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# Competitive interactions between parasitoid larvae and the evolution of gregarious development

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**Abstract** We report experiments using two closely related species of alysiine braconids directed at understanding how gregarious development evolved in one subfamily of parasitoid wasps. Theoretical models predict that once siblicide between parasitoid wasps has evolved, it can only be lost under stringent conditions, making the transition from solitary to gregarious development exiguous. Phylogenetic studies indicate, however, that gregariousness has independently arisen on numerous occasions. New theoretical models have demonstrated that if gregarious development involves reductions in larval mobility, rather than a lack of fighting ability (as in the older models), the evolution of gregariousness is much more likely. We tested the predictions of the older tolerance models (gregariousness based on non-fighting larval phenotypes) and the reduced mobility models (gregariousness based on non-searching larval phenotypes) by observing larval movement and the outcome of interspecific competition between *Aphaereta genevensis* (solitary) and *A. pallipes* (gregarious) under multiparasitism. Differences in larval mobility matched the prediction of the reduced mobility model of gregarious development, with the solitary *A. genevensis* having larvae that are much more mobile. The proportion of hosts producing the solitary species significantly declined after subsequent exposure to females of the gregarious species. This contradicts the prediction of the older models (fighting vs non-fighting phenotypes), under which any competitive interactions between solitary and gregarious larvae will result in a highly asymmetrical outcome, as the solitary species should be competitively superior. The observed outcome of interspecific competition offers evidence, with respect to this subfamily, in favour of the new models (searching vs non-searching phenotypes).

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## Introduction

A major ecological phenomenon determining the course of evolution is competition by organisms for scarce resources like food, territory or mating opportunities. Many of the traits that organisms possess are adaptations that aid success in competitive interactions (Darwin 1859; Schluter 2000). Competition frequently takes place in open systems, so individuals have the option of either competing over the immediate resources present or of leaving the resource patch and going elsewhere (Hamilton and May 1977; Stephens and Krebs 1986).

Competition can also occur in closed systems in which organisms cannot leave the resource patch. In many species, juvenile organisms have a limited ability to move to another resource patch. Many species utilize a developmental nursery characterized as a spatially and structurally discrete unit in which progeny complete their development (Mock and Parker 1997). Organisms under such conditions can compete in one of two broad ways: they either attempt to exclude other competitors in a contest process or in a scramble process (sensu Nicholson 1954) gain as much of the disputed resource as possible but without pro-actively interfering with other competitors. In a developmental nursery, several juveniles (frequently siblings) can be placed together and the competition for resources may often be intense. Sibling rivalry has been the subject of intensive research by evolutionary biologists (see Cheplick 1992; Elgar and Crespi 1992; Mock and Parker 1997). The predominant conceptual framework derives from Hamilton's classic work on social evolution (Hamilton 1963, 1964a, b).

Kin selection can be applied to a wide range of social phenomena including aggression and fighting behaviour (Hamilton 1964a, b). Hamilton suggested that a high level of relatedness could arise because of limited dispersal during life (or a phase of life) and that such population

viscosity would facilitate the evolution of co-operative or non-aggressive traits (Hamilton 1972). However, the increased intensity of competition between relatives can potentially reduce or remove any kin selected benefits of co-operative or non-aggressive behaviour (Wilson et al. 1992; Griffin and West 2002; West et al. 2001, 2002). If the value of the disputed resource is high enough (and/or the costs of resource monopolization are low), it can be in the evolutionary interests of sibs not to help or assist each other. Sibs can often act aggressively towards each other resulting in conflict between offspring (Mock and Parker 1997) and parent–offspring conflict (Trivers 1974; Godfray 1995).

A conspicuous example of sibling rivalry and parent–offspring conflict is found in the behaviour and development of parasitoid wasps (Hymenoptera) which lay their eggs on or in a host organism (usually the non-adult stage of another insect). The developing wasps feed upon the still-living body of their host eventually killing it (Godfray 1994; Quicke 1997). The majority of recorded species of parasitoid wasps develop solitarily (Mayhew and Hardy 1998). In solitary species, if more than one egg is laid the offspring are involved in antagonistic interactions for possession of the host (with rivals typically destroyed by direct physical combat or alternatively via physiological suppression) (see Salt 1961; Godfray 1994), until only one individual remains to complete its development. In contrast to the stark "ultra-siblicide" of solitary species (Mock and Parker 1997), gregarious species are characterized by the observation that multiple offspring can successfully develop together from a single host. In gregarious species, females can potentially optimize their clutch size decisions. In solitary species, females may be forced to reduce their average clutch size to avoid wasting eggs (and hence resources) on offspring that will inevitably fail to complete development (Skinner 1985; Waage and Godfray 1985), thereby decreasing the fitness of adult females.

