

Costs and consequences of superparasitism in the polyembryonic parasitoid *Copidosoma koehleri* (Hymenoptera: Encyrtidae)

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Abstract. 1. Polyembryonic wasps provide dramatic examples of intra-specific developmental conflict. In these parasitoids, each egg proliferates into a clonal lineage of genetically identical larvae. If more than one egg is laid in a host (superparasitism), individuals of different clones may compete for food resources.

2. In the polyembryonic encyrtid *Copidosoma koehleri*, one larva per clone can differentiate into a sterile soldier. It is shown that soldiers are always females, and that they attack intra-specific competitors.

3. Research hypotheses were that (a) clones that develop in superparasitised hosts suffer heavier mortality than clones that develop in singly parasitised hosts, and (b) female clones cause higher mortality to their competitors than male clones, hence larval survival is lower in superparasitised hosts that contain females than in male-only broods.

4. The potential frequency of superparasitism in *C. koehleri* was manipulated by varying parasitoid–host ratios and exposure durations.

5. As parasitoid densities and exposure durations increased, the frequency of superparasitism rose, brood sizes increased, but the number of hosts that completed development was reduced. The number of offspring per parasitoid female decreased with increasing parasitoid–host ratios. Offspring size and longevity were inversely correlated with brood size. As superparasitism rates increased, fewer all-male broods were produced. Male–female broods were female-biased, suggesting selective killing of males by female soldiers. All-female broods were significantly smaller than all-male broods at high parasitoid densities only, possibly reflecting aggression among soldiers of competing clones.

6. The results support the working hypotheses, and suggest that female larvae outcompete males in superparasitised hosts.

Key words. *Copidosoma*, intra-specific competition, polyembryony, sex ratio.

Introduction

Host selection by parasitoids has major effects on their fitness (Hassell, 2000). Parasitoids that develop in high-quality hosts, which provide ample food resources, have higher survival and

fecundity than parasitoids that develop in low-quality hosts (e.g. Bernal *et al.*, 1999; King, 2000). When high-quality hosts are not available, a parasitoid can either accept low-quality hosts or continue searching for good hosts (Van Alphen & Vet, 1986). Hosts that had already been parasitised (either by the same parasitoid female or by a different one) are generally of lower quality, as embryos developing within them suffer intra-specific competition for food resources (Godfray, 1994). Models and experiments show that parasitoids modify their host selectivity

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according to previous foraging experience and internal physiological state. They are more likely to accept parasitised hosts after they had encountered them repeatedly, when carrying large egg loads, or when nearing the end of their reproductive period (Bouskila *et al.*, 1995; Strand & Obrycki, 1996; Clark & Mangel, 2000).

The fate of larvae developing in superparasitised hosts varies among species. In solitary parasitoids, only one individual emerges from each host, and the development of other larvae is aborted. In gregarious parasitoids, all larvae within a host can complete development and emerge. Polyembryonic parasitoids show a unique variation on gregarious development. In these parasitoids, one or more eggs may be laid within one host. The embryo that originates from each egg cleaves several times during early development within the host body to form a clone of many genetically identical individuals (Grbić *et al.*, 1998). A brood (all individuals developing in one host) may consist of one or more clones. When food resources become limiting for the developing brood, competition between clones may ensue. This competition may reduce the number, longevity, and/or fecundity of individuals in some or all of the competing clones. Thus, a wide range of possible outcomes of superparasitism is possible. This feature makes polyembryonic parasitoids attractive subjects for studying the consequences of superparasitism.

The polyembryonic genus *Copidosoma* has the additional interesting feature of phenotypic plasticity among clone-mates. In several *Copidosoma* species, the genetically identical embryos develop into two larval castes that differ in morphology and physiology (Doutt, 1947; Grbić *et al.*, 1992; Hardy, 1996). Precocious soldier larvae differentiate at an early stage of host development, and die before reaching sexual maturity. They form a sterile defender caste that protects clone-mates from intra-specific (Grbić *et al.*, 1992) and inter-specific (Harvey *et al.*, 2000) competitors. Reproductive larvae develop later, and mature into normal adults.

In the present study the costs associated with superparasitism in *Copidosoma koehleri*, in terms of number and quality of offspring, were measured. First, the number of eggs laid by a female per oviposition was determined. If several eggs are laid in each oviposition, then most hosts are expected to be superparasitised. If eggs are oviposited one at a time, superparasitism should only occur in hosts that receive two ovipositions (by one or more females). The frequency of superparasitism may then vary according to foraging conditions. Next, *C. koehleri*'s soldier caste was characterised. The aim was to verify that *C. koehleri* resembles the better-studied species *C. floridanum* in having mainly female soldier larvae, and that these larvae attack intra-specific competitors. The sex-related asymmetry in larval development suggests that the consequences of intra-specific competition in *Copidosoma* may depend on larval sex. Following this rationale, it was hypothesised that female clones (which are the main producers of soldiers) will cause higher mortality to their competitors than male clones. Hence lower larval survival is expected in superparasitised hosts that contain females (male–female or female-only broods) than in male-only broods. In male–female broods, more females are expected to complete their development than males. In female–female

broods, more competition-induced larval mortality, resulting in smaller adult broods, is expected as compared with male–male broods.

The study question can be framed within the more general contexts of foraging theory and sex allocation theories. From a foraging point of view, a prey choice problem is considered, namely the trade-off between feeding on poor prey items (parasitised hosts) and continuing to search for good items (non-parasitised hosts). From a sex allocation point of view, the present study deals with an interesting situation, where local resource competition between developing offspring may directly affect secondary sex ratios.

The hypothesis was tested by creating nine combinations of host–parasitoid ratios and exposure durations, and characterising the number and sex of offspring that developed in each treatment. Higher frequencies of superparasitism are expected under increased parasitoid densities and prolonged exposure durations to hosts. Under these conditions, the research hypothesis predicts (a) a lower number/quality of offspring per parasitoid female, due to higher resource competition, and (b) fewer individuals in female-containing broods than in male-only broods, due to aggressive behaviour of female soldiers.

Methods

Study species

Copidosoma koehleri (Blanchard) is an egg-larval proovigenic parasitoid of the potato tuber moth *Phthorimaea operculella* (Zeller) (Gelechiidae: Lepidoptera). Parasitoids lay their eggs in host eggs of all ages (0–5 days), and kill their host at its last larval instar. Development within the host body requires \approx 25 days at optimal temperature (Horne & Horne, 1991), and the median longevity of fed adults is 7–10 days. All individuals in a brood emerge from the host on the same day. Adult females mate and start ovipositing immediately after emergence. The egg complement per female is \approx 100 eggs (Kfir, 1981; and Keasar, pers. obs.). There is no clear size dimorphism between males and females.

The insectary stock of *C. koehleri* was initiated from a field collection from South Africa in 2003. The potato tuber moth stock originated from repeated field collections in Israel during 2002 and 2003. Parasitoids and their hosts were reared using modifications on Berlinger and Lebiush-Mordechi's (1997) and Kfir's (1981) protocols.

The number of eggs released per oviposition

The presence of multiple clones in a host may arise from the release of several eggs in a single oviposition, or from repeated ovipositions by one or several females (superparasitism). To determine the number of eggs laid in one oviposition event, ovipositions by 21 mated females were observed. The females were removed at the end of a single oviposition, and the freshly parasitised hosts were dissected under a light microscope. The number of *C. koehleri* eggs within each host was recorded.

Determination of soldier sex

The study's working hypothesis is based on the assumption that soldier larvae in *C. koehlerii* are primarily females. Testing of this assumption made use of the fact that virgin females produce only male offspring, while fertilised females produce both male and female broods. The frequency of soldier production in hosts parasitised by virgin vs. mated *C. koehlerii* females was compared. The frequency of soldier larvae should be higher among broods produced by mated females than among offspring of virgin females, if most soldiers are females. Virgin females and mated females were presented with a host, and were observed until they completed one oviposition. Each female was allowed to parasitise up to five hosts. Fifty hosts were parasitised by virgin females, and 78 were parasitised by mated females. The parasitoids were then removed, and the hosts were reared until the larval stage. Hosts were dissected under a light microscope, and the presence and number of soldier larvae was recorded.

Determination of soldier aggression

The tendency of soldiers to attack intra-specific competitors was assessed using *in-vitro* and *in-vivo* methods. In the *in-vitro* assays, 19 soldiers were dissected out of their hosts. Each of them was paired with a polymorula originating from a different host in 1 ml culture wells containing TC-100 medium (Sigma Aldrich, St. Louis, MO). Soldier behaviour was observed for 1.5 h. An attack on the competitor larva was scored when a soldier gripped the polygerm with its mandible, and consumption of tissue by the soldiers was readily visible. In the *in-vivo* assays, parasitoid clones were labelled with the fluorescent marker carboxyfluorescein diacetate succinimidyl ester (CFSE). Labelled polygerm was injected into seven parasitised hosts, which were dissected 24 h later. The proportion of digestive tracts of soldier larvae in these broods, which were fluorescently labelled, was recorded.

Manipulation of superparasitism rates

The frequency of superparasitism in *C. koehlerii* was manipulated by varying parasitoid–host ratios and exposure durations. Four, 14, or 28 parasitoids were exposed to 50 hosts for 6, 24, or 48 h. This resulted in nine combinations of density and exposure time, each in nine replicates. Each replicate contained an equal number of male and female parasitoids, randomly collected from laboratory stock.

Forty-eight-hour-old parasitoid males and females were used. The hosts were 24 h-old eggs of potato tuber moth. The experiment was conducted in 500 ml plastic containers, covered with gauze cloth. The containers were kept at 27 ± 2 °C and 70% relative humidity with a 16:8 h L:D cycle. The hatched moth larvae were kept on potato tubers in the same containers. Parasitised hosts die at their last larval instar, and can be distinguished from normal healthy hosts at that stage, 18–22 days after they were parasitised. Parasitised host mummies were separated at this stage. Each mummy was kept in a separate vial until the emergence of the parasitoid offspring. These offspring

were counted and sexed, as measures of reproductive success and sex ratio in the different experimental treatments.

Brood size and offspring size

Head width was measured for individuals that originated from 36 broods randomly taken from insectary stock. Nine broods were all male, 23 were all female, and four contained male and female wasps. Five individuals from each single-sex brood, and five individuals of each sex in the male–female broods were measured. The dead wasps were photographed under a light microscope at $\times 100$ magnification. Measurements were performed on the photographed images using Analysis™ (Münster, Germany) software (Soft Imaging System, 2002).

Brood size and offspring quality

The effect of brood size on parasitoid survival was studied in a sample of 98 broods of both sexes (three broods male-only, 21 broods female-only, 74 broods mixed), randomly taken from the insectary stock. Wasps were fed with honey and housed at 13 °C. The number of surviving individuals was recorded twice a week. Median survival, in days, was calculated for each brood. Median survival values were then correlated with brood sizes.

The relationship between brood size and future female fecundity was characterised in 33 additional female-only broods. One female from each brood was mated to one male randomly selected from insectary stock, and exposed to 50 hosts at room temperature. Hosts were replaced every other day until the female's death. The number of parasitised hosts provides a direct measure of a female's lifetime fecundity. The females' fecundity was correlated with the size of the brood in which they had developed.

The relationship between brood size and male reproductive success was characterised by measuring the time to first copulation, and the number of copulations per 15 min, in 20 males that originated from six all-male broods. Each male was placed in a Petri dish with 10 virgin females at room temperature. The time to first mating, and the number of copulations observed, were correlated with the brood size from which the males had originated.

Data analysis

Two-way ANOVA was used to study the effects of parasitoid density and host exposure duration on the following variables: number of parasitised hosts, frequency of superparasitism, brood size, number of offspring per parasitoid female, frequency of male and female broods and number of hosts that completed development. Median longevity of broods was defined as the number of days that elapsed until 50% of the individuals in the brood died. Linear regression was used to relate brood size with median longevity and female fecundity. The relationship between brood size and the number of male copulations was examined using Pearson's correlation. Statistica™ 6.0 software (StatSoft Inc., 2005) was used for the analyses.

Results

The number of eggs released per oviposition

Three hosts that experienced a single *C. koehlerii* oviposition did not contain any parasitoid eggs, 16 hosts contained one egg, and each of the remaining two hosts contained two eggs. The mean number (\pm SD) of eggs per host was 0.952 ± 0.450 .

Soldier larvae in offspring of virgin and mated females

None of the 50 clones mothered by virgin females contained any soldier larvae. A single soldier larva developed in 47.4% of the 78 clones produced by mated female. Two soldiers developed in 1.3% of these clones. The remaining clones mothered by mated females did not produce any soldiers.

Soldier aggression

Soldier larvae attacked a genetically unrelated intra-specific competitor in seven of 19 *in-vitro* assays (37%). The fluorescent label was found in the guts of four of seven (57%) soldiers in *in-vivo* assays.

Competition for hosts and superparasitism

The extent of superparasitism was estimated via the frequency of male–female broods, as such broods must have originated from at least two ovipositions – a male and a female egg – within the same host. This is a conservative estimate of superparasitism, because it does not include broods composed of two or more clones of the same sex. The frequency of male–female broods increased with increasing exposure time (two-way ANOVA, $F_{2,48} = 24.04$, $P < 0.0001$) and parasitoid density ($F_{2,48} = 4.83$, $P = 0.012$) (Fig. 1).

Competition for hosts and number of offspring

Brood size increased with increasing parasitoid densities and exposure durations ($F_{2,48} = 70.81$, $P < 0.0001$ for density, $F_{2,48} = 34.28$, $P < 0.0001$ for duration, Fig. 2). The total number of parasitised hosts was not significantly affected by parasitoid density and exposure time. The total number of offspring was divided by the number of adult females in each replicate to obtain the mean number of offspring per female (Fig. 3). This measure decreased with increasing parasitoid density (two-way ANOVA, $F_{2,48} = 9.72$, $P < 0.0001$), and was not significantly affected by the duration of exposure ($F_{2,48} = 0.85$, $P = 0.43$).

Competition for hosts and host development

The number of hosts that developed (resulting in either parasitised larvae or in non-parasitised pupae) significantly decreased

with increasing parasitoid densities and exposure durations (two-way ANOVA, $F_{2,48} = 5.01$, $P = 0.009$ for parasitoid density, $F_{2,48} = 6.44$, $P = 0.003$ for duration of exposure, Fig. 4). The remaining hosts died at the egg stage or at one of the larval stages.

Brood size and sex

The frequency of all-male broods decreased with increasing parasitoid density ($F_{2,48} = 6.016$, $P = 0.005$), while the frequency of all-female broods was not significantly affected by parasitoid density and exposure duration. Thus, the increase in male–female broods that accompanied increased parasitoid densities (Fig. 1) was mainly at the expense of male broods. The sex of the brood interacted significantly with density ($F_{4,1061} = 10.34$, $P < 0.0001$) and duration ($F_{4,1061} = 4.41$, $P < 0.0001$) in their effect on brood size: female broods were larger than male broods at low parasitoid densities and short exposures, and male broods were larger than female broods at high densities and long exposure durations (Fig. 5). The mean sex ratio (\pm SE) in male–female broods in all treatments was 0.32 ± 0.01 .

Brood size and offspring quality

Brood size was inversely related to offspring head width ($y = -0.4x + 403.87$, $R^2 = 0.16$, $F_{1,210} = 40.40$, $P < 0.0001$). There was a significant negative correlation between brood size and the median survival of the brood ($r = -0.3$, $P = 0.003$, $n = 98$). Fecundity, however, was not affected by brood size ($r = -0.17$, $P = 0.35$, $n = 33$). Similarly, the number of copulations obtained by males in 15-min observations was not affected by the brood size from which these males had originated.

Discussion

The results show that *C. koehlerii* females readily superparasitise hosts in the laboratory, and that the frequency of superparasitism increases with increasing parasitoid density and exposure duration (Fig. 1). This suggests that superparasitism may be a

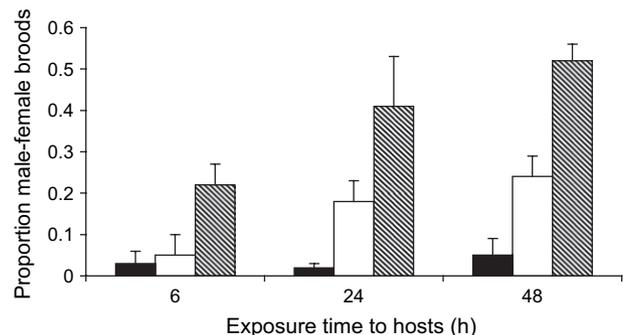


Fig. 1. The mean proportion (\pm 1 SE) of broods containing both males and females, a measure of the frequency of superparasitism.

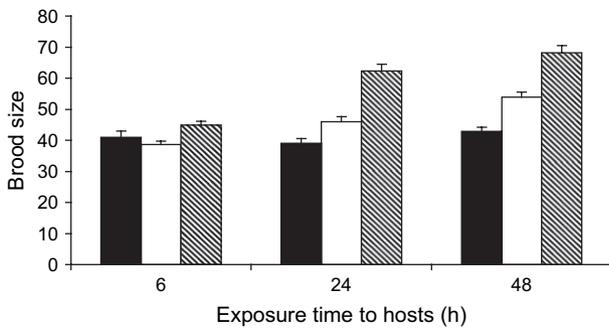


Fig. 2. The mean number of individuals (+1 SE) per brood.

consequence of host limitation. Superparasitism may result from successive ovipositions by a single female into the same host (self-superparasitism), or by ovipositions into hosts that had been previously parasitised by a different female (Doutt, 1947). In a preliminary experiment, only 4.2% of 497 broods produced by single mated females ($n = 26$), each presented with 50 hosts, were mixed-sex (data not shown). Direct observations in the present study confirm that females usually lay one egg per oviposition. These data are compatible with the notion that females tend to lay a single egg per host when hosts are abundant. Host limitation, which the parasitoids experienced in most of the experimental treatments, may increase the frequency of self-superparasitism (Ode & Strand, 1995).

The response variable used in the present study, the frequency of mixed-sex broods, clearly underestimates the true extent of superparasitism. The experiment's main aim is therefore to study the implications of varying rates of superparasitism, rather than to measure these rates precisely.

Superparasitism reduces the number of progeny per female (Fig. 3) in two ways, both presumably through intra-specific competition. First, as competition between parasitoids increases, more hosts fail to develop (Fig. 4). Possibly, these hosts are mechanically mutilated so severely by multiple ovipositions that they do not initiate or complete their development. Alternatively, host tissues may be consumed prematurely by the large number of larvae, preventing the completion of their

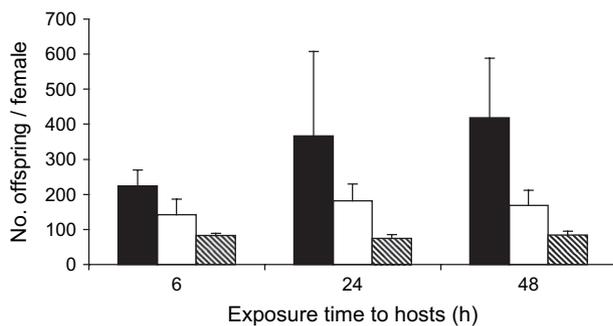


Fig. 3. The mean number (+1 SE) of offspring per female, calculated as the total number of parasitoids emerging in each replicate divided by the number of potential mothers (two, seven or 14, depending on treatment).

development. The failure of some hosts to develop could explain why the total number of parasitised hosts was affected by the experimental manipulations. Second, the number of wasps per brood increases as parasitoid density and exposure duration are increased, but this increase is less than linear (Fig. 2). This pattern may reflect mortality of some of the developing larvae in multiply parasitised hosts, because of insufficient food resources or direct aggression. *Copidosoma koehleri* lays ≈ 30 eggs within 48 h after emergence, but some eggs may be oviposited later (Kfir, 1981). Thus, egg limitation is not likely to have limited the increase in superparasitism rates in the 48-h treatments.

Superparasitism also likely reduces the quality of emerging parasitoids, as wasps that originated from large broods were smaller, and survived a shorter time, than individuals from small broods. This negative correlation between brood size and survival may reflect increased competition for food among adults in large broods. The wasps typically did not consume all the honey supplied to them, reducing the likelihood of this explanation. Brood size did not affect female fecundity or male mating success in the present experiment, but Ode and Strand (1995) report reduced reproductive ability in small male and female *C. floridanum* originating from large broods. A possible reason for the difference between the present study and Ode and Strand (1995) is that total offspring number was used as a measure of female reproductive success in the present experiment. Other measures, such as egg production, or offspring survival, may differ for females originating from large or small broods.

The experimental results indicate that *C. koehleri* suffer substantial fitness costs from superparasitism, which may result from competition between broods for limiting host resources. Individual oviposition patterns of females were not observed in the present study, and therefore direct information on the genetic composition of broods is lacking. The following indirect evidence suggests that the outcome of between-brood competition is affected by the sex of the brood: (a) male–female broods contained more females than males (also reported by Doutt, 1947), suggesting that females can outcompete males (b) when competition for hosts was potentially high (high parasitoid densities, long exposure durations), all-female broods were smaller than all-male broods. The opposite pattern appears when high-quality hosts were abundant (Fig. 5). Under weak host competition,

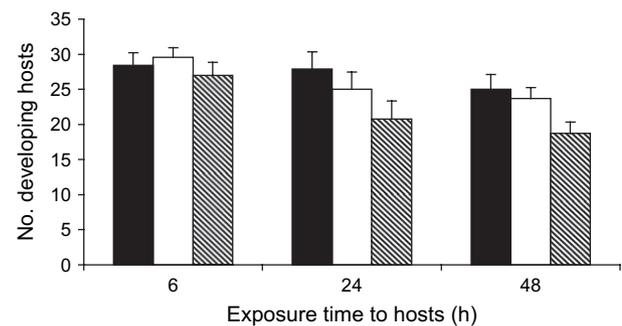


Fig. 4. The mean number (+1 SE) of hosts that developed into either normal pupae or parasitised mummies. Black bars = two parasitoid females; white bars = seven females; hatched bars = 14 females.

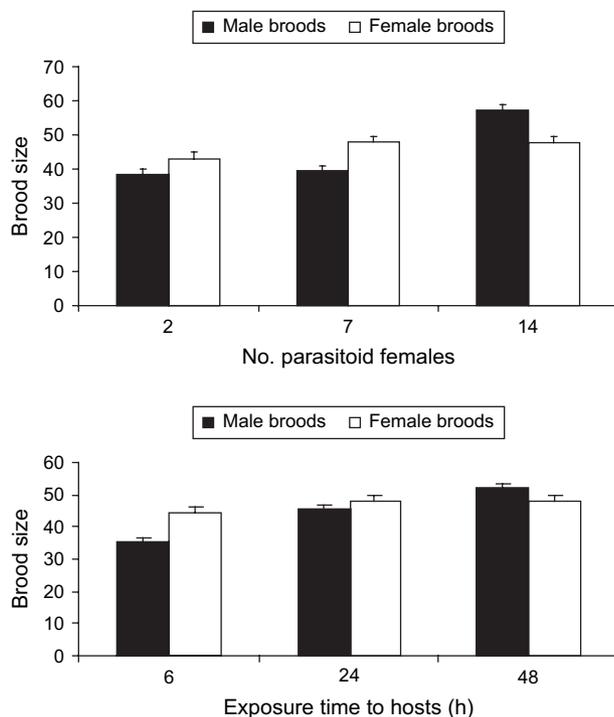


Fig. 5. The mean number (± 1 SE) of individuals in male (black bars) and female (white bars) broods. Top: with increasing parasitoid densities (two, seven, or 14 parasitoid females exposed to 50 hosts). Bottom: with increasing host–parasitoid exposure durations (6, 24, or 48 h).

most unisexual broods probably arise from a single egg. Under severe competition for hosts, many unisexual broods probably arise from two or more eggs of the same sex that had been laid into the same host. Direct larval competition may lead to larger mortality in female–female broods than in male–male broods, accounting for the difference in the number of emerging adults.

Sex-related asymmetry in larval competitive ability is consistent with the census of soldier larvae in offspring of virgin vs. mated *C. koehlerii* females. While soldier larvae were non-existent in male-only broods, they developed in 48.7% of the broods produced by mated females (also reported by Douth, 1952). Sampling for soldiers destroyed these broods, hence they could not be sexed. Nevertheless, assuming that the sample contained male and female broods in similar proportions, and that no soldiers develop in male-only broods, a high incidence of soldiers in female broods is implied. Observations of larval aggression further support the notion that female larvae are better competitors than males, as female soldiers actively attacked and cannibalised intra-specific competitors both *in vitro* and *in vivo*. The small sample sizes of the *in-vivo* and *in-vitro* assays allow only a qualitative demonstration of the aggressive behaviour of soldiers in *C. koehlerii*. Larger-scale experiments are needed to quantify the frequency and intensity of soldier aggression under varying competition regimes.

Soldier larvae in *Copidosoma* do not reproduce, yet they improve the reproductive prospects of their clone-mates. Hence

they are considered an altruistic caste (Grbić *et al.*, 1992). In *C. floridanum*, the soldiers' intensity of aggression increases with decreasing genetic relatedness between the competing clones, as predicted by kin selection theory (Giron *et al.*, 2004). The results of the experimental manipulations of wasp densities and exposure durations suggest that *C. koehlerii*'s soldiers likewise attack unrelated competitors in superparasitised hosts more than related individuals. Genetic relatedness between competing clones is expected to decrease as the number of potential mothers increases (because the probability for self-superparasitism is reduced), but should not depend on parasitoid–host exposure durations. Thus, increasing parasitoid density is expected to affect development in superparasitised hosts more severely than increasing exposure durations. In agreement with this prediction, mean fecundity per female in the present experiment significantly decreased with increasing parasitoid density, but not with increasing exposure duration (Fig. 3).

Clark and Mangel (2000) modelled the oviposition behaviour of proovigenic parasitoids, and showed that host discrimination ability should optimally depend both on internal state and on information state. In agreement with this theory, parasitoids tend to accept low-quality (already parasitised) hosts if they perceive that no good hosts are available (Roitberg *et al.*, 1992; Visser *et al.*, 1992). They also become less selective as they age (Fletcher *et al.*, 1994) and/or carry larger egg complements (Henneman *et al.*, 1995; Van Randen & Roitberg, 1996; Takatoshi, 1999).

The manipulated experimental variables combined effects on information state and internal state. By increasing parasitoid density, encounter rates among parasitoids were increased, which could be used as a cue to parasitoids and affect their information state. Another outcome of increasing parasitoid density is an increase in the fraction of parasitised hosts. If parasitoids are able to discriminate parasitised hosts from fresh hosts (Nufio & Papaj, 2001), they could also use their rate of encounter with parasitised hosts as an information cue. Host encounter rates also affect oviposition decisions in other polyembryonic parasitoids (Hardy *et al.*, 1993; Hoffmeister *et al.*, 2000).

The second experimental manipulation, increasing the wasps' duration of exposure to hosts, also increased the fraction of parasitised hosts and thus could have affected the wasps' information state. Increasing parasitoid–host exposure time also resulted in older parasitoids, i.e. also affected their internal physiological state. As predicted (Clark & Mangel, 2000), both experimental variables increased the parasitoids' tendency to superparasitise.

Females that oviposit into hosts that are already parasitised may gain higher fitness from laying female eggs than from laying male eggs, if females are more successful in between-brood conflicts. The results indirectly suggest that females indeed produced more daughters as hosts became limited: the frequency of male–female broods increased (Fig. 1), while the frequency of all-male broods decreased. A possible interpretation of this finding is that females preferably laid female eggs in hosts that already contained a male brood. The sex ratio data does not suffice to infer whether females also laid mostly female eggs in hosts that have already been parasitised by a female brood. The increase in female brood size with increasing

parasitoid density and exposure time supports this possibility (Fig. 2). In addition, host-limited females may increase their reproductive success by laying more female eggs when encountering unparasitised hosts, as daughters are more likely to survive if the same host is superparasitised later. The possibility that females modify primary sex ratios in response to host limitation was not tested directly in the present study, because females' ovipositions were not observed and controlled. While consistent with the findings, this possibility remains to be explored in more detailed experiments.

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