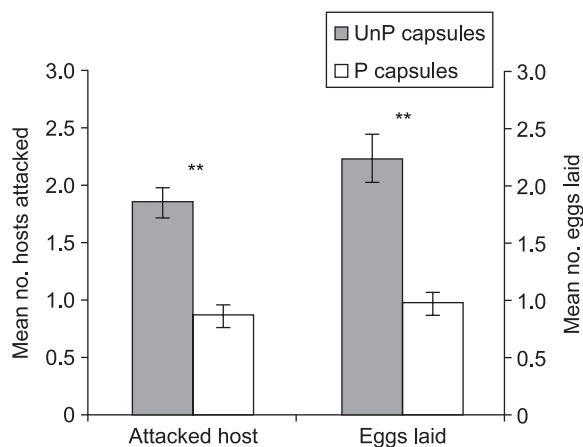


Figure 1 Mean (\pm SE) number of *Callosobruchus maculatus* hosts attacked and eggs laid by mated *Anisopteromalus calandrae* females per patch ($n = 11$) when ovipositing on unparasitized (UnP) or previously parasitized (P) hosts. ** $P < 0.01$.

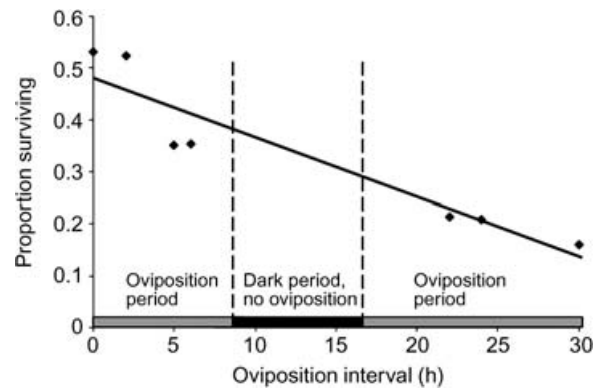


Figure 2 Relationship between the proportion of surviving *Anisopteromalus calandrae* eggs deposited under superparasitism conditions and the time elapsed since the first egg was laid. Proportion of surviving eggs observed (diamond) and linear regression (solid line). No differences were found between the two strains.

(R and B; GLM: Deviance = 0.29, d.f. = 1, $P = 0.59$), and their variations with oviposition intervals were not significantly different (GLM: deviance = 2.03, d.f. = 1, $P = 0.15$). The incidence of survival decreased as oviposition intervals increased (GLM: deviance = 34.83, d.f. = 1, $P < 0.001$; Figure 2). The probability of survival of two eggs laid simultaneously on the same host was the same (0.53; $\chi^2 = 0.26$, d.f. = 1, $P = 0.61$), whereas it decreased to 0.16 for an egg deposited 30 h after the first egg (Figure 2). In our experiments, 15% of B eggs and 18% of R eggs failed to develop under single-development conditions, with no difference between the two strains ($\chi^2 = 0.21$, d.f. = 1, $P = 0.65$).

Discrimination of the age of eggs

In the choice test, 9.8% of females did not examine any capsule. There was no significant difference in the first capsule visited, but the females selected the hosts parasitized for 2 h significantly more often than those parasitized for 28 h (Table 1). About half (49.1%) of these females did not lay eggs on parasitized hosts, but when they did, it was significantly more often on those parasitized for 2 h (Table 1). Forty-eight percent of ovipositing females laid an egg on a host after rejecting the other one; most of them (73%) rejected the 28-h capsule and laid eggs preferentially on the 2-h parasitized host ($\chi^2 = 4.49$, d.f. = 1, $P = 0.03$). Duration of oviposition was similar for 2- and 28-h parasitized hosts (53.3 ± 5.03 min for 2 h and 52.5 ± 10.46 min for 28 h; $Z = 0.47$, $P = 0.64$).

The sex ratios of eggs laid on 2- and 28-h parasitized hosts were similar (0.12 and 0.15, respectively; Fisher's test: $P = 0.49$) and female biased ($\chi^2 = 13.5$, d.f. = 1, $P < 0.001$).

Table 1 Behavioural and oviposition strategies of *Anisopteromalus calandrae* females confronted with *Callosobruchus maculatus* hosts parasitized once, at 2 or 28 h previously. Number of host larva types visited first, selected, and oviposited upon by the female wasps

	Host parasitized 2 h previously	Host parasitized 28 h previously	χ^2	d.f.	P-value
First capsule visited	33	22	2.20	1	0.138
Host selected	34	13	9.38	1	0.002
Egg laid	19	8	4.48	1	0.034

Under superparasitism conditions, the mortality rate of the first egg laid (22%) was not significantly different from the control (16.5% for single parasitism) under our experimental conditions ($\chi^2 = 0.87$, d.f. = 1, $P = 0.35$).

Discussion

The present study demonstrates that *A. calandrae* females adapt their level of acceptance of a parasitized host according to the development of the first egg laid on the host. In solitary parasitoids, where only one adult can emerge from a host whatever the number of eggs initially laid, it is adaptive for females to distinguish between unparasitized and parasitized hosts. However, the quality of parasitized hosts may vary due to the development of the first juvenile, and the female may gain an advantage by discrimination based on that quality.

As expected, *A. calandrae* females discriminated unparasitized from parasitized hosts and laid more eggs on unparasitized hosts that could be considered to be of better quality. This discrimination ability has been demonstrated in most parasitoid species tested (Gauthier et al., 1996; Santolamazza et al., 2004; Darrouzet et al., 2007). For *A. calandrae* females, the benefits of recognizing parasitized hosts translate into a higher probability of egg survival on unparasitized hosts. The sex ratios produced by isolated females when in indirect competition are female-biased and not different from those produced by females that are not in a competition context. These results run counter to the LMC theory (Werren, 1980), which predicts that under indirect competition conditions females will produce more sons in order to inseminate daughters of other females. However, *A. calandrae* is a solitary species, and it is possible that females do not adjust their sex ratio as predicted by the LMC theory, as already observed in *Anaphes nitens* Girault (Santolamazza & Rivera, 2003). In our experiment, all the eggs offered to females were male; under this condition, if females are able to recognize the sex of eggs previously laid, laying more males is with no benefit to them when no female eggs have been laid in the vicinity. A similar result has previously been reported by Werren (1984) with *Nasonia vitripennis* (Walker) females, in which the sex

ratio was not influenced by the competition context when the first female was virgin.

Our main results indicate that *A. calandrae* females can recognize whether the hosts they encounter have been parasitized by eggs just laid or by eggs about to hatch. They can therefore adapt their oviposition according to the probability of their offspring reaching adulthood. When two eggs are laid at the same time on the same host, their chance of survival is about 50%, but the incidence of survival of the second egg deposited on a parasitized host decreases to about 16% when the time interval between the two ovipositions increases. Hosts with an egg that is about to hatch are thus of lower quality for the female, who therefore avoids these hosts, ovipositing preferentially on a recently parasitized host, that is, one on which their offspring has a greater chance of survival. This result suggests that *A. calandrae* females have developed highly sensitive discrimination capacities to maximize their fitness. However, the present study analysed the oviposition strategies of females confronted with hosts containing eggs at developmental extremes. We did not analyse their capacity to discriminate between hosts containing eggs at intermediate developmental stages, although this affects the probability of their offspring winning the competition.

How can a female assess the developmental stage of a previously deposited egg? Is it the same cue used by a female to discriminate unparasitized from parasitized hosts? Host discrimination is often mediated by a chemical marker (van Alphen & Visser, 1990) deposited on the seed or host by ovipositing females. This substance originates from the Dufour gland and is deposited after oviposition (Marris et al., 1996; Jaloux et al., 2005). Under our experimental conditions, we observed that half the females that laid an egg oviposited after visiting and rejecting the other host, which was usually the 28-h parasitized host. Before rejecting a host, many females examined the capsule with their antennae, without introducing their ovipositor into the capsule (S Lebreton, personal observations). This suggests that there could be a chemical cue at the surface of the capsule, probably perceived by receptors on the antennae. The females' ability to discriminate based on the time elapsed since a previous oviposition could be based on a

quantitative or qualitative modification of this marker. It is also possible that different cues give sequential information about the parasitized status of the host over time (Outreman et al., 2001) and could be responsible for the observed discrimination of egg age. Further studies are thus necessary to determine the nature of the cue involved in discrimination by *A. calandrae*.

When encountering parasitized hosts, females could also kill the first egg (ovicide) prior to ovipositing their own egg. However, the low mortality level of the first egg laid suggests that *A. calandrae* females do not perform ovicide. In fact, ovicide is only adaptive when the time and energy costs to the female who performs it are low compared to the benefit gain for her egg (Netting & Hunter, 2000). The fact that ovicide is not performed by *A. calandrae* females could be due to their inability to localize previously deposited eggs precisely.

To conclude, the present work furthers our understanding of the recognition and avoidance of already parasitized hosts. *Anisopteromalus calandrae* females detect the parasitized status of hosts and act accordingly. In superparasitized hosts, the probability of the first parasitoid larvae winning the contest with conspecifics increases with the time interval between ovipositions. Females have therefore developed highly sensitive discrimination capacities regarding host quality in order to increase their fitness.

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