Egg load and adaptive superparasitism in *Anaphes nitens*, an egg parasitoid of the *Eucalyptus* snout-beetle *Gonipterus scutellatus*

Serena Santolamazza Carbone* & Adolfo Cordero Rivera

Departamento de Ecoloxía e Bioloxía Animal, Universidade de Vigo, Spain

Abstract

We studied egg production and the occurrence of adaptive superparasitism in *Anaphes nitens*, an egg parasitoid of the *Eucalyptus* snout beetle *Gonipterus scutellatus*. First, we determined whether *A. nitens* females were synovigenic or pro-ovigenic. Newly emerged females were allowed to lay eggs alone during 3 days on six fresh egg capsules. A first group of females (n = 25) were killed by freezing and the remaining females (n = 21) were maintained during two extra days with food, but without hosts. Their fecundity was measured by dissection of host eggs and females' ovarioles. We found that the second group of females increased their fecundity by about 20%, suggesting they were weakly synovigenic. To test for the occurrence of adaptive superparasitism in relation to competitors' density, we compared the oviposition behaviour of females kept alone, in pairs, or in groups of four during patch visit. Results indicated that the females superparasited significantly more often in this last treatment. Synovigeny and the ability to modulate the use of superparasitism could be mentioned as important attributes that allow *A. nitens* to efficiently control the pest population.

Introduction

In the context of biological control, egg limitation has broad implications on the capability of the parasitoid to depress pest populations (Heimpel & Rosenheim, 1998). A high reproductive capacity has traditionally been thought to be one of the most important qualities of parasitoid species employed as biocontrol agents, because it influences the rate of establishment of parasitoid populations and allows a more rapid response to changes in the abundance of the pest population (DeBach & Rosen, 1991). Nevertheless, this position has been recently disputed from a comparative study that suggested that a high fecundity does not always ensure a high control, and that in contrast to fecundity, other features (e.g. searching efficiency, longevity, mobility, host taxon) can contribute to the success of biological control plans (Lane et al., 1999).

Correspondence: Serena Santolamazza Carbone, Departamento de Ecoloxía e Bioloxía Animal, Universidade de Vigo, EUET Forestal, Campus Universitario, 36005 Pontevedra, Spain. E-mail: serena@uvigo.es

In any case, fecundity and the trade-off between time and egg limitation remain among the most important traits from an evolutionary point of view (Rosenheim, 1996, 1999). From this perspective, parasitoid species can be divided into two fundamental physiological categories: pro-ovigenic and synovigenic (Godfray, 1994; Heimpel & Rosenheim, 1998). Synovigenic species can mature oocytes during their adult life, while pro-ovigenic species are born with a fixed complement of mature eggs that, when exhausted, limit female reproductive success (reproductive senescence; Mangel & Heimpel, 1998). Synovigenic females can experience short-term egg limitation (Heimpel & Rosenheim, 1998; Rosenheim, 1999) on a daily time scale, depending on the number of suitable hosts encountered, but they will rarely suffer lifetime fecundity limitation (Lane et al., 1999).

Egg limitation could involve the existence of a different egg allocation strategy. Unlike synovigenic females, it is expected that pro-ovigenic females will lay fewer eggs in every host and that they will become more selective when deciding whether to parasitize or superparasitize, preferring good quality hosts (Iwasa et al., 1984; Heimpel et al., 1996; Sirot & Krivan, 1997; Heimpel & Rosenheim, 1998; van Baalen, 2000).

Frequently, parasitoids shape clutch size and offspring sex ratio using contact with other females or with their chemical traces as a cue to assess competitor density (Godfray, 1994). The frequency of superparasitism usually increases with the number of females foraging in the patch (Visser et al., 1990; Visser & Driessen, 1991; Cronin & Strong, 1993b), and the theory predicts that parasitized hosts will at first be rejected, but later accepted (Visser et al., 1992b; Visser, 1995). Despite the existence of fitness penalties induced by superparasitism, especially in solitary parasitoids (Anderson & Paschke, 1969; Harvey et al., 1993; Potting et al., 1997), self- and conspecific superparasitism could be adaptive in some special circumstances, as a form of competing for resources and overcoming host defences (van Alphen & Visser, 1990), stabilizing the host-parasitoid dynamic (Sirot & Krivan, 1997).

Anaphes nitens Girault (Hymenoptera: Mymaridae) is an egg parasitoid of the *Eucalyptus* snout beetle, *Gonipterus scutellatus* Gyllenhal (Coleoptera: Curculionidae). Both species have been studied since the beginning of the 20th century because of the important damage produced by the coleopteran to the cultivations of eucalypts and the use of the parasitoid for biological control (Tooke, 1955). *Anaphes nitens* was introduced to Galicia (north-west Spain) at the end of 1993 as an agent of pest control, which very quickly obtained excellent results (Mansilla & Pérez Otero, 1996). *Gonipterus scutellatus* females lay egg capsules of approximately eight eggs on young eucalypt leaves, covered by a dark secretion, mainly composed of faeces, which becomes very hard after some hours. This situation makes *A. nitens* a solitary parasitoid whose offspring develops in a quasi-gregarious situation, because even if each larva developed alone in the egg, the adults would emerge together from the host egg mass, mating immediately (Tooke, 1955). This makes this species specially challenging to study because it is impossible to know which of the eggs hidden inside the capsule were visited by the investigated female or by the others. With an external inspection, the female might infer how many eggs contains the capsule (Hanks et al., 2000), its age (Santolamazza Carbone, 2002) and previous parasitism (Santolamazza Carbone, 2002), but she cannot be sure that all the eggs inside the capsule are parasitized until she probes them with her ovipositor. Despite *A. nitens* being mentioned as one of the few cases of successful biological control achieved in different continents by using an egg parasitoid acting alone (Greathead, 1986; DeBach & Rosen, 1991; Lane et al., 1999), no studies have been performed to investigate the details of the mechanism that ensures such an ability.

This study aimed to explore the reasons for the success of *A. nitens* as a biological control agent. In the first experiment we used a relationship between egg load, size and age to establish if *A. nitens* females are synovigenic or pro-ovigenic. Then, in the second experiment we studied the influence of the presence of a variable number of females on the decision to superparasitize the host patch.

Methods

All the adult parasitoids employed in the experiments were reared in the laboratory from egg capsules collected in the field. *Gonipterus scutellatus* fresh egg capsules were obtained from adults collected in the field and reared in the laboratory.

Egg supply

We first tested the effect of female size on egg load at birth, because it is likely to correlate with egg production in insects (Labarbera, 1989). In *A. nitens*, newly eclosed females immediately start to mate and oviposit, suggesting that the eggs do not need a maturation period (Tooke, 1955). Newly emerged females ($n = 27$) were killed by freezing and their egg load estimated by dissecting ovarioles and counting the number of eggs. All the eggs found seemed fully developed from this point. Female size was estimated from right forewing length. The wing was mounted on a glass slide and its image was digitized and measured using Global Lab® 3.0 software.

To test for syn- or pro-ovigenic egg development, newly emerged females, mated just before the beginning of the experiment, were maintained in a patch constituted by a 10 cm diameter Petri dish containing two fresh egg capsules (24 h old, on average) on a disk of wet filter paper. We provided droplets of honey *ad libitum* as a food source. Adult lifespan in the laboratory, without access to food at 21 °C, was 3.4 ± 0.06 days for males ($n = 14$) and 3.0 ± 0.03 for females ($n = 23$) (Santolamazza Carbone, 2002), while if they can feed, longevity increases to 6 days for males and 8 days for females at 20–23 °C, reaching 15 days for males and 18 for females at 10 °C (Vidano et al., 1979).

All females had the opportunity to oviposit alone through 3 days on six fresh egg capsules (each day they received two egg capsules). A first group (treatment 1; $n = 27$) were immediately killed by freezing and a second group (treatment 2; $n = 23$) were maintained during two extra days in the Petri dish, provided with food but without hosts. At the end of the experiment, the host eggs were dissected under the microscope in a droplet of water to count the number of parasitoid eggs laid. The remaining females were killed by freezing and dissected to count the residual eggs in the ovarioles. Forewing length was measured as described above. Two females in each treatment died during the experiment, and therefore final sample sizes were 25 for treatment 1, and 21 for treatment 2. We predict that

if the females are synovigenic, older females (treatment 2) should carry more unlaid eggs (residual eggs) than younger females (treatment 1).

We compared the number of laid and unlaid eggs between treatments with a Generalized Linear Model (GLM) with Poisson errors and a log link using GENSTAT release 4.2, 5th edition (GenStat, 2000). Means are presented with SE.

Adaptive superparasitism

Preliminary test. Visser et al. (1990) suggested that contact with other females during the rearing period (prepatch experience) can influence the tendency to self-superparasitize by the inexperienced females searching alone in the patch, as a form to saturate the host capacity if future conspecific superparasitism is predictable.

We know that *A. nitens* attacks a gregarious host, so that emerged adults could be in contact prior to dispersal. To verify if *A. nitens* experiences such a pre-experimental effect, we performed a preliminary test using only females isolated from the pupal stage in sterile plastic vials. It seems important to detect this effect, particularly to avoid any extraordinary behaviour inside the control group (see below).

We analysed the difference in terms of self-superparasitism rate between 24 h old mated females (n = 13) kept in isolation, and females that after isolation were kept in groups of four individuals for 24 h prior to the test (n = 12), and then exposed individually to the hosts. The first group of females were never in contact with other adults, with the exception of their mating male (for every female we used a virgin male). Females were fed during the test with droplets of honey. Each female of both groups was maintained for 24 h in a 10 cm diameter Petri dish containing two egg capsules (24 h old) on a disk of wet filter paper. At the end of the experiment each host was dissected under a microscope, counting the eggs laid by the parasitoid.

Experiment. To assess the relationship between female density and (self- and conspecific) superparasitism occurrence, we used 24 h old *A. nitens* females (n = 75) previously mated, and fresh egg capsules of *G. scutellatus*. Rosenheim & Rosenl, (1991) pointed out that when carrying out experiments on ovipositing behaviour, the effects of the experience and egg load can be confused. Therefore we decided to isolate the females during the 24 h between one assay and the other to reduce the effect of the precedent experience (Miura et al., 1994), and under the possibility of synovigeny, to allow the maturation of more eggs.

After emergence, parasitoids were kept separated for 1 day (day 0) in a plastic test-tube at room temperature (18–20 °C, 60–70% r.h.), to eliminate any memory of the previous contact with conspecifics (Visser et al., 1990). During this isolation period, as well as during the experiment, all females had access to drops of honey as food.

The experiment was done on days 1, 3, and 5, and the females remained isolated on days 2 and 4 to avoid the possibility of previous experience influencing egg allocation on the next day. The patch was a 10 cm diameter Petri dish containing a disk of wet filter paper with two fresh egg capsules.

On day 1, each female was kept individually in the Petri dish with the opportunity to visit two fresh egg capsules. After 24 h, the egg capsules were dissected to verify the number of eggs laid into the hosts and quantify the percentage of self-superparasitism. On day 3, females received three different treatments: in treatment A, females were kept alone in a Petri dish with two fresh egg capsules (n = 11); in treatment B (n = 20), the females were maintained in pairs, but to maintain the same host/parasitoid proportion we offered four egg capsules; in treatment C (n = 44), the females remained in groups of four (eight egg capsules offered). During the 5th day, all females were isolated again, as during the first day, and maintained with two fresh egg capsules. Since three females died during the isolation period on day 4, sample sizes were reduced to 11, 19, and 42 females for treatments A, B, and C, respectively.

To count the eggs laid by the parasitoid, host eggs were extracted from the capsule and dissected in a droplet of water under a microscope. All means are presented with SE. Results were analysed with an ANOVA where the response variable was the number of parasitoid eggs found inside each host egg (transformed as square-root of value + 0.5). Treatment effects were compared between females or groups of females (included as a random factor) on days 3 and 5. A repeated-measures ANOVA could not be calculated because some females were in groups on day 3, but all were alone on day 5. Analyses were made with GENSTAT release 4.2, 5th edition (GenStat, 2000).

Results

Egg supply

We found that the newly emerged females carried an average of 45.6 ± 3.73 eggs (n = 27). Figure 1a shows that larger females were born with more eggs (regression equation: $y = 114.7x - 70.004$; $r = 0.68$, $P < 0.0001$).

Females of both treatments were similar in wing length ($t_{44} = -0.68$, $P = 0.502$) and laid a similar number of eggs (treatment 1: 31.4 ± 2.58; treatment 2: 32.7 ± 3.22; GLM, deviance ratio = 0.11, $P = 0.744$), but there were significant
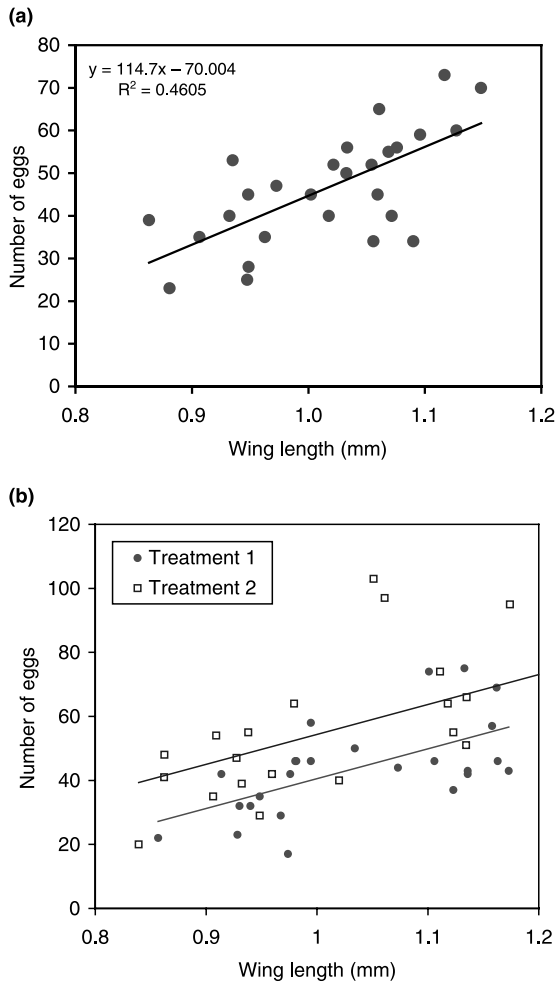
(a)

(b)

Figure 1 (a) The effect of size (wing length) on egg load at birth. n = 27 females. (b) The relationship between size and fecundity (eggs laid + eggs unlaid) for females of treatment 1 (short lived) and 2 (long lived).

differences in the number of eggs remaining in the ovarioles at the end of the experiment (Figure 1b; treatment 1: 12.5 ± 1.60, treatment 2: 22.9 ± 2.89; GLM, deviance ratio = 10.89, P = 0.002).

Adaptive superparasitism

Preliminary test. There were significant differences in the average number of eggs per host between both treatments (isolated females: average eggs laid per host = 0.35 ± 0.03; grouped females: 0.69 ± 0.03; $\chi^2 = 56.14$, P < 0.0001). Isolated females parasitized less than grouped females, but there were no differences in the percentage of eggs self-superparasitized during the two treatments (Figure 2; 8/113 in isolated and 18/191 in grouped; $\chi^2 = 0.242$,

P = 0.621). Consequently, we think that it is unnecessary to subject the females of the adaptive superparasitism experiment to the treatment of pupal stage isolation, considering a sufficient precaution to be an isolation during the 24 h prior to assay.

Experiment. In this experiment we tested the effect of the number of competitors inside the patch on superparasitism rate (self- and conspecific), measuring the occurrence of multiple egg clutches. Self-superparasitism occurs when females visit alone the patch, while probably a mix of self-and conspecific superparasitism occurs when there is more than one female foraging. We found a significant effect of the experimental treatment on day 3 (Figure 3, ANOVA, $F_{2,29} = 9.59$, P < 0.001). Least significant differences between means indicated that treatment A (control group of isolated females) did not superparasitize less than treatment B females (in pairs), but there were significant differences between treatments A and C (groups of four females).

After 24 h of isolation, on day 5 of the experiment, there were no significant differences in terms of self-superparasitism rate among the females that had again had the possibility to visit the patch alone (ANOVA, $F_{2,67} = 0.65$, P = 0.478), suggesting that there was no memory of the treatment.

Females were apparently egg-limited at the end of the experiment, because on the last day, when they were about 7 days old, the number of eggs laid generally diminished (ANOVA with day as the factor, treatment A, P < 0.001; treatment B, P = 0.043; treatment C, P < 0.001).
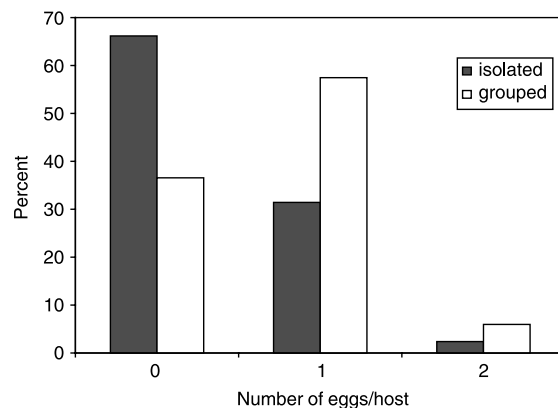

Figure 2 Histogram of distribution of clutch size for females maintained in isolation from pupal stages, without contact with conspecifics (number of hosts examined n = 439 eggs, average larvae per host = 0.35) and females maintained in groups of four before the experiment (n = 302 eggs, average larvae per host = 0.69).
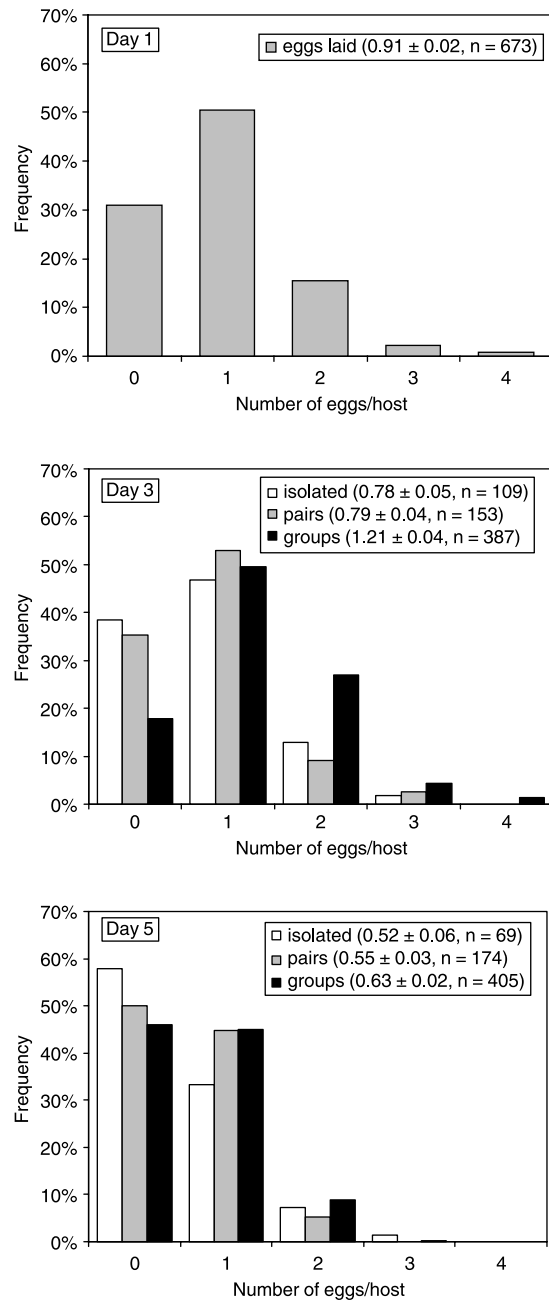
Figure 3 Histogram of distribution of the number of eggs per host for female *A. nitens*. On the first day all females laid eggs alone; after 24 h of isolation they were randomly assigned to three treatments on day 3: alone, in pairs, or in groups of four. Again, after 24 h of isolation they were maintained alone with hosts on day 5, but are reported separately here to test for memory effects of the treatment received on day 3. The average number of eggs per host (± SE, n = number of hosts) is also shown.

Discussion

Our experiment on egg production showed that after three days, all females have laid almost the same number of eggs (see Results), but that after 5 days older females with no access to hosts (treatment 2) carried significantly more eggs. Further investigation is required to verify the rate of egg maturation and eventually egg resorption in *A. nitens*, but at the moment our results throw serious doubts on the pro-ovigenic nature of *A. nitens*, as a member of a family where pro-ovigenic development is a common feature (Cronin & Strong, 1993a; Jervis et al., 2001). Moreover, Jervis et al. (2001) considered that synovigeny should be linked with some life-history strategies as idiobiosis (i.e., the host attacked do not continue to develop), which is a characteristic of the egg parasitoids. Along the continuum existing between pro-ovigeny (all the eggs are mature at emergence) and extreme synovigeny (no eggs are mature at emergence) there are some 'weakly synovigenic species', which include those females born with a conspicuous egg complement, but that can mature some eggs during adulthood. We think that *A. nitens* should be considered as a member of this last biological category.

Visser et al. (1990) and Rosenheim & Hongkham (1996) reported that contact with conspecifics prior to the assay led to a larger clutch. Nevertheless, in the preliminary test of the experiment on adaptive superparasitism (see Methods), we found a low percentage of self-superparasitism in both experimental groups, and it was not higher for the females that were maintained in contact with conspecifics prior to the test, as predicted by the theory on the rate of gain of self-superparasitism when conspecific superparasitism in the near future is likely (Visser et al., 1990).

The only variable introduced in the experiment was female density, while the host/parasitoid ratio was kept constant during the 3 days of the test, even if patch quality varied along the 24 h as the females depleted the patch. On day 1, after isolation and host deprivation, all females showed a high oviposition rate (Figure 3) with 18% self-superparasitism, probably as an effect of the initial lack of hosts and high egg load. On day 3, according to some theoretical models on foraging behaviour (Bakker et al., 1985; van Alphen, 1988; Visser et al., 1990, 1992b), we expected a higher percentage of superparasitism when females search in company. Effectively, females in groups of four superparasitize significantly more often than when they are alone. The fact that host eggs are concealed inside the capsule prevents us from knowing if during treatment C the increased superparasitism was due to self- or conspecific superparasitism, even if both strategies were adaptive under such conditions.

Female oviposition strategy is predicted to be related to the number of competitors in the patch, and to vary if the

host encountered has been parasitized by a conspecific or by the female herself in a previous visit. When a female searches alone, she should accept a parasitized host always (if unparasitized hosts are scarce) or never (if unparasitized hosts are abundant), and in the latter case she should leave the patch when unparasitized hosts become rare. For solitary parasitoids, if there is no competition in the patch, the pay-off from self-superparasitism is predicted to be zero (because it provokes competition among siblings), while in the presence of competitors it has a fitness return. The optimal strategy when the patch is simultaneously depleted by a number of females is a game theoretical problem, where it is expected that when the environment becomes sufficiently poor, the ESS switches from rejecting parasitized hosts to accepting them (Visser et al., 1992b).

Considering the percentage of self-superparasitism found in group A (15%), where females foraged alone, one could propose that the increase of multiple egg clutches in group C (32%) is fundamentally due to an increment in conspecific superparasitism. Unlike treatment C females, treatment B females (paired females) did not exhibit an increase in multiple egg clutches (11%), compared with control group A. It is possible that females foraging in pairs on four egg capsules do not experience enough competition to result in a higher superparasitism rate.

Figures 2 and 3 show that the percentage of unparasitized hosts remained high among the groups during the whole test. This might be explained if the females followed a bet-hedging strategy, spreading the risk of oviposition among patches (Messina & Fox, 2001). One could also argue that a lack of host discrimination ability could account for this behaviour. Nevertheless, in a no-choice test we found that *A. nitens* females discriminated between parasitized and unparasitized egg capsules in terms of acceptance/rejection rate and visit duration, while they did not seem to distinguish between egg capsules parasitized by themselves or by conspecifics (Santolamazza Carbone, 2002).

This lack of ability in self-discrimination could explain the existence of superparasitism when the female forages alone (see Figure 2), which must be classified as self-superparasitism. We supposed that if *A. nitens* females can produce eggs during their adult life, they could have a tendency to allocate more than one egg in the host egg during the same visit (multiple egg clutch; Rosenheim & Hongkham, 1996) or as a consequence of two visits (self-superparasitism), if this behaviour has some fitness return. In solitary parasitoids, the period between ovipositions is crucial in deciding which individual takes possession of the host (Vinson, 1980). Considering that in the genus *Anaphes* only the mymariform first instar larva is strongly

mandibulate (Tooke, 1955; Boivin et al., 1993), it is reasonable to assume that younger larvae have the better chance of destroying older ones (Visser et al., 1992a). This means that superparasitizing mothers have a more than zero chance of gaining an offspring.

It is important to consider that conspecific-superparasitism increases individual fitness (i.e., the female may gain an offspring), under extreme competition. From the applied point of view, self- and conspecific-superparasitism could lead to a decrease in control efficiency, because: (i) it provokes an increase in parasitoid preimaginal mortality (Harvey et al., 1993), (ii) it could represent a waste of eggs, hosts, and searching time (Sirot & Krivan, 1997), and (iii) it might be the reason for a decrease in parasitism rate (van Dijken & van Alphen, 1991). Nevertheless, self-superparasitism could be positive for pest control if it allows it to overcome host defences and if it has a value of insurance from any form of larval mortality (Rosenheim & Hongkham, 1996). Superparasitism can also act as an stabilizing factor in the dynamics of host-parasitoid systems if it decreases parasitoid efficiency (Sirot & Krivan, 1997), stimulates females to disperse (Cordero Rivera et al., 1999), and localizes other host populations. In any case, superparasitism could carry fitness penalties for *A. nitens* females, causing a significant reduction in female size and increasing pre-imaginal mortality (Santolamazza Carbone, 2002).

In conclusion, our results indicate that in contrast to the general prediction for members of the Mymarid family, *A. nitens* should be considered as a weakly synovigenic species, and that females use superparasitism as an adaptive tool to compete for resources only under strong competition.

Acknowledgements

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