The reduction of clutch size (and the restriction of brood size to one) is an example of parent–offspring conflict over the level of resources received by offspring, in which the offspring have control. Examined from a genetic perspective it is clear that offspring "selfishness" can evolve, even if it is detrimental to parental fitness (Godfray 1995), as many population-genetic models have shown that a gene causing offspring to take more resources than the parental optimum can spread (reviewed in Mock and Parker 1997). The parent–offspring conflict in parasitoid wasps represents the contradictory nature of divergent optimization "goals" during different stages of life: larval monopolization of limited resources during development versus the maximization of brood size per host as an adult, with the genes expressed in parents and their offspring having different optima. Solitary development reflects (in many species) an evolutionary process rather than the consequence of a physical (proximate) constraint upon brood size. Hosts do represent a limited set of resources, but it is not usually the case that there are insufficient resources or space for more than a single individual to successfully complete development (see Khoo et al. 1985; le Masurier 1987; also see Harvey et al. (2000a), for a discussion of parasitoids that only partially feed on upon their hosts).

Models for the spread of either a rare fighting allele or a rare non-fighting allele suggest that siblicidal behaviour can evolve in small gregarious broods (up to clutches of around four individuals) whereas the clutch size required for the spread of a non-fighting allele in a siblicidal population is much larger. Consequently, a bimodal distribution of emerging brood sizes should be observed with many solitary species, many gregarious species with very large brood sizes and an absence of gregarious species with small gregarious broods (Godfray 1987). The existence of this threshold difference in the conditions required for the spread of tolerant and intolerant phenotypes has resulted in the evolution of solitary development being cited as an example of directionally biassed/irreversible evolution (Harvey and Partridge 1987; Williams 1992).

However, the phylogenetic distribution of solitary and gregarious development indicates that solitary development is likely to be the ancestral condition in the parasitic Hymenoptera: gregariousness has independently evolved at least 43 times in 26 different families of the Hymenoptera (Rosenheim 1993; Mayhew 1998a). In addition, in many parasitoid taxa small gregarious broods are observed and are apparently stable (Mayhew 1998b). The tolerance model of Godfray (1987) assumes that a tolerant larva, when in the presence of an intolerant larva, will always be killed as tolerant larvae cannot fight. Thus the intolerant (fighting) and tolerant (non-fighting) phenotypes constitute a dichotomy without intermediate behavioural phenotypes.

This orthodox view was recently tested in multiparasitism experiments involving a sympatric pair of egg endoparasitoid sister-species from the genus *Anaphes* (Boivin and van Baaren 2000). In the field, *A. listronoti* produces small gregarious broods of around two individuals (Boivin 1986). These species are frequently found together in the field and have strongly overlapping host species ranges. First instar larvae in both species have well-developed mandibles and are distinguishable from their different segmentation (van Baaren et al. 1997). Physical fights between first instar larvae in the solitary *A. victus* eliminate supernumerary larvae (Nénon et al. 1995).

When a larva of each species was in a host the proportion of individuals of either species emerging indicated that both types of larvae had an equal probability of surviving under interspecific competition (Boivin and van Baaren 2000). Boivin and van Baaren (2000) proposed that, rather than the loss of fighting ability in *A. listronoti*, the phenotypic change resulting in gregarious development was a reduction in larval mobility/searching intensity. Observations (in vitro) confirmed that the first instar larvae of these species significantly differed in movement in the predicted way (Boivin and van Baaren 2000). Dissections of multiparasitized hosts confirmed the presence of scars and wounds (derived from physical combat) in the first instar larvae of both species.

Recent models have demonstrated the relative ease (in small gregarious clutches of two to four larvae) with which a rare "immobility" allele can spread in a population of fully mobile fighters, assuming that reduced mobility larvae have an equal chance of winning any fight with a fully mobile larva (Pexton et al. 2003). Relatively few empirical studies have focused upon the behavioural and evolutionary ecology of immature parasitoids (compared with adults) (see Brodeur and Boivin 2004). Hence, the aim of this study was to test the asymmetric aggression hypothesis (tolerant/intolerant phenotypes) against the asymmetric larval mobility hypothesis (searching/non-searching phenotypes) with a pair of congeneric sister-species from the braconid genus *Aphaereta*. In doing so we provide another independent contrast (see Harvey and Pagel 1991) and contribute towards a greater understanding of the behavioural ecology of juvenile parasitoid wasps.

## Materials and methods

### Insects

The genus *Aphaereta* belongs to the subfamily Alysiinae that contains over 1,000 described species in around 70 genera (Shaw and Huddleston 1991). *Aphaereta* occupies a derived position with the genera *Asobara* and *Phaenocarpa* (Wharton 1980; Gimeno et al. 1997). The Alysiinae itself forms a monophyletic group with the subfamily Opiinae (itself containing over 1,300 species; Quicke and van Achterberg 1990; Quicke 1993; Belshaw et al. 1998). All the members of this clade are koinobiont endoparasitoids of cyclorrhaphous Diptera. With the exception of a recently described species of *Phaenocarpa* (van Achterberg 1998), *Aphaereta* is the only genus in both subfamilies known to contain gregarious species (Wharton 1977, 1980, 1984; Shaw and Huddleston 1991). This suggests that gregarious development is the derived state within this subfamily.

*Aphaereta genevensis* and *A. pallipes* are morphologically almost indistinguishable as adults [adult females can only be distinguished by small (overlapping) differences in the relative length of the ovipositor; males are indistinguishable at present (see Wharton 1977)]. Mayhew and van Alphen (1999) confirmed that the first instar larvae of both species possess mandibles at this developmental stage. Mayhew and van Alphen (1999) also demonstrated that in *A. genevensis*, if more than one larva was present in a host, the sharp sclerotized mandibles were used in lethal physical combat until a single larva remained (with fights recorded during the first instar stage only). Despite the same first instar larval morphology there was no evidence for *A. pallipes* larvae engaging in combat, with no fighting observed amongst larvae. We used these species in multiparasitism experiments with the null hypothesis that the proportion of hosts from which *A. genevensis* emerged should be robust under multiparasitism (when *A. pallipes* also had the opportunity to oviposit into hosts). In contrast, the proportion of hosts from which *A. pallipes* emerged should not be robust when female *A. genevensis* also had the opportunity to oviposit into hosts.

### Cultures

Laboratory populations of *A. genevensis* and *A. pallipes* were used in this study. The cultures were collected in New York State (USA), during 1995 and 1996, in a region where the species are sympatric (see Mayhew and van Alphen 1999). Since 1997, both species have been reared on *Drosophila virilis*. The hosts were reared in glass bottles on standard yeast based medium. Wasps were reared in 5 cm diameter glass jars with foam stoppers. A 2 cm layer of nutrient agar was poured into the base of a jar and allowed to set. Several 5–8 day old *D. virilis* larvae were added along with a dab of viscous yeast medium. Groups of mated females (2–5 females) with no prior experience of hosts were introduced to the jars and left until death. Jars were placed in secure plastic boxes to ensure that both species were kept completely apart within a single culturing room. The cultures were maintained at 20°C, constant light and ambient humidity. All experiments described in this study were also performed under these environmental conditions.

### Larval mobility

The mobility of first instar larvae was measured in our study species. *D. virilis* larvae parasitized during the process of culturing were carefully dissected 5 days after their initial exposure to female wasps. Upon dissection (in a drop of Ringer solution) in vitro observation of the first larva seen to move was performed under a light microscope (60x magnification) for a continuous period of 5 min. The number of folds (vertical bending) and twists (lateral torsion) was recorded in blocks of 1-min periods. If more than one larva was present only data from the first larva was noted and used for subsequent analysis (in order to avoid pseudoreplication). All moves whether large or small were recorded (so that partial folds and twists were also included in the dataset).

### Post hoc identification of wasps

Given the morphological similarities of the species and that no molecular markers exist for these species, a post hoc method of identification (i.e. post emergence from a replicate of a multiparasitism experiment) is necessary. For females, this is relatively easy because there is a clear distinction in the average brood per host [*A. genevensis* females has an average brood size of approximately one per host, whilst *A. pallipes* has an average brood size of approximately 2.5 per host (either two or three wasps per host)]. Females emerging from multiparasitism experiments could be identified by placing individual females in

a tube (75 mm ×25 mm) with between 20–40 second/third instar *D. virilis* larvae and leaving the female wasp until death occurred. The *Drosophila* larvae were left to develop allowing for both flies and wasps to emerge. After the development in identification tubes the number of flies, parasitoids and pupae could be counted and the average brood size calculated as simply: (no. wasps)/(no. pupae − no. flies).

For males, mating preferences potentially provide a method for identifying male wasps. Male wasps of both species have a stereotypical pre-mating behaviour upon encountering a female/attempting to mate. Males vibrate their antennae and beat their wings very rapidly (see Matthews 1975; Wharton 1984; Carton et al. 1986), before attempting to mount a female. In order to test this method of identification, preliminary experiments on mating preferences were performed.

Males up to 1 week old (previously stored at 4°C) were individually placed into a sterile and previously unused glass tube (50 mm ×25 mm). A female of a known identity (either of the male's species or of the other species) was placed into another unused tube of the same size. The male was then carefully introduced into the tube with the female. Observation of male behaviour was conducted over a 5-min period. The number of individual bouts of wing beating, attempted mounts and matings was recorded. After 5 min the male was removed and placed into another fresh and unused tube of the same size. The experimental male was allowed to rest for 30 min. A second female of the opposite identity to that of the first female was placed into a previously unused and sterile tube (again 50 mm ×25 mm). The experimental male was transferred to this second tube and introduced to the second female. As before mating behaviour was recorded over a 5-min period. All treatments were randomized with respect to the order in which males encountered females of either their own species or the other species. Mating preferences were observed (see Results) so this method was subsequently used to identify males that emerged from the multiparasitism experiments.

## Multiparasitism experiments

In order to test the hypothesis that gregarious larvae are unable to compete with solitary larvae under multiparasitism, it was necessary to perform experiments in which both species oviposit into hosts. If gregarious larvae are truly tolerant (that is they cannot fight at all) then, all other things being equal, only the solitary species should survive and emerge. An indirect statistical approach was used in the experiments. Patches of hosts (12 late second instar/early third instar *D. virilis* larvae) were placed into sterile petri dishes (35 mm diameter ×10 mm depth), that already had a 5 mm layer of nutrient agar and a spot of liquid yeast on the agar. Groups of between 3 and 5 females (between 4 and 10 days old) were introduced to the patch and left for 2 h. Eight treatments were carried out (each with approximately 50 replicates): two control treatments per species (four in total; two dissection and two developmental) and two experimental treatments per species (four in total; two dissection and two developmental) in which the first set of females (from one species) was initially placed onto the patch for two hours then removed (in the controls) (see Table 1). In the multiparasitism treatments a second set of females (of the other species) were placed immediately onto patches and left for 2 h.

Once the *Drosophila* larvae had fully pupated (in the treatments that were allowed to develop), pupae were carefully removed from each replicate and individually placed in appropriately labelled tubes (75 mm ×25 mm) with around 2–3 cm of cotton wool soaked with 35% sucrose solution and a circle of filter paper covering the cotton wool. This procedure insured that emerging brood size could be recorded for individual hosts, emerging wasps had a source of food (so would be unlikely to die should they have emerged in the time between the daily checks of the tubes) and that the proportion of hosts producing wasps could be accurately recorded per replicate. In the multiparasitism treatments as soon as a wasp(s) or fly had emerged from a host this event was recorded. In addition, the number and sex of the wasps was recorded. Some hosts failed to produce either a fly or a wasp. These events were recorded as developmental failures (included in this category are the small number of hosts from which a wasp emerged, but that were found dead the next day). Tubes with newly emerged wasps were stored at 4°C in a refrigerator (hugely extending the lifespan of both male and female wasps). The post hoc identification procedures were performed (males were identified first because they have a shorter lifespan than females).

## Statistical analysis

Data analysis was performed with the GLIM statistical package or by using standard techniques within SPSS as appropriate. General linear modelling in the GLIM statistical package (see Crawley 1993) was used in the analysis of the multiparasitism data. For count data Poisson error variance and for proportional data binomial error variance were initially assumed. Significance was assessed by the change in deviance by stepwise subtrac-

**Table 1** The range of treatments used in this study

| Treatment type | Species order | Subsequent treatment |
|---|---|---|
| Control | AG only | Dissection after 5 days |
| Control | AP only | Dissection after 5 days |
| Experimental | AG1+AP2 | Dissection after 5 days |
| Experimental | AP1+AG2 | Dissection after 5 days |
| Control | AG only | Development completed |
| Control | AP only | Development completed |
| Experimental | AG1+AP2 | Development completed |
| Experimental | AP1+AG2 | Development completed |

tion from a full model of all potential explanatory variables. Significance was assessed under normal errors by an *F*-test or under Poisson and binomial errors by a $\chi^2$-test. The appropriateness of the error structure was assessed by a heterogeneity factor, equal to the residual deviance divided by the residual degrees of freedom. If the heterogeneity factor was greater than 1.3, indicating over dispersion, the model was rescaled using the value of Pearson's $\chi^2/df$ (see Crawley 1993).

## Results

### Larval mobility

Larval movement was observed in the two species (Fig. 1a), with the larvae from the solitary species *A. genevensis* showing significantly more movement than *A. pallipes* larvae. One difficulty of in vitro observations is that endoparasitoid larvae can (ordinarily) only live for a limited time outside of their hosts. In order to check the results were not an artefact of larvae starting to die within the observation period, a further analysis of the movement of larvae over time was done (for both movement types in both species), by analysing the average number of moves on a minute-by-minute basis (Fig. 1b,c). In both species, the level of larval movement was consistent over time.

### Mating preferences

The behaviour of males from both species, on average, indicated mating preferences for females from their own species (Fig. 2). In both species there appears to be a strong bias in males towards more mating activity with females of their own species, irrespective of the order in which females were presented. This is especially true of *A. genevensis* males that only very rarely attempted to mate (or enter into mating behaviour) when in the presence of an *A. pallipes* female.

### Multiparasitism experiments (dissection treatments)

Those treatments that involved dissections allowed for statistical comparisons to be made between the treatments when a group of females from one species had been allowed onto a patch and the situation when groups of females from both species had been allowed onto patches. The tendency for females to oviposit can be measured in a variety of ways: the average clutch size in those hosts parasitized, the total number of eggs found within all hosts in a replicate and the proportion of hosts parasitized within replicates (Table 2). All three measures of oviposition activity were significantly higher in those treatments in which a second group of females had been placed onto patches, indicating that multiparasitism occurred.

At all stages of development there is the risk of mortality. A potentially confounding variable is the possibility that *D. virilis* larvae suffered different levels of mortality in the alternate treatments, perhaps due to the greater length of time exposed to female wasps in the two species replicates [or for another unknown reason(s)]. The average number of *Drosophila* larvae surviving (per

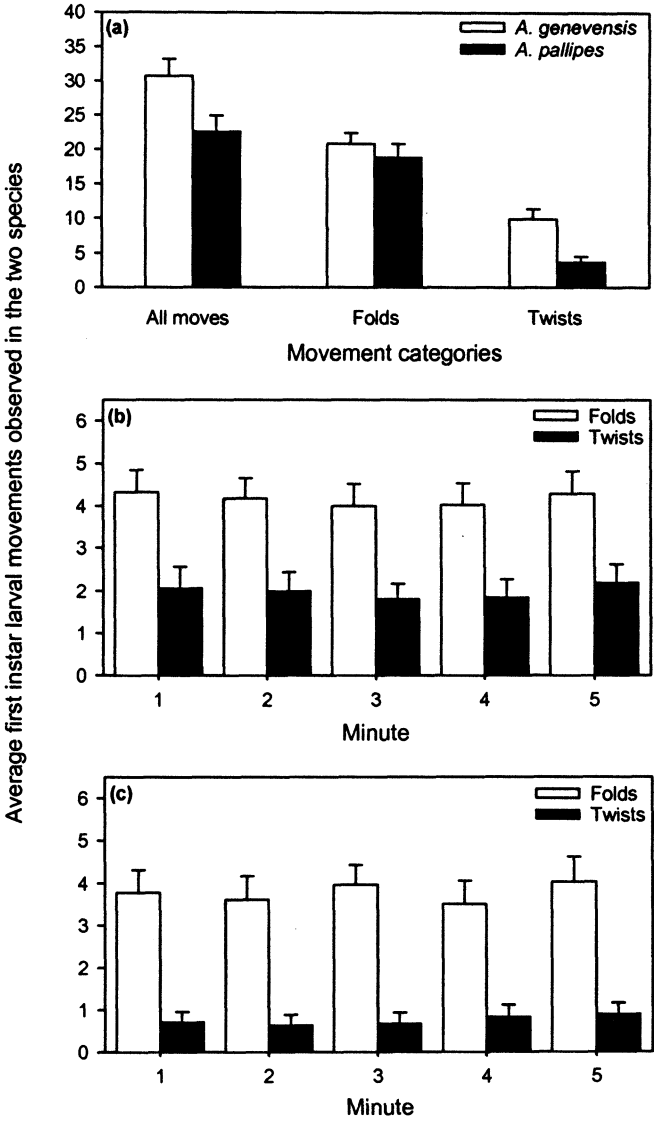


**Fig. 1a–c** First instar larval movements observed in *A. genevensis* and *A. pallipes*: **a** the mean number of first instar larval movements observed over 5 min; **b** the mean number of folds and twists observed per minute in *A. genevensis* first instar larvae across the observational period and, **c** the mean number of folds and twists observed per minute in *A. pallipes* first instar larvae across the observational period (upper 95% confidence limits displayed in all categories). Overall movement (folds and twists combined) was significantly different between the species (*t*-test, $t$=4.81, $df$=89, $P$<0.001). The mean number of folds did not significantly differ between the species (*t*-test, $t$=1.54, $df$=89, NS); in contrast, the mean number of twists did significantly differ between the species (*t*-test, $t$=7.64, $df$=89, $P$<0.001). In *A. genevensis*, the number of folds and twists were not significantly different over time: (folds) ANOVA (repeated measures), $F_{4,176}$=0.76, NS; (twists) ANOVA (repeated measures), $F_{4,176}$=0.54, NS. Within *A. pallipes*, again the number of folds and twists were not significantly different over time: (folds) ANOVA (repeated measures), $F_{4,180}$=0.33, NS; (twists) ANOVA (repeated measures), $F_{4,180}$=0.48, NS

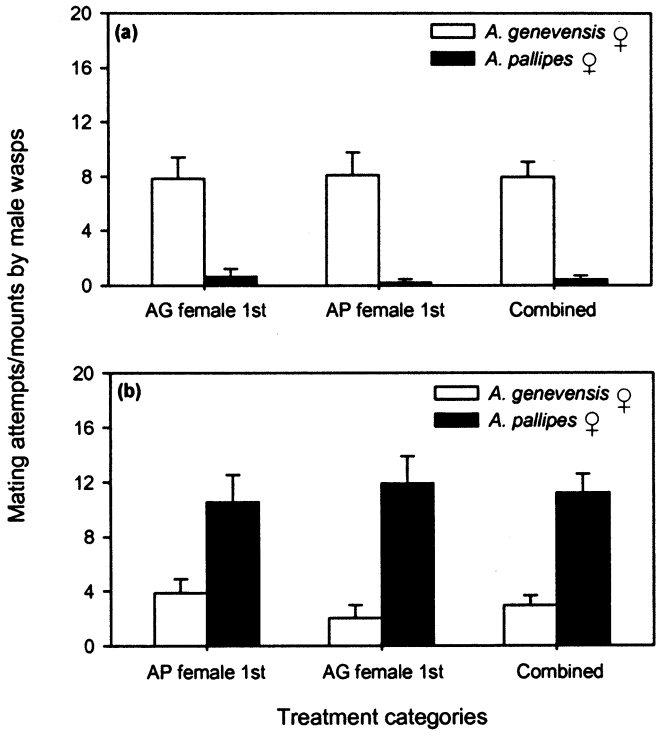


**Fig. 2a,b** The mean number of mating attempts by males of both species: **a** the mean number of mating bouts observed in *A. genevensis* males when presented with *A. genevensis* or *A. pallipes* females (upper 95% confidence limits shown in all cases) ($n$=40); **b** the mean number of mating bouts observed in *A. pallipes* males when presented with *A. genevensis* or *A. pallipes* females (upper 95% confidence limits shown in all cases) ($n$=44). Irrespective of the order in which individual males encountered females, both species displayed relatively robust mating preferences for females from their own species. *A. genevensis* males initially presented with an *A. genevensis* female: paired $t$-test; $t$=9.89, $df$=19, $P$<0.001. *A. genevensis* males initially presented with an *A. pallipes* female: paired $t$-test; $t$=9.78, $df$=19, $P$<0.001. Mating preferences for *A. genevensis* males irrespective of the order in which females are presented: paired $t$-test; $t$=13.99, $df$=39, $P$<0.001. A similar pattern exists in the gregarious species: *A. pallipes* males initially presented with an *A. pallipes* female: paired $t$-test; $t$=9.58, $df$=21, $P$<0.001. *A. pallipes* males initially presented with an *A. genevensis* female: paired $t$-test; $t$=8.95, $df$=21, $P$<0.001. Mating preferences for *A. pallipes* males irrespective of the order in which females are presented: paired $t$-test; $t$=11.99, $df$=43, $P$<0.001

replicate) (±SE, $n$) until dissection in the AG only treatment was: 10.33 (±0.25, $n$=52); and in the AG1+AP2 treatment was: 9.57 (±0.16, $n$=56). This difference was not significant: $F_{1,107}$=2.97, NS. The average number of larvae surviving (per replicate) (±SE, $n$) until dissection in the AP only treatment was: 9.63 (±0.26, $n$=49); and in the AP1+AG2 treatment was: 9.36 (±0.31, $n$=50). This difference was not significant: $F_{1,98}$=0.11, *NS.*

## Multiparasitism experiments (developmental versus dissection treatments)

The control treatments, when organisms were allowed to complete their development, can be compared to the control treatments that were dissected after 5 days. In the AG only treatment, the average number of wasps emerging (per replicate) (±SE, $n$) from pupae producing wasps, was: 1.003 (±0.003, $n$=44). This was significantly lower that the average number of eggs laid in those larvae parasitized by groups of *A. genevensis* females in the AG only control dissection treatment: 1.668 (±0.079, $n$=52); $F_{1,95}$=37.11, $P$<0.01. In the AP only treatment, the average number of wasps (per replicate) (±SE, $n$) from pupae producing wasps was: 2.446 (±0.150, $n$=42). This was not significantly different from the average number of eggs laid in those larvae parasitized by groups of *A. pallipes* females in the AP only control dissection treatment: 2.273 (±0.153, $n$=49); $F_{1,90}$=0.64, *NS.* Therefore, significant brood reduction occurs in *A. genevensis* but no brood reduction was recorded in *A. pallipes*, under these conditions. This corresponds with the findings of Mayhew and van Alphen (1999) and it also confirms that emerging brood size in *A. genevensis* and *A. pallipes* is significantly different: $F_{1,85}$=44.51, $P$<0.01. The average emerging brood size of females is a robust identification technique, with *A. genevensis* females never producing more than the odd brood size greater than one (in the AG only developmental controls a single brood of two was recorded).

## Multiparasitism experiments (developmental treatments)

The number of *D. virilis* larvae that survived until pupation could have been a confounding variable if the

**Table 2** Mean oviposition activity per replicate and statistical comparisons between dissection treatments

| Variable | Treatments and sample sizes | | | | Comparisons between treatments | |
|---|---|---|---|---|---|---|
| | AG only ($n$=52) | AG1+AP2 ($n$=56) | AP only ($n$=49) | AP1+AG2 ($n$=50) | AG only vs AG1+AP2 | AP only vs AP1+AG2 |
| Proportion of dissected hosts containing eggs (±SE) | 0.633(±0.033) | 0.746(±0.028) | 0.509(±0.028) | 0.824(±0.028) | $\chi^2_1$=5.33* | $\chi^2_1$=49.99*** |
| Eggs per host when hosts contained at least one egg (±SE) | 1.67(±0.08) | 2.99(±0.16) | 2.27(±0.15) | 3.71(±0.19) | $F_{1,107}$=36.55** | $F_{1,98}$=22.19** |
| Total number of eggs found in dissected hosts (±SE) | 11.27(±0.92) | 22.41(±1.85) | 12.67(±1.50) | 28.24(±1.66) | $\chi^2_1$=31.86*** | $\chi^2_1$=44.18*** |

*$P$<0.025, **$P$<0.01, ***$P$<0.001

**Table 3** Mean outcome of development for each treatment as a proportion of the number of hosts recorded in each replicate

| Variable | Treatments and sample sizes | | | |
|---|---|---|---|---|
| | AG only (*n*=44) | AG1+AP2 (*n*=57) | AP only (*n*=44) | AP1+AG2 (*n*=57) |
| Proportion of hosts producing AG (±SE) | 0.519(±0.037) | 0.263(±0.029) | NA | 0.173(±0.023) |
| Proportion of hosts producing AP (±SE) | NA | 0.121(±0.025) | 0.509(±0.041) | 0.359(±0.033) |
| Proportion of hosts producing a fly (±SE) | 0.087(±0.023) | 0.122(±0.023) | 0.342(±0.035) | 0.102(±0.017) |
| Proportion of hosts not producing an organism(s) (±SE) | 0.394(±0.023) | 0.495(±0.036) | 0.150(±0.028) | 0.351(±0.037) |

alternate treatments inherently resulted in differential mortality for the still growing and developing *D. virilis* larvae. The average number of larvae surviving until pupation (per replicate) (±SE, *n*) in the AG only treatment was: 7.04 (±0.38, *n*=44); and in the AG1+AP2 treatment was: 6.58 (±0.42, *n*=57). The difference was not significant: $F_{1,100}$=0.07, *NS*. The average number of larvae surviving until pupation (per replicate) (±SE, *n*) in the AP only treatment was: 8.24 (±0.34, *n*=42); and in the AP1 +AG2 treatment was: 9.00 (±0.29, *n*=55). The difference was not significant: $F_{1,96}$=0.61, *NS*.

Pupae from each replicate were, after development had proceeded, classified as either producing wasps (*A. genevensis* or *A. pallipes* as appropriate), or a fly and finally as a developmental failure (if nothing was produced) (see Table 3 for the proportions of each of the categories in both the control and the developmental treatments). In some experimental replicates a single wasp had partially emerged and died, or had emerged and died in the post emergence period, but before the tubes had been rechecked. In these cases, as only a single individual had emerged, there was no way to identify the wasp. These individuals were recorded and initially classified as

**Table 4** The proportion of wasps emerging from multiparasitism treatments under different assumptions and statistical comparisons with the proportion of wasps emerging from control treatments. (*a* dead wasps classed as AG, *b* removal of replicates that did not produce any organisms from datasets, *c* singly emerging AP males reclassed as AG, *d* singly emerging AP females reclassed as AG)

| Variable | Mean proportion of wasps emerging from hosts calculated using the total number of pupae per replicate (±SE)(*n*) | | | | Mean proportion of wasps emerging from hosts calculated using the number of pupae that successfully pupated per replicate (±SE) (*n*) | | | |
|---|---|---|---|---|---|---|---|---|
| Changes to the datasets | AG from AG1+AP2 | Comparison with AG only[a] | AP from AP1+AG2 | Comparison with AP only[b] | AG from AG1+AP2 | Comparison with AG only[§] | AP from AP1+AG2 | Comparison with AP only[b] |
| None | 0.263 (±0.030) (57) | $\chi^2_1$=34.87**** | 0.368 (±0.033) (55) | $\chi^2_1$=6.22** | 0.482 (±0.048) (57) | $\chi^2_1$=37.80**** | 0.517 (±0.039) (55) | $\chi^2_1$=0.27 |
| a | 0.319 (±0.035) (57) | $\chi^2_1$=25.37**** | 0.368 (±0.035) (55) | $\chi^2_1$=6.22** | 0.534 (±0.047) (57) | $\chi^2_1$=33.66**** | 0.509 (±0.039) (55) | $\chi^2_1$=0.46 |
| b | 0.283 (±0.030) (53) | $\chi^2_1$=35.15**** | 0.389 (±0.033) (52) | $\chi^2_1$=4.67* | 0.518 (±0.048) (53) | $\chi^2_1$=37.80**** | 0.546 (±0.037) (52) | $\chi^2_1$=0.27 |
| c | 0.301 (±0.031) (57) | $\chi^2_1$=24.25**** | 0.325 (±0.032) (55) | $\chi^2_1$=11.06**** | 0.556 (±0.048) (57) | $\chi^2_1$=32.03**** | 0.457 (±0.039) (55) | $\chi^2_1$=2.87 |
| a+c | 0.357 (±0.035) (57) | $\chi^2_1$=16.73**** | 0.325 (±0.032) (55) | $\chi^2_1$=11.06**** | 0.599 (±0.047) (57) | