Impact des combats larvaires sur les stratégies de ponte des femelles

Contexte de l’étude

Comme nous l’avons vu dans la partie précédente, les stratégies employées par les parasitoïdes influencent la sélection de l’hôte. Ainsi, en fonction de leur état physiologique, un certain nombre de femelles choisissent préférentiellement des hôtes déjà parasités (notamment afin de s’y nourrir), mais finissent par pondre dessus. La probabilité de survie de ces œufs est réduite de par la présence de compétiteurs. Cela se traduira par des combats larvaires aboutissant à la mort de toutes les larves sauf une. Cependant, tous les hôtes parasités ne sont pas de qualité égale et la probabilité de survie d’un deuxième œuf pondu peut varier d’un hôte à l’autre. L’intervalle de temps entre les deux pontes ainsi que le sexe des larves sont des paramètres déterminant pour la probabilité de survie du deuxième individu. En effet, on observe généralement une forte diminution de la probabilité qu’une deuxième larve gagne le combat dès lors que l’intervalle de temps entre les deux pontes augmente. Un œuf pondu sur un hôte sur lequel une larve est déjà présente aura par conséquent une probabilité de survie très faible qui pourra parfois avoisiner le zéro. Les heures suivant la première ponte jusqu’à l’éclosion du premier œuf sont donc une période clé pour une femelle choisissant de pondre sur un hôte déjà parasité. Par conséquent, si les femelles parasitoïdes sont capables de déterminer l’intervalle de temps écoulé depuis une première ponte, elles devraient préférentiellement pondre sur les hôtes parasités depuis peu de temps de manière à maximiser les chances de survie de leurs descendants.

Concernant l’impact du sexe des larves, il a déjà été montré dans d’autres études que l’aptitude d’une larve à combattre pouvait dépendre de son sexe (van Baaren et al., 1999). Une femelle parasitoïde pourra donc adapter le sexe des œufs qu’elle pond en fonction de celui qui est le meilleur compétiteur lors des combats larvaires. Mais le sexe des descendants des autres femelles peut également affecter la ponte d’un parasitoïde. En effet, si on se réfère à la théorie de la Local Mate Competition d’Hamilton (1967), une femelle parasitoïde doit ajuster son sex-ratio de ponte lorsqu’elle est en présence de compétitrices en produisant une proportion plus importante de mâles. Ceci dans le but que ses fils soient assez nombreux pour pouvoir inséminer à la fois ces propres filles, mais également celles des autres femelles. Ce modèle peut également s’appliquer dans le cas où une femelle est confrontée à un patch de ponte déjà exploité (compétition par exploitation). Mais dans ce cas, et si la femelle parasitoïde est capable de discriminer le sexe des œufs déjà pondus, elle devrait adapter son sex-ratio de ponte, non pas en fonction du nombre d’œufs présents, mais en fonction du sexe de ces œufs. C’est ce que Hamilton (1967) prédit dans son modèle « Sex Ratio Games ».

Ainsi, chez des espèces solitaires qui se reproduisent sur des agrégats d'hôtes telles qu'*A. calandrae*, ces deux facteurs (asymétrie dans les combats larvaires et Sex Ratio Games) devraient influencer les stratégies de ponte (sélection de l'hôte et adaptation du sex-ratio de ponte) des femelles en situation de compétition intraspécifique par exploitation.

L'objectif de cette troisième partie est, dans un premier temps, d'étudier l'influence de l'intervalle de temps entre deux pontes sur la survie d'un deuxième individu ainsi que l'impact que cela aura sur les stratégies de ponte des parasitoïdes (Article 2). Dans un deuxième temps, nous essaierons de comprendre les mécanismes impliqués dans cette discrimination et notamment d'identifier les signaux perçus par les femelles, leur permettant de distinguer ces différents types d'hôtes (Article 3). Enfin, nous étudierons l'influence du sexe des larves sur leurs aptitudes à combattre. Nous verrons comment ce facteur peut influencer la ponte d'une femelle exploitant un patch d'hôtes déjà parasité et nous discuterons nos résultats par rapport à la théorie du « Sex Ratio Games » (Article 4).

Article 2

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 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). Our results show that *A. calandrae* females can discriminate between parasitized and unparasitized hosts, as they lay more eggs on the latter. However, they did not adjust their offspring sex ratio on already parasitized hosts compared to unparasitized one. 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 which 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; they can recognize whether the hosts have already been parasitized or not, and whether previously laid eggs are fresh or older.

Introduction

Parasitoid females are expected to adapt their oviposition strategies in different ways in order to maximise their reproductive success. For example, they could adjust their offspring sex ratio to environmental factors (Godfray, 1994). One such factor which has been studied in depth is the level of intraspecific 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 which 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, i.e., 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 immatures. 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 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 (van Alphen & Visser, 1990; Waage, 1986). 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-Carbone 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, i.e., 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 which 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, i.e., 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; 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, i.e., 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. calandrae females do not oviposit (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 16**). 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.

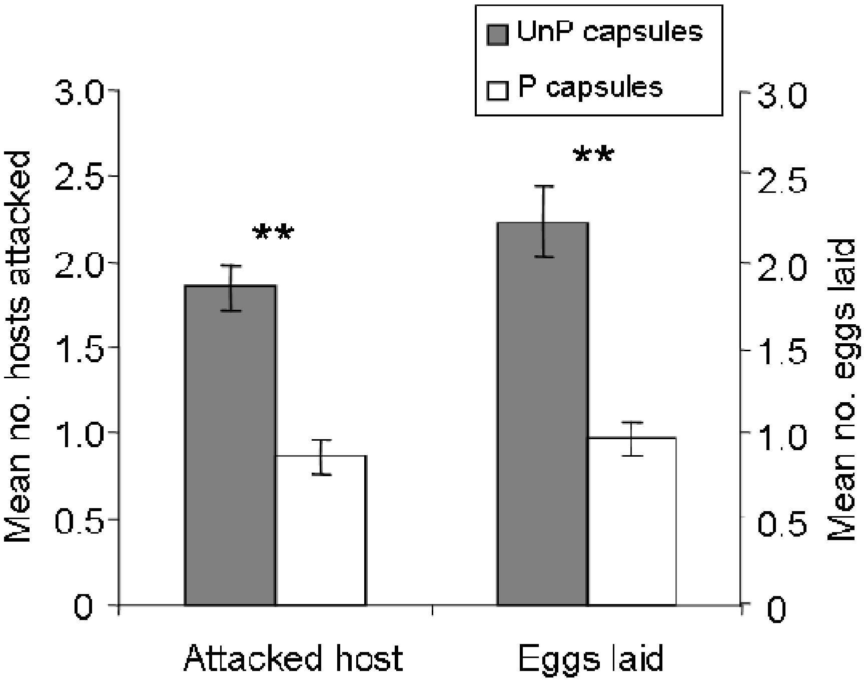

Figure 16 Mean (± SE) number of *Callosobruchus maculatus* hosts attacked and eggs laid by mated *Anisopteromalus calandrae* females per patch (n = 11) when ovipositing on unparasitized (UnP) or previously parasitized (P) hosts. ** P<0.01.

Survival of supernumerary eggs

The proportion of surviving eggs deposited under superparasitism conditions was similar for 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 17**). 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 17**). 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).

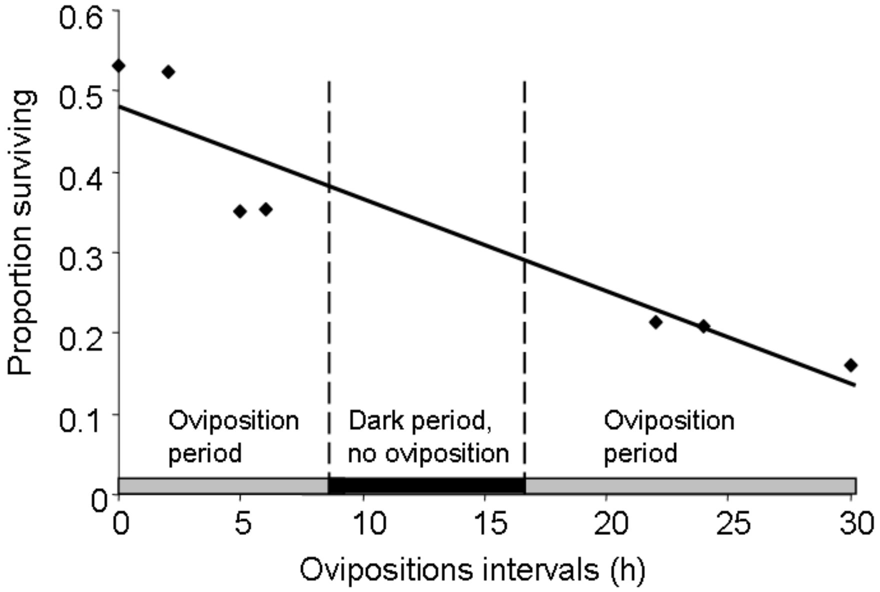

Figure 17 Relationship between the proportion of surviving *Anisopteromalus calandrae* eggs deposited under superparasitism conditions and the time elapsed since the first egg was laid. Proportion of surviving eggs observed (diamond) and linear regression (solid line). No differences were found between the two strains.

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-h 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-h 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). 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).

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

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 which could be considered to be of better quality. This discrimination ability has been demonstrated in most parasitoid species tested (Gauthier et al., 1996; Santolamazza-Carbone 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 which 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, as already observed in *Anaphes nitens* Girault (Santolamazza-Carbone & Cordero 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 which is about to hatch are thus of lower quality for the female, who therefore avoids these hosts, ovipositing preferentially on a recently parasitized host, i.e., 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 (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 which perform 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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Article 3

Modifications of the chemical profile of hosts after parasitism allow parasitoid females to assess the time elapsed since the first attack

Lebreton, S., Christidès, J.-P., Bagnères, A.-G., Chevrier, C. & Darrouzet, E.
(en préparation pour *Journal of Chemical Ecology*)

Abstract

In solitary parasitoids, only one adult can emerge from a given host. In some of these species, when several eggs are laid on the same host, supernumerary individuals are eliminated by lethal larval fights. In the solitary parasitoid *Anisopteromalus calandrae,* the probability a second larva win the fight strongly depends on the time elapsed since the first oviposition. The most the first egg is old at the moment at the second egg is laid, the less the second egg have chance to win the competition. As a consequence, females of this species lay preferentially their eggs on recently parasitized hosts rather than on hosts parasitized by an egg about to hatch.

A. calandrae females parasite bruchid larvae located into cowpea seeds. In a series of choice test experiments using an artificial seed system, we demonstrated that the cue perceived by parasitoid females allowing them to discriminate hosts parasitized for different times is not brought by the seed nor by the egg previously laid but by the host. Moreover, pentane extracts of bruchid larvae elicited a similar response than hosts themselves. This cue is perceived at short range distance, indicating that chemicals involved in this discrimination are probably partly volatile. Interestingly, although parasitism stops hosts' development, their cuticular profiles continue to evolve, but in different way than those of unparasitized hosts. Indeed, chemical analyses of pentane extracts show differences in the cuticular profiles of hosts after parasitism evolving with time and probably at the origin of the discrimination by parasitoid females.

Introduction

Parasitoid females have to localize hosts to lay their eggs. This location is often mediated by semiochemicals which could have different origins, such as volatiles released by plants when attacked by herbivores, chemical markers left by host females when they oviposite or host sex or aggregative pheromones (For a review see (Hilker & McNeil, 2008). For example, the Aphid parasitoid *Aphidius ervi* (Hymenoptera: Braconidae) is attracted by volatiles emitted by the blackcurrant when attacked by Aphids (Birkett et al., 2000). In the same way, the Pteromalid wasps *Dinarmus basalis* and *Anisopteromalus calandrae* could localize their hosts, Bruchid larvae, into cowpea seeds thanks to chemical marker deposited by the Bruchid females after oviposition (Kumazaki et al., 2000; Onodera et al., 2002).

After locating their hosts, females have to decide which hosts to parasite. Indeed, all the hosts are not of equal quality and one of the most important parameter influencing this quality is the parasitized status of hosts. Already parasitized hosts are often considered as worse quality hosts compared to unparasitized ones, because of the competition between larvae resulting from several clutches laid on the same host (Godfray, 1994). In some solitary parasitoids, when several eggs are laid in or on the same host, larvae fight until the death of all but one immature. Only one adult can therefore emerge from a given host in these species (Hubbard et al., 1987). Parasitoid females are generally able to discriminate these two categories of hosts (Gauthier et al., 1996; Weber et al., 1996; Darrouzet et al., 2007; Lebreton et al., 2009b). This discrimination capacities could be based on different chemicals like markers deposited by the first parasitoid during parasitisation (Völkl & Mackauer, 1990) as well as alarm pheromones produced by the host itself after having being stung for the first time (Outreman et al., 2001). It seems thus disadvantageous for a parasitoid female to lay several eggs on the same host or to lay eggs on already parasitized hosts. This behaviour called superparasitism has though been demonstrated to be adaptive under certain circumstances especially when unparasitized hosts are very scarce or when travels between oviposition patches are too long (van Alphen & Visser, 1990).

When females are confronted to several parasitized hosts, the survival probability of their offspring could vary from one host to another depending on different factors. Among these factors, the time elapsed between the two ovipositions is one of the most important (Visser et al., 1992; van Baaren & Nénon, 1996; Goubault et al., 2003; Lebreton et al., 2009b). In fact, at the moment when the second egg is laid, the older the first egg is, the less the second egg will have a chance to win the competition. This is due to the previous hatching of the first egg. The earlier the first egg hatched, the more the larvae originating from this first egg will have time to kill the second egg prior to its own hatching (Godfray, 1994). In a previous study, we demonstrated that, in the solitary ectoparasitoid *Anisopteromalus calandrae*, the survival probability of a second egg strongly decrease when the time interval between the two ovipositions increases (Lebreton et al., 2009b). Whereas two eggs laid in the same time interval have about the same probability to win the competition, the survival probability of the second egg reach only fifteen percent when it is laid few times before the hatching of the first egg. As a consequence, females lay preferentially their eggs on host recently parasitized rather than on those parasitized by an egg being about to hatch (Lebreton et al., 2009b). The present study aimed to identify the stimuli at the origin of this discrimination capacity. *A. calandrae* females parasite larvae of bruchids contained into cowpea seeds. When a female encounters a seed containing a host, she taps the surface of the seed with her antennae to precisely locate the host, and then, she inserts her ovipositor into the seed in order to probe the host. The cue perceived by females could thus come from an external marker deposited by the first female after oviposition. But it could also be due to an internal marker originating from the host in response to the first sting or from the presence of the egg itself.

In the present study, we wished to investigate whether the cue perceived by females and involved in the discrimination between hosts parasitized for different times is originated from the seed, the host or the egg. For this purpose, using an artificial seed system (Gauthier & Monge, 1999; Darrouzet et al., 2003), we performed a series of choice tests, in which we presented to females either parasitized seeds, eggs previously laid or parasitized hosts. After locating the cue, we identified the chemical signal involved by gas chromatography and mass spectrometry analyses.

Materials and Methods

Insects Rearing

Anisopteromalus calandrae were reared in the laboratory on larvae and pupae the bruchid *Callosobruchus maculatus* (Coleoptera: Bruchidae). Both *C. maculatus* and *A. calandrae* originated from Ivory Coast (collected in 2000) and were mass-reared under conditions close to that of their area of origin: 12 h light at 29 °C, 12 h dark at 22 °C, and 65% r.h.

Behavioural analyses

In order to obtain parasitized hosts, we used an artificial seed system composed by gelatine capsules which contain a bruchid larva (Gauthier & Monge, 1999; Darrouzet et al., 2003). The bruchid L4 larvae were placed inside the capsules after removal from seeds by dissection and selection by size. Five capsules were proposed to a group of five virgin females during a two hour period, in a climatic chamber. Because Hymenopteran parasitoids reproduce by arrhenotokous parthenogenesis, virgin females were able to lay only male eggs which avoid a bias due to the sex of the eggs during the choice test. At the end of the exposure period, eggs on parasitized hosts were located under a dissecting microscope and parasitized artificial seeds were selected for choice tests.

Choice tests were composed by two artificial seeds, one parasitized for two hours (recently parasitized capsule) and the other parasitized for 28 hours (parasitized by an egg being about to hatch) in a Petri dish (Lebreton et al., 2009b). In order to determine whether the cue perceived by females is located on the capsule, on the egg, or on the host, three categories of tests were performed by proposing the three different parts of the artificial seed system to the females. In test 1, choice tests consist in the presentation of 2 hours and 28 hours old parasitized capsules containing an unparasitized host. In test 2, 2-hour-old and 28-hour-old eggs were proposed in a clean capsule, on an unparasitized host. Finally, in test 3, hosts parasitized for 2 hours and 28 hours, on which eggs had been previously removed, were proposed in a clean capsule.

In a previous study, we demonstrated that mated females distinguish 2h and 28h parasitized artificial seeds (Lebreton et al., 2009b). In order to avoid a bias due to the mating status of females, in the present study, we also confronted mated females to these choice tests in a climatic chamber (29 °C, 65% r.h.). All the oviposition sequence was observed (first capsule visited, capsule with oviposition). The tests ended as soon as females lay an egg on the selected capsule but lasted no more than two hours to avoid the hatching of the first egg during the test. After the test, females were removed from the Petri dish. The first capsules visited and the selected capsules were noted. The proportion of females selecting each category of capsules in each category of test was then calculated and compared to those obtained in a control choice test from a previous study (with capsules containing all the parts of the system: the parasitized host with its egg into the parasitized capsule) (Lebreton et al., 2009b). Statistical analyses were performed with a χ^2*-test*.

Chemical Analysis of hosts' cuticular profile

We managed to identify chemical compounds present on hosts' cuticle. For this purpose, samples of one bruchid larva were extracted in 10 µL of pentane, in which 2 µL of an internal standard (C20) were added, for 1 min. Samples were analyzed with a Perkin-Elmer Autosystem XL GC (Perkin-Elmer, Wellesley, MA, USA) equipped with a flame ionization detector (FID) and interfaced with Turbochrom workstation software. Each sample was dried up to 2 µL. These 2 µL were then injected in the GC-FID injector heated at 220°C in splitless mode, and analyzed using a BP1capillary column that was temperature programmed from 50°C (2 min hold) to 310°C at 7°C/min with a final hold of 10 min. Five categories of hosts were analyzed: Larvae parasitized for 2h (P 2h) or 28h (P 28h) and unparasitized Larvae stored in the same conditions than 2h (UnP 2h) or 28h parasitized hosts (UnP 28h). As after 28h, some of unparasitized larvae evolve to a pre-pupa stage, unparasitized pre-pupae (UnP Pre-pupae) were also analyzed. The components were then identified by GC-MS analysis, performed using a Hewlett-Packard 5890 GC system coupled to a 5989A MS, controlled by a HPUX chemstation. A "2h parasitized hosts" and a "28h parasitized hosts" samples were injected in the GC/ MS injector following the same program as described above. In order to analyze whether there was a difference in the cuticular profile of the different categories of hosts, we performed a discriminant analysis based on the relative amount of the 30 major peaks.

Bioassays with hosts chemical extracts

In order to confirm that compounds observed in GC-MS are responsible for the discrimination of the time elapsed since the first oviposition by parasitoid females, we performed another series of choice tests. In these tests, mated females (n=42) were confronted to two capsules, each containing an unparasitized host on which we deposited an extract of parasitized hosts. These extracts were obtained by immersing individually 100 hosts parasitized for 2h or 28h in 200 µl of pentane for one minute. Then, 2µl of each extract (2h or 28h; equivalent to one host) were deposited on an unparasitized host previously rinsed in pentane to remove its own chemical compounds. One host of each category (with the “2h parasitized” or “28h parasitized” extract) were used to perform the choice tests. Tests took place in a climatic chamber as previously described. First capsule visited and capsule with oviposition were noted. The proportion of each category of capsules visited in first and on which females oviposited was then calculated. Statistical analyses were performed with a χ^2-*test*.

Results

Behavioural Analysis

In tests 1 and 2, parasitoid females visited in first as many capsules of each category (test 1, $\chi^2 = 0.03$, dl = 1, P = 0.87; test 2, $\chi^2 = 0.61$, dl = 1, P = 0.43) and laid as many eggs in each category of capsules (**Figure 18**; test 1, $\chi^2 = 1.58$, dl = 1, P = 0.21; test 2, $\chi^2 = 0.03$, dl = 1, P = 0.85). In test 3, most of females (69.2%) visited in first the capsules containing the 2h parasitized host rather than the 28h parasitized host ($\chi^2 = 7.69$, dl = 1, P = 0.006). Moreover, most of them (72.7%) selected the 2 hours parasitized hosts to lay their eggs (**Figure 18**; $\chi^2 = 6.82$, dl = 1, P = 0.009). This result is in accordance with that observed when females were confronted to the whole system (**Figure 18**; control; $\chi^2 = 4.48$, dl = 1, P = 0.034).

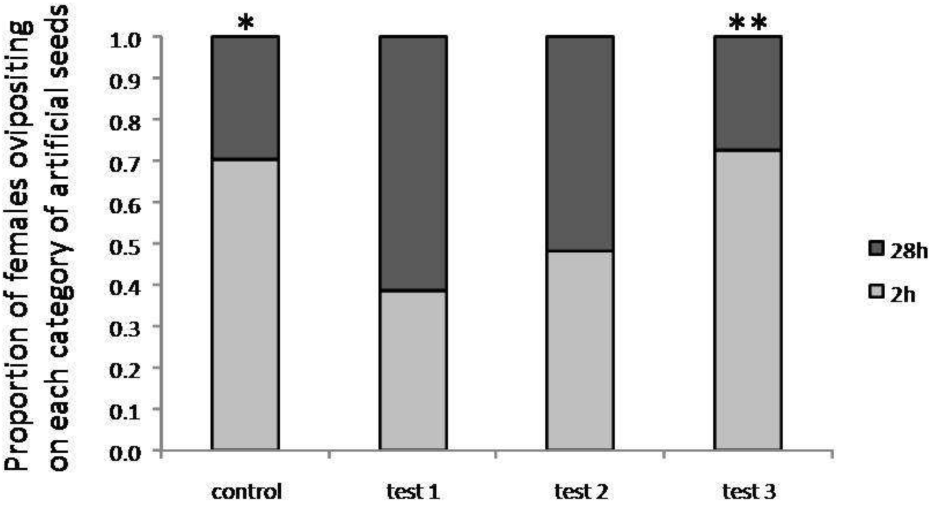

Figure 18 Proportion of females selecting each category of artificial seeds to lay their eggs (2h or 28h parasitized) when confronted to each category of tests: the whole parasitized system (control), parasitized capsules with an unparasitized host (test 1), eggs on an unparasitized host into a clean capsule (test 2) and parasitized hosts on which eggs were previously removed, in a clean capsule (test 3). Marks show significant differences between proportions observed and a random distribution of 50:50. * $p<0.05$; ** $p<0.01$.

Chemical Analysis of hosts' cuticular profile

The GC-MS analysis shows the presence of 44 major peaks (**Figure 19**) and 71 identified component (**Table 2**) on hosts. These include a series of n-alkanes and methyl-branched alkanes (C_{25}-C_{35}; **Table 2**). The same compounds were found on the different categories of hosts but in different proportions (**Table 2**). The discriminant analysis underlines a difference between UnP Pre-pupae and UnP Larvae (UnP 2h: F = 3.53, dl = 30.1, p = 0.020; UnP 28h: F = 3.35, dl = 30.1, p = 0.023) and no differences were found between the two categories of UnP Larvae (F = 1.21, dl = 30.1, p = 0.39; **Figure 20**). Whereas P 2h were not different from UnP 2h (F = 1.28, dl = 30.1, p = 0.30), P 28h were different from P 2h (F = 3.64, dl = 30.1, p = 0.018) and had a tendency to diverge from UnP 28h (F = 2.39, dl = 30.1, p = 0.073; **Figure 20**). P 28h were also different from UnP Pre-pupae (F = 3.12, dl = 30.1, p = 0.031; **Figure 20**).

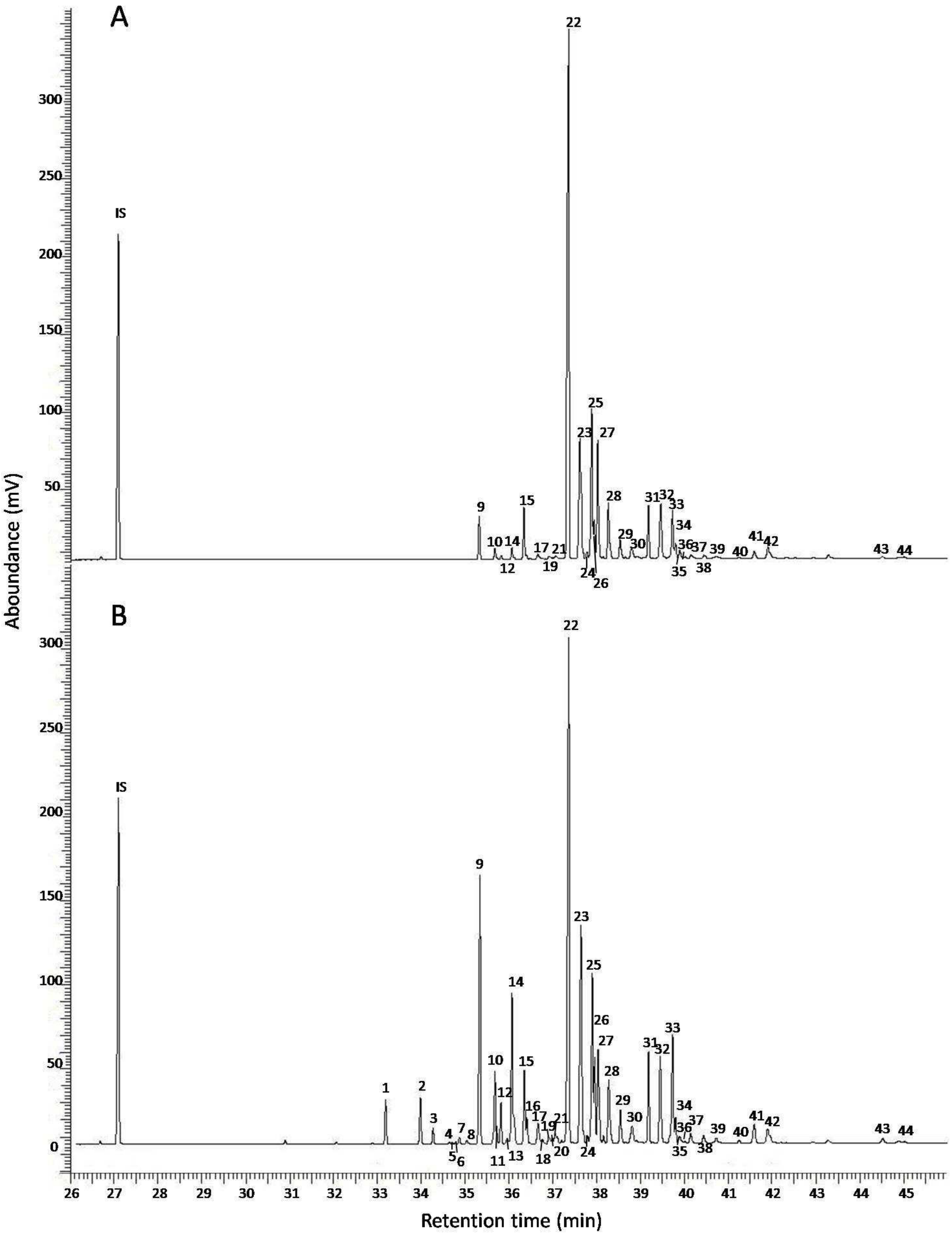

Figure 19 Gas chromatogram of the cuticular pentane extract of hosts parasitized for 2h (A) or 28h (B). IS= internal standard.

Table 2 Cuticular hydrocarbons present on the host's cuticle with their relative amount on each category of host

Peak	**Hydrocarbons**	**ECL**[a]	**CN**[b]	**Relative amount (% of total)**				
				UnP 2h	UnP 28h	P 2h	P 28h	UnP pre-pupae
1	*n*-C25	25.00	25	0,23	0,25	0,21	0,55	0,59
2	3-MeC25	25.68	26	0,07	0,10	0,14	0,40	0,44
3	*n*-C26	26.00	26	0,10	0,22	0,17	0,30	0,46
4	9MeC26	26.3	27	0,00	0,01	0,03	0,06	0,04
5	6-MeC26	26.47	27	0,00	0,00	0,00	0,03	0,00
6	5-MeC26	26.49	27	0,03	0,01	0,01	0,02	0,05
7	4-MeC26	26.51	27	0,01	0,03	0,03	0,10	0,10
8	3-MeC26	26.67	27	0,02	0,03	0,04	0,13	0,13
9	*n*-C27	27.00	27	4,88	9,99	6,29	8,58	10,19
10	9-MeC27	27.30	28	0,75	0,83	1,45	2,63	2,18
11	7-MeC27	27.32	28					
12	5-MeC27	27.43	28	0,11	0,23	0,35	0,65	0,57
13	9,15-DiMeC27	27.59	29	0,11	0,19	0,18	0,32	0,25
14	3-MeC27	27.69	28	0,78	1,54	1,62	3,54	3,36
	5,9-DiMeC27	27.72	29					
15	*n*-C28	28.00	28	4,00	4,77	4,05	3,46	3,70
16	3,9-DiMeC27	28.05	29	0,21	0,20	0,15	0,09	0,24
17	10-MeC28	28.26	29	0,27	0,32	0,46	0,77	0,65
18	6-MeC28	28.30	29	0,01	0,04	0,03	0,14	0,06
19	4-MeC28	28.50	29	0,11	0,20	0,16	0,32	0,28
20	2-MeC28		29	0,03	0,07	0,09	0,10	0,04
21	3-MeC28	28.64	29	0,07	0,26	0,29	0,51	0,41
22	*n*-C29	29.00	29	56,95	48,43	47,55	34,47	36,66
23	15-MeC29	29.28	30	5,25	4,79	7,32	10,44	7,52
	13-MeC29	29.28	30					
	11-MeC29	29.28	30					
	9-MeC29	29.30	30					
	7-MeC29	29.32	30					
24	5-MeC29	29.39	30	0,14	0,15	0,26	0,33	0,27
25	9,13-DiMeC29	29.49	31	5,26	5,45	5,72	7,06	6,05
26	7,11-DiMeC29	29.60	31	0,80	1,45	1,11	1,45	1,93
	7,13-DiMeC29	29.60	31					
27	3-MeC29	29.68	30	2,44	4,14	3,75	4,08	4,08
28	*n*-C30	30.00	30	4,02	3,32	3,44	3,20	3,67
	3,9-DiMeC29	30.05	31					
	3,11-DiMeC29	30.05	31					
	3,13-DiMeC29	30.05	31					
29	14-MeC30	30.28	31	0,80	0,82	1,05	1,23	1,11
	12-MeC30	30.28	31					
30	10,14-DiMeC30	30.48	32	0,62	0,79	0,84	0,92	0,95
	9,13-DiMeC30	30.48	32					
	8,12-iMeC30	30.48	32					
	8,18-DiMeC30	30.48	32					
31	*n*-C31	31.00	31	4,96	4,03	3,86	3,65	3,76

32	15-MeC31	31.28	32	2,27	1,98	2,97	3,34	2,47
	13-MeC31	31.28	32					
	11-MeC31	31.28	32					
	9-MeC31	31.30	32					
	7-MeC31	31.30	32					
33	9,13-DiMeC31	31.46	33	2,43	2,81	3,23	3,48	3,75
34	7,11-DiMeC31	31.59	33	0,44	0,24	0,39	0,49	0,52
	7,13-DiMeC31	31.59	33					
	3-MeC31	31.66	32					
	5,13DiMe-C31	31.64	33					
35	*unknown*			0,30	0,38	0,38	0,33	0,51
36	*n*-C32	32.00	32	0,04	0,08	0,06	0,06	0,15
37	3,9,13-TriMeC31	32.05	34	0,25	0,29	0,30	0,34	0,46
38	14-MeC32	32.28	33	0,18	0,20	0,26	0,28	0,30
	12-MeC32	32.28	33					
39	10,14-DiMeC32	32.46	34	0,11	0,15	0,21	0,20	0,16
40	*n*-C33	33.00	33	0,13	0,13	0,10	0,15	0,29
41	15-MeC33	33.28	34	0,31	0,30	0,48	0,59	0,50
	13-MeC33	33.28	34					
42	11,15-DiMeC33	33.48	35	0,48	0,64	0,75	0,89	0,94
	9,13-DiMeC33	33.48	35					
	7,11-DiMeC33	33.60	35					
43	*n*-C35	35.00	35	0,04	0,09	0,13	0,15	0,09
	13-MeC35	35.29	36					
44	13,17-DiMeC35	35.46	37	0,02	0,06	0,12	0,17	0,09
	11,15-DiMeC35	35.48	37					
	9,13-DiMeC35	35.48	37					

[a]ECL = Equivalent Chain Length
[b]CN = Carbon number

Bioassays with hosts chemical extracts

Most of females (70.7%) visited the capsule containing the “2h parasitized” extract in first ($\chi^2 = 7.04$, dl = 1, P = 0.008). Ovipositing females have even a tendency to lay a little more eggs on these hosts (64.3% vs. 35.7% on hosts with the “28h parasitized” extract) but the difference is not significant ($\chi^2 = 2.29$, dl = 1, P = 0.13).

Discussion

Chemicals are known to play a crucial role for host location in parasitoids (Afsheen et al., 2008). Host cues could have many different origins such as oviposition markers (Kumazaki et al., 2000; Onodera et al., 2002), chemical residues left by adult hosts on the substrate (Colazza et al., 1999; Peri et al., 2006; Colazza et al., 2007), chemicals originally from host faeces (Meiners et al., 1997; Steidle et al., 2003; Steiner et al., 2007; Inoue & Endo, 2008) or even possibly carbon dioxide (Hilker & McNeil, 2008). Some of these chemicals arise from adults' activity and are often used by egg or larval parasitoid females to locate their hosts. However, other chemicals could directly come from larvae and these compounds could not only give the information of the presence of a host, but also of the parasitized status of this host (Outreman et al., 2001). In our study, we show a new level of information as semiochemicals could inform the parasitoid on the time elapsed since the first parasitism.

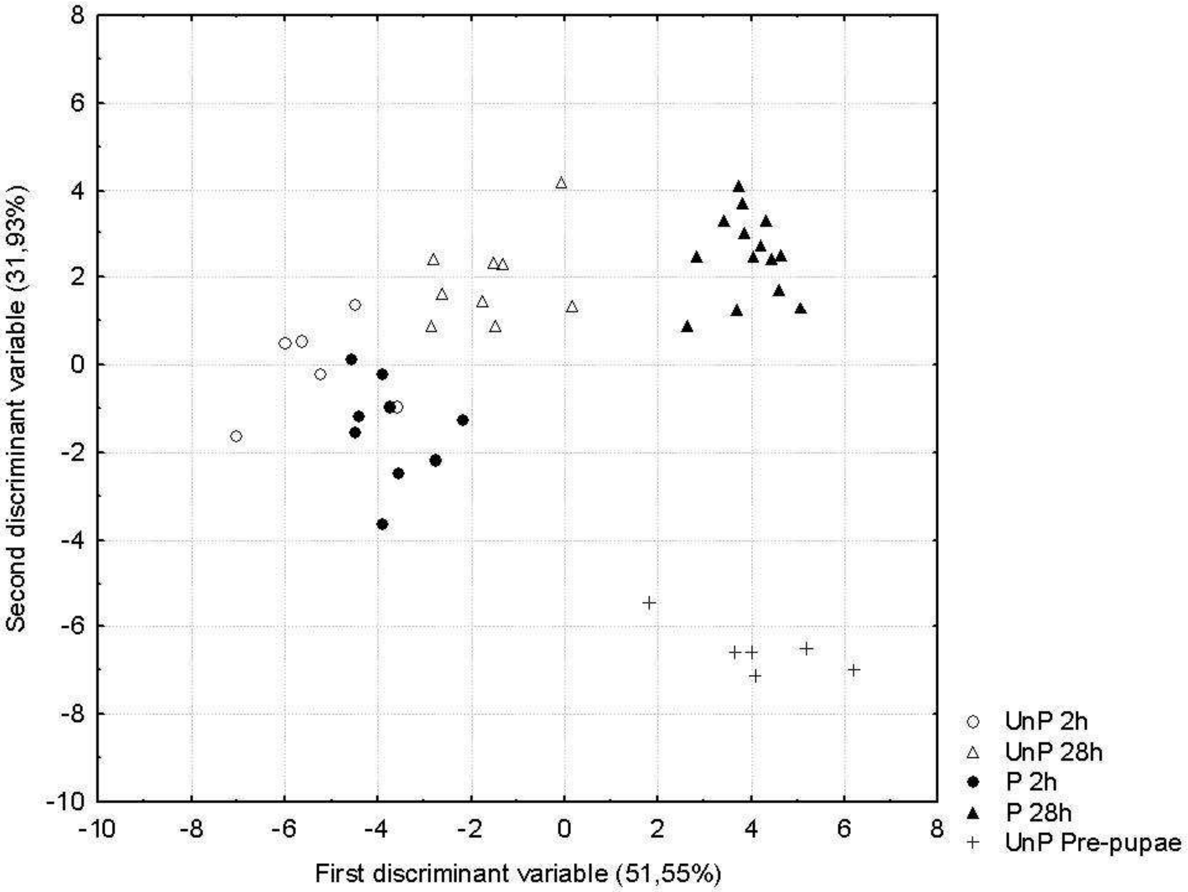

Figure 20 Discriminant analysis based on the relative amount of the 30 major peaks of the cuticular profile of the different categories of hosts (L4 larvae parasitized for 2h or 28h, unparasitized L4 larvae stored in the same conditions than 2h and 28h parasitized hosts, and unparasitized pre-pupae).

With a series of choice tests, we demonstrated that the cue perceived by females to inform them on the time elapsed since the first oviposition is nor located on the seed, neither produced by the egg, but situated on the host. Our results show that most of tested females visited the capsule containing the 2 hours parasitized host in first (test 3, 70%), indicating that compounds implicated in this discrimination capacity are not detected by contact but at a distance. When females were introduced in the choice test, they came near the capsules and reached out their antennae before choosing a capsule (Lebreton, personal observation). This observation indicates that the cue is perceived at a short range distance. In a previous study (Lebreton et al., 2009b), when parasitoid females were confronted to the complete system (parasitized capsule, parasitized host and egg), the proportion of them which visited the 2 hours parasitized host in first was not so marked than those observed in the present study (60%). In this previous experiment, females were confronted to a more “noisy” environment, as other cues could be present on or into the seeds such as oviposition markers or chemicals produced by the egg. In fact, infochemicals have to be distinguishable from the noisy background (Turlings et al., 1995; Hilker & McNeil, 2008). These other cues could in some extent mask the compounds emitted by the host (Schroeder & Hilker, 2008).

When females were confronted to the chemical compounds only (experimentally deposited on an unparasitized host), they were attracted by the host exhibiting the “most recently parasitized” profile. However, although they have a tendency to lay their eggs onto these hosts, this choice is not as marked as for hosts really parasitized (Lebreton et al., 2009b). This result suggests that several cues could actually be necessary for a female to perfectly assess the quality of hosts. During the first parasitism, *A. calandrae* females also paralyze their hosts by injecting venom into these hosts (Lebreton et al., 2009a). In this experiment, hosts are unparasitized and are therefore not paralyzed. It is possible that hosts firstly perceived by females as “recently parasitized hosts” at a distance, are then considered as not really parasitized because of their non-paralyzed status.

In a previous study, Outreman and coworkers (2001) showed that an alarm pheromone produced by Aphids after being parasitized allows parasitoid females to determine whether a host is already parasitized or not. They correlated an antennal rejection of parasitized host with the presence of the pheromone in the hours following the first parasitism. Then, as the pheromone disappeared, the antennal rejection makes way for a sting rejection. This result indicates that several cues could succeed to give the same information. In our study, the difference in the cuticular profile of hosts is mostly visible after 28 hours. It is thus possible that this cue could not inform parasitoids on the parasitized status of hosts just after the parasitism whereas other cues, such as oviposition markers deposited by parasitoids, give the information for shorter periods. Actually, when *A. calandrae* females oviposite on unparasitized hosts, they generally deposit a substance with their ovipositor after laying an egg (Lebreton, personal observation). Moreover, when confronted to parasitized capsules (test 1), females have a tendency to select a little more 28 hours parasitized capsules even if this difference is not statistically significant from a random choice. This observation could be in accordance with an oviposition marker more detectable shortly after its deposit. Oviposition markers are originally from the Dufour gland. In *A. calandrae*, the Dufour gland contains a mix of *n*-alkanes (C_{30} to C_{39}) (Howard & Baker, 2003).

Our results show differences in the cuticular profiles of hosts analyzed. First, concerning unparasitized hosts, their profiles depend on their developmental stages, as larvae and pre-pupae exhibit different profiles. Concerning parasitized hosts, their profile also evolves with time, as it is different between hosts parasitized for 2h and 28h. However, it evolves in a different way than in unparasitized hosts. Although unparasitized pre-pupae and 28h parasitized hosts' profiles closed in according to the first discriminant variable, they diverged from each other according to the second discriminant variable. This result suggests that parasitism affects the evolution of hosts' profile. The compounds identified (a series of linear and methyl alkanes) were in accordance with those previously identified by Howard (2001). Although these hydrocarbons consist in heavy long chains, in our choice test experiments, parasitoid females were able to detect the time elapsed since the first oviposition at a short range distance, i.e., outside the capsule. Nevertheless, considering the high temperature in our experimental procedure (29°C), some of these compounds could be semi-volatiles (Saïd et al., 2005). It is thus possible that only the lighter component play a role in this discrimination. This hypothesis is in accordance with our results which show that the major difference between 2h and 28h parasitized hosts is located on these compounds. However, this hypothesis remains to be tested. Another possibility should have been the spontaneous oxidation of these heavy cuticular lipids to volatile aldehydes or alcohols, as already observed in other insect species (Bartlelt & Jones, 1983; Bartlelt et al., 2002). However, the compounds involved in this discrimination are pentane-soluble by immersing bruchids into the pentane solution and are therefore apolar and probably not so volatile.

To conclude, the present study suggests that different cues could be useful to totally assess the wholeness quality of a host. It highlights an evolution of the hosts' cuticular profile after having being parasitized, allowing following parasitoid females to assess the time elapsed since the first parasitism. Even if this evolution is perceptibly different from an unparasitized host, it seems to follow the same global direction. Interestingly, whereas parasitism stops the growth of hosts and disables them to reach the pupation, it does not stop the evolution of their cuticular profile. This result thus opens new questions on the incidence of parasitism on host metabolism.

Article 4

Sex allocation strategies in response to conspecifics offspring sex ratio in solitary parasitoids

Lebreton, S., Chevrier, C. & Darrouzet, E.
(en révision pour *Behavioral Ecology*)

Abstract

Parasitoid females adjust their offspring sex ratio in order to maximize their fitness. The optimal sex ratio they produce varies with several factors, but especially with competition level. In solitary species, only one adult can emerge from a given host, whatever the number of eggs laid. In some species, the mortality of supernumerary individuals could be due to larval combats. This ability to fight could vary from one sex to another within species. In this way, when females explore an already parasitized host patch, the sex ratio of previous eggs can influence their fitness. These two factors could thus strongly influence females' sex allocation strategies. However, this prediction assumes that parasitoid females can assess the sex of eggs previously laid by conspecifics. We used host acceptance and sex ratio behavior to test this capacity, and our experimental data provide the first evidence for this capacity in a parasitoid species. Females of the solitary ectoparasitoid *Anisopteromalus calandrae* discriminated the sex of eggs already laid by a conspecific, but only when these eggs had reached a certain developmental stage. They adapted their offspring sex ratio as predicted by Hamilton's "sex ratio games" model, allocating the sex of their eggs differentially according to the sex of eggs already on the hosts on which they oviposited. In this way they prevented a lethal larval fight between their sons and the females they could potentially mate after their own emergence, simultaneously increasing the fitness of both the mothers and their sons.

Introduction

Sex ratio and sex allocation strategies have a direct impact on the fitness of individuals. While a number of organisms produce equal numbers of males and females (Fisher, 1958), the females of a wide range of species have been shown to adjust their offspring sex ratio, for example, birds (Komdeur et al., 1997), protozoan parasites (West et al., 2001) and parasitoid wasps (Godfray, 1994). In parasitoid Hymenoptera, females adjust their offspring sex ratio through arrhenotokous parthenogenesis, whereby fertilized (diploid) eggs develop into females and unfertilized (haploid) eggs into males. Females adapt their sex allocation strategies in response to several factors such as host quality (Charnov et al., 1981), environmental constraints (King, 1987), overlapping generations (Charnov, 1982) and competition level, which is one of the most important factors (Hamilton, 1967; Werren, 1980; West et al., 2000; King, 2002). In his Local Mate Competition (LMC) model, Hamilton (1967) showed that isolated females produce a female-biased sex ratio, producing the minimum number of sons able to inseminate their daughters. If the number of egg-laying females increases, they adapt their sex allocation strategies, producing more sons in order to inseminate the daughters of other females. This theory applies to a population of females, and the cues they perceive to evaluate the level of competition could be either direct (the presence of n females) or indirect (e.g. the presence of eggs previously laid in a patch) (Werren, 1980; Shuker & West, 2004; Darrouzet et al., 2008). As the presence of eggs on hosts has been shown to be the primary mechanistic cue of sex ratio adaptation in a competition context (Shuker & West, 2004), the features of a previous clutch should thus influence the sex allocation strategy of a second female. One important parameter is the sex ratio of the first clutch; females should adjust the number of sons they produce according to the number of female eggs laid by other females. In this case, in order to maximize their fitness, females should assess the sex ratio of the first clutch. This capacity has never been demonstrated in any parasitoid species. However, in gregarious parasitoids, Hamilton (1967) predicted that if a female is able to recognize the sex of eggs already present on hosts, the optimal sex ratio of a female which superparasitizes these hosts will depend on the sex ratio of the first clutch. This “sex ratio games” model was further adapted by Werren (1980) who incorporated the relative clutch size. The main predictions of these superparasitism models are that, for a given clutch size, females will lay a higher proportion of male eggs when a majority of females have already been laid on the host. The proportion of male eggs should then decrease as the number of female eggs on hosts decreases. More recently, these superparasitism models have been extended, incorporating the characteristics of specific species. In *Melittobia* spp, for example, the model has been extended to cover lethal combat among adult males to explain extremely female-biased sex ratios (Abe et al., 2003). Likewise, Shuker et al. (2005) included asymmetrical competition for mates between males because of asynchronous emergences that occur in the wasp *Nasonia vitripennis*.

These models were primarily developed for gregarious species in which several individuals could emerge from one host. While it appears that it is less relevant for solitary species which generally lay single eggs, it could be extended to those which reproduce on an aggregate of hosts. In fact, an aggregate of hosts could be considered as a single host on which several parasitoids develop at its expense (even if, in fact, only one individual emerges from a given host of this aggregate). The fact that only one adult can emerge from a host, whatever the number of eggs initially laid, is another important constraint in solitary species. This mortality is due either to lethal larval combats or physiological suppression (Godfray, 1994). In species where larval fights occur, the first instars actively search for eggs and larvae on the hosts and try to eliminate these competitors by attacking them with their mandibles (van Alebeek et al., 1993). However, this ability to fight varies from one sex to another within species (van Baaren et al., 1999). Differences in competitive abilities could be explained by morphological differences between male and female larvae, such as the number of bristles involved in mobility, an important feature in larval fights (van Baaren et al., 1997; 1999). This asymmetry could strongly affect the oviposition strategy of a female performing superparasitism (laying an egg on an already parasitized host). We could thus expect that ovipositing females would avoid laying eggs on hosts already parasitized by an egg belonging to the better sex competitor and/or have a tendency to lay their better sex competitor on already parasitized hosts.

In this study, we investigated the ability of females of the solitary ectoparasitoid *Anisopteromalus calandrae* Howard (Hymenoptera, Chalcidoidea, Pteromalidae) to distinguish the sex of eggs previously laid by a conspecific. Assessing the exact nature of previously laid eggs is especially important in *A. calandrae* because females, depending on their physiological state, tend to select already parasitized hosts (Lebreton et al., 2009a). We investigated: (1) the larval combat ability of males and females in this species, and (2) whether ovipositing females could assess the sex of eggs previously laid, and how they adjust their sex allocation strategies, based on both the “sex ratio games” model and lethal larval fights between sexes.

Materials and Methods

Rearing conditions

Anisopteromalus calandrae Howard (Hymenoptera, Chalcidoidea, Pteromalidae) is a solitary ectoparasitoid of a wide range of coleopteran pest larvae and pupae (Ahmed, 1996), used throughout the world for biological control of pests in seed stocks. Two homozygous strains of *A. calandrae* were isolated: one red-eyed mutant (noted as R) and one black-eyed wild-type (noted as B) (Do Thi Khanh et al., 2005). The use of these two strains enabled us to monitor the offspring of a given female. Parasitoids were mass-reared on larvae of the bruchid *Callosobruchus maculatus* in a climatic chamber: 12 h light at 28 °C, 12 h dark at 22 °C, and 65% r.h.

Sex competition in larval combat

To obtain eggs, *A. calandrae* females were allowed to lay on an artificial seed system composed of gelatine capsules (Darrouzet et al., 2003). The bruchid larvae were placed inside the artificial seeds 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.

To analyze whether females or males were better competitors in larval combat, virgin R and mated B females were allowed to lay eggs on *C. maculatus* larvae for two hours. In a previous study, no differences were found in the competitive abilities of the two strains (Lebreton et al., 2009b) and therefore only one combination was performed in this study. Mated females were obtained by placing a 2-hour-old virgin female with a 24-hour-old virgin male of the same strain in a Petri dish. Mating was confirmed by direct observation. Random eggs of the B strain (males or females) were deposited individually with a random egg of the R strain (male) on the same host (n=284) and placed in a separate cell in a Plexiglas sheet closed by a Plexiglas cover-slide until emergence of the parasitoid adults (Darrouzet et al., 2003; Darrouzet et al., 2007). At emergence, the phenotype and sex of the surviving individual was noted.

The survival rate of an egg laid on an already parasitized host decreases sharply as the time between the two ovipositions increases (Lebreton et al., 2009b). To investigate how the fighting abilities of male or female larvae vary with the time interval, random eggs of the B strain (males or females) were deposited individually with a random egg of the R strain (male) laid with a 28-hour time interval (the first egg about to hatch) on the same host as described above (R egg laid first: n=217; B egg laid first: n=217). At the same time, we determined precisely the proportion of each strain winning a male/male combat by placing two eggs laid by a virgin female (one of each strain) on the same host for the two intervals described above (eggs laid at the same time: n=79; eggs laid with a 28h interval: n=86).

When eggs laid by B mated and R virgin females are placed together, two categories of larval competition occur: one between a B female and an R male, the second between two male larvae (one R and one B). Individuals emerging after larval fights are thus either B females, B males or R males (**Table 3**). As we were unable to determine precisely the proportion of R males emerging from each category of fight, we had to make an estimate, as shown in **Table 3**. From the number of females and the estimated number of males emerging from a female/male combat, it was then possible to determine which sex was the better competitor in larval competitions.

Table 3 Estimation of the number of R males emerging in each category of larval fight.

		Competition categories		
		B female/R male	B male/R male	total
Emerging strain	B	$\mathbf{B_{female}}$	$\mathbf{B_{male}}$	$\mathbf{B_{tot}}$
	R	$R_{tot} - \left[\frac{B_{male}}{P_B} - B_{male}\right]$	$\frac{B_{male}}{P_B} - B_{male}$	$\mathbf{R_{tot}}$

In bold, the observed number of emerging individuals. P_B represents the proportion of B male winning a male/male fight with an R male, obtained with competition between offspring of virgin females.

Egg sex discrimination

Isolated mated R females were exposed to 2 hosts in a choice test experiment, using the same artificial seed system as previously described. One host had previously been parasitized by a male egg (laid by a virgin B female) and the other by a male or female egg (laid by a mated B female). To examine whether females' oviposition strategies evolved with the embryonic development of the egg already on the host, two series of experiments were performed, one with two-hour-old eggs (obtained just after oviposition) and the second with 28-hour-old eggs (just before hatching). The choice tests took place in a climatic chamber (L12:D12, 30°C: 22°C, and 71% r.h.) and ended as soon as the females laid an egg, with a maximum time of 2 hours. The oviposition sequence was observed: the first capsule visited, the capsule selected (corresponding to the capsule on which the female settled after examining the patch), and the capsule with oviposition. 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 in order to avoid larval fights. After emergence, the phenotype (R or B) and sex of individuals were noted, enabling us to differentiate between eggs offered to females during the choice tests and those laid by tested females. Female/male (2h, n=23; 28h, n=43) and male/male (n=32) choice tests were then analyzed separately. For each category of test, the hosts selected for oviposition were noted, and the sex ratio of eggs laid by tested females (proportion of males) was calculated. Because other recent models incorporate characteristics of other parasitoid species or parameters which are not applicable to *A. calandrae*, we compared these sex ratios with those predicted by Werren's model (X^*), which included asymmetry in clutch size:

$$X^* = \frac{\sqrt{2X_0(T+1)} - 2X_0}{2T}$$

where X_0 is the sex ratio of the first clutch and T the ratio of the eggs laid by the first and second females (Werren 1980).

Statistical analyses

To analyze which sex is the better competitor in larval fights, we used a χ^2-test to compare the proportion of females and males emerging from each category of fight to a theoretical proportion of 50:50. To compare the offspring sex-ratios laid by tested females on each category of host (previously parasitized by a male or female egg), a χ^2-test was also performed. Next, to compare these sex-ratios with those predicted by Werren's model, we performed a Fisher's exact test. Analyses were carried out with R software (R 2.1.1, R Development Core Team, Free Software Foundation Boston, MA, USA) and a threshold level of $P<0.05$ was set.

Results

Sex competition in larval combat

Following larval combats between males laid at the same time, each individual had an equal probability of winning the competition, as 53% of B males and 47% of R males emerged ($\chi^2=0.26$, df =1, p=0.61; Fig. 1). Thus, the proportion of R males surviving R male/B male combats with eggs from B-mated females should also represent about 47% of these combats. We can thus estimate, as described in the methods section (**Table 3**), that the number of emerging males represents only about 23.5% of male/female combats (**Figure 21**). These results demonstrate that female larvae have supremacy over male larvae in larval fights ($\chi^2=35.5$, df =1, p<0.001) and have a higher probability of winning than a male laid under the same conditions (0.74 vs 0.53; $\chi^2=15.3$, df =1, p<0.001; **Figure 21**). Under our experimental conditions, in 8.8% of cases, no individuals reached adulthood.

When eggs were laid with a 28h interval, the probability of the second male winning the competition against a first male was only 25.75% (**Figure 21**). The estimated percentage of females laid 28h after a male egg which emerged from these combats is thus 53.12%. Under these conditions, females lose their supremacy against a male laid 28h before ($\chi^2=0.0017$, df =1, p=0.97) but have a higher survival rate than a male egg laid under the same conditions (0.25; $\chi^2=7.48$, df =1, p=0.006; **Figure 21**). When the female is laid first, the estimated survival probability of a second male is 18.7%, which is not different from its probability of winning the fight when the two eggs are laid at the same time (23.5%; χ^2=1.34, df =1, p=0.25). Under our experimental conditions, in 16.0% of cases, no individuals reached adulthood.

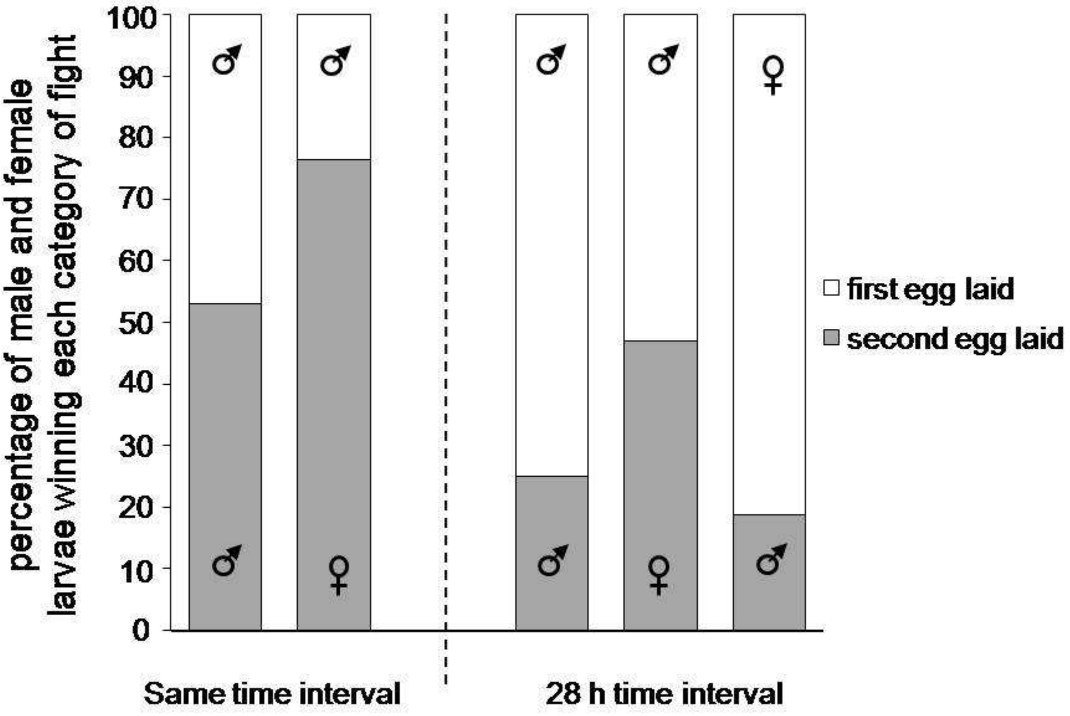

Figure 21 Percentage of larvae of each sex emerging after a fight in a two-larvae contest. Both male/male and female/male competition were investigated with either individuals laid at the same time or with a 28 hours time interval. The outcomes of male/male fights were observed directly, while those of female/male fights had to be estimated (for more details, refer to the method section). With regard to eggs laid at the same time, first and second eggs were chosen arbitrarily.

Egg sex discrimination

With regard to host selection, when confronted with mixed patches containing hosts previously parasitized by a male and a female egg, *A. calandrae* females first visited an equal number of each category of capsules, whatever the developmental stage of the eggs provided (2 hours old: χ^2=0.03, df =1, p=0.87; 28 hours old: χ^2=0.01, df =1, p=0.90). While they selected an equal number of hosts parasitized by male and female eggs when confronted with 2-hour parasitized hosts (χ^2=0.0, df =1, p=1.0), they selected more hosts parasitized by a female egg when confronted with 28-hour parasitized hosts (65.4% of selected hosts; χ^2=4.90, df =1, p=0.03). Nevertheless, they laid as many eggs on hosts parasitized by a female as by a male egg, whatever their developmental stage (**Figure 22**; 2-hour-old, χ^2=0.04, df =1, p=0.83; 28-hour-old, χ^2=2.80, df =1, p=0.09).

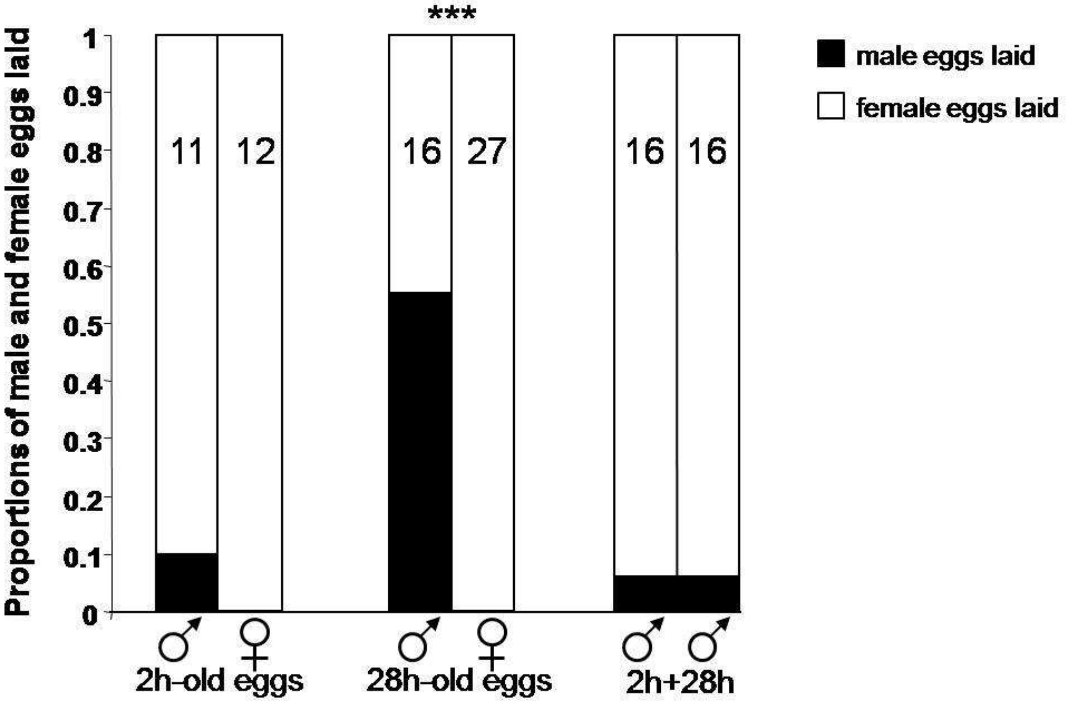

Figure 22 Adjustment of the females' offspring sex ratio according to both sex and the developmental stage of the eggs of a previous clutch. Proportions of male and female eggs laid on previously parasitized hosts obtained in a male/female choice test with 2-hour-old (just after oviposition) or 28-hour-old (about to hatch) eggs, or a male/male choice test (in male/male choice tests, as results are similar whatever the developmental stage of first eggs (Fisher test, p=1.0), results with 2-hour-old and 28-hour-old eggs have been pooled). Marks show a statistical difference between the sex ratio of eggs laid on hosts previously parasitized by a male egg and by a female egg (*** p<0.001). The values in each bar represent the number of tested females oviposited on each category of host.

With regard to sex allocation, when confronted with a host parasitized by 2-hour-old male or female eggs, the females allocated the sex ratio (proportion of males) of their offspring (SR=0.10) in the same way as those confronted with a host containing only male eggs (SR=0.06) (**Figure 22**; Fisher test, p=0.93). However, females allocated sexes differentially on hosts parasitized by a male or female egg when these eggs were 28 hours old (about to hatch, **Figure 22**). In this case, they produced more sons on hosts already parasitized by a male egg (SR=0.55) than on those parasitized by a female egg (SR=0; **Figure 22**; Fisher test, p<0.001).

When *A. calandrae* females were confronted with patches containing two hosts, one parasitized by a female and one by a male egg about to hatch (X_0=0.5), they laid eggs with a sex ratio of 0.17, which is similar to the value predicted by Werren's model (X*=0.22; Fisher test, p=1.0). When there were only male eggs on hosts (X_0=1), the observed sex ratio (0.06) was also similar to that predicted by the model (X^*=0; Fisher test, p=1.0).

Discussion

In some solitary parasitoids, because of asymmetries in larval combats (van Baaren et al., 1999), the survival probability of an egg laid under superparasitism conditions depends largely on the sex of other eggs laid on that host. Therefore, in these species, the ability of a female to assess the sex of eggs previously laid could have a direct impact on her fitness. The present study provides evidence of this capacity in a parasitoid species.

In *A. calandrae,* when two eggs are laid during the same time interval, female larvae win more larval combats than males, with about 75% of fights ending in a female victory. Therefore, when the previous eggs have been recently laid, females often lay a female egg (which has a higher probability of winning the competition) under superparasitism conditions. Interestingly, in this study, females never laid a male egg on a host already parasitized by a female egg. Because males are poorer competitors in larval combats, a male egg laid on a host already parasitized by a female egg has a low probability of reaching adulthood. Therefore, a female should avoid laying a male egg in this situation. Moreover, if the male wins the competition, it means that it killed the female larvae, and by killing females before their emergence, it reduces its own reproductive success at the adult stage. Therefore, by not laying male eggs on hosts already parasitized by female eggs, *A. calandrae* females avoid a lethal fight between their sons and females which they could possibly mate after their own emergence. In this way, females increase both their sons' reproductive success and their own fitness. However, the loss of fitness induced by killing a female would be negligible compared to the low probability of winning the fight. This would therefore be the main factor explaining why females did not lay any male eggs on hosts previously parasitized by a female egg.

Although the supremacy of females in larval fights could be mainly attributed to fighting abilities, we cannot exclude the possibility that an intrinsic differential mortality between sexes during their egg or larval development partly explains this competitive asymmetry. However, without fight, under our experimental conditions, the mortality of males was generally low (less than 10%, Lebreton, personal observation) and not significantly higher than the mortality of clutches containing both male and female eggs. This cannot therefore be the only factor responsible for the asymmetry.

Nevertheless, females did not avoid laying eggs on hosts parasitized by female eggs (the better sex competitor) as might be expected. They even had a tendency to select a capsule containing a host parasitized by a female egg when the eggs were about to hatch. However, when female eggs were laid on a host which had been parasitized for a long time by a male egg (28h), they lost their supremacy in larval fights, with only 50% of females emerging from a male/female competition, although they still had a higher probability of winning the competition than a male egg laid under the same conditions (50% vs 25%). Consequently, being laid 28h after a first male reduces the probability of winning the competition by 25%, whatever the sex of the second individual. This decrease is mainly due to the previous hatching of the first egg, as the larva from this first egg could kill the second egg before it hatches (Godfray, 1994). But when the first individual laid is a female, the probability of a second individual winning the fight if it is laid 28h later seems to decrease only slightly (less than 5% for a male). This result could thus be explained either by an inability of females to localize other eggs or by a weaker aggressiveness of females towards these eggs. In any event, this result could partly explain why ovipositing females have a tendency to select more hosts parasitized 28h before by a female egg. In fact, on these hosts, their offspring have little risk of being killed before hatching.

Nevertheless, hosts which have been parasitized for 28 hours, especially by a male egg, could thus be considered as worse quality hosts, which could explain the higher proportion of sons laid on these hosts, as predicted by the “host quality model” (Charnov et al., 1981). Moreover, the loss of female supremacy in larval fights could induce less benefit in laying only female eggs. Under this condition, and if the second individual has a non-null probability of winning the fight, it could be better for the female to lay a male egg, which is less costly to produce as it does not use spermatozoa. Indeed, *A. calandrae* females mate only once, and after successive matings, males do not transfer enough spermatozoids to females to produce female eggs throughout their reproductive life (Do Thi Khanh, 2005). In this case, it is possible that females become sperm limited. It could thus be more profitable for females to adjust their sex allocation strategies according to the sex ratio of the first clutch (in order to maximize their offspring’s reproductive success) rather than to their offspring’s fighting abilities. *A. calandrae* females have previously been shown to respond to both Local Mate Competition and host quality (Nishimura & Jahn, 1996).

Previous experimental studies failed to demonstrate an egg sex discrimination ability in parasitoids (van Baaren et al., 1999), in spite of a number of theoretical studies dealing with this topic (Hamilton, 1967; Suzuki & Iwasa, 1980; Werren, 1980; Abe et al., 2003). Werren (1980) suggested that the relative size of the two clutches is more important in sex ratio adaptation than the sex ratio of the previous clutch. However, according to this model, for a given relative clutch size, the sex ratio of the first clutch plays a primary role in sex allocation strategies. In 1999, van Baaren *et al.* were unable to demonstrate this discrimination capacity in a solitary parasitoid species (*Anaphes victus*), but the hosts offered to the second females in their study had been parasitized less than one hour before. It is possible that eggs have to reach a certain developmental stage for females to be able to distinguish their sex. The present study demonstrated that *A. calandrae* females adjust their sex allocation strategies only when they are confronted with 28-hour-old eggs (about to hatch) and not when eggs are 2 hours old (recently laid). While we showed that females may have no benefit in adjusting their offspring sex ratio when they are confronted with newly laid eggs, this lack of laying adjustment could also be due to an inability to discriminate the sex of newly laid eggs. Ovipositing females could thus perceive a cue which evolves as the eggs develop. However, there are no visible morphological differences between male and female eggs in *A. calandrae* prior to hatching (personal observation). It is therefore possible that eggs produce a sex-related cue during their development which could be perceived by ovipositing females. However, to our knowledge, differences in the chemical profile of eggs according to their sex have never been shown.

While sex ratio games have already been identified in some experimental studies (Werren, 1980; Werren, 1984; Shuker et al., 2005; Abe et al., 2007), the present study confirms the model first described by Hamilton, i.e., an adjustment of offspring sex ratio according to those of conspecifics. In line with the models of Hamilton (1967) and Werren (1980), *A. calandrae* females only laid a higher proportion of male eggs when females were laid in the vicinity. In fact, females have no benefit in laying male eggs if there are no female eggs nearby to inseminate after their emergence. At the same time, when females compete with virgin conspecifics, they are not expected to adjust their offspring sex ratio in the same way as when confronted with only mated competitors. This hypothesis is supported by the constrained model (Godfray, 1990), which predicts a more female-biased offspring sex ratio when females are confronted with constrained conspecifics (virgin, sperm-depleted or mated with a sterile male) in order to compensate for the excess of males in the population. This factor could be important in *A. calandrae*; as females mate only once, the proportion of constrained females could be significant. The present study suggests that, if this theory is valid in this species, the main mechanistic cue perceived by mated females could be the sex of eggs laid by virgin females rather than their mating status. Indeed, they do not produce more males when confronted with male offspring of mated females; our results show that ovipositing females responded to the sex of eggs previously laid, not to the mating status of their mother.

To conclude, sex allocation has already been shown to be adjusted in response to several factors, such as the number of females, relative brood size, dispersal status, etc. (West et al., 2005). Here, we demonstrated an extra level of complexity, showing that females can adapt their offspring sex ratio in response to those of clutches laid by a previous conspecific. Through their high discrimination capacity, females can detect the reproductive strategy of conspecifics, thereby increasing their sons' mating success, and at the same time their own fitness.

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Conclusion de la partie

Les femelles *A. calandrae* ont développé d'importantes capacités de discrimination leur permettant de distinguer des hôtes parasités de différentes qualités. Elles sont ainsi capables de discriminer d'une part un hôte récemment parasité (depuis 2h) d'un hôte parasité par un œuf sur le point d'éclore (depuis 28h) et d'autre part un hôte parasité par un œuf mâle ou femelle. Cette aptitude est fondamentale chez les espèces solitaires, pour lesquelles la ponte de plusieurs œufs sur un même hôte se traduira par des combats mortels entre larves. Chez *A. calandrae*, les femelles sont de meilleures compétitrices lors des combats larvaires. Plus l'intervalle de temps entre deux pontes augmente et moins le deuxième individu pondu aura de chance de gagner le combat. Ainsi, lorsqu'une femelle pond sur un hôte déjà parasité depuis 28h par un œuf mâle, la probabilité que son descendant remporte l'affrontement est réduit de 25% quel que soit le sexe de celui-ci. Mais lorsque le premier œuf pondu est une femelle, la probabilité de survie d'un individu (mâle en l'occurrence) pondu 28h après ne diminue que de 5% par rapport aux chances de survie qu'il aurait s'il était pondu en même temps. Ce résultat semble indiquer que les larves femelles éliminent moins leurs compétiteurs au stade œuf que les larves mâles.

Lorsqu'elles sont confrontées à des hôtes parasités par des œufs mâles, les femelles parasitoïdes choisissent préférentiellement des hôtes récemment parasités pour y pondre, augmentant ainsi la probabilité de survie de leurs descendants. Cette discrimination est rendue possible grâce à des modifications du profil cuticulaire de l'hôte après le premier parasitisme qui s'accentuent avec le temps ; les femelles *A. calandrae* étant capables de percevoir ces différences.

En ce qui concerne le sexe des œufs précédemment pondus, les parasitoïdes ont besoin que ceux-ci aient déjà atteint un certain stade de développement pour pouvoir déterminer leur sexe. A ce moment là, les femelles pondent une plus grande quantité de fils sur des hôtes parasités par des œufs mâles, mais uniquement lorsque des œufs femelles sont déjà pondus sur d'autres hôtes à proximité. Elles adaptent donc leur propre sex-ratio de ponte en fonction du sex-ratio des œufs précédemment pondus dans l'environnement. La ponte des fils sur des hôtes parasités par des œufs mâles et non par des œufs femelles peut s'expliquer par l'existence des combats larvaires. En effet, un mâle pondu sur le même hôte qu'une femelle n'aurait que peu de chance de gagner le combat du fait de la suprématie des femelles ; et s'il remportait la victoire, cela signifierait qu'il aurait tué une femelle avec laquelle il aurait pu éventuellement s'accoupler après son émergence. D'autres études seront nécessaires quant à l'identification du ou des signaux permettant aux femelles de distinguer le sexe des œufs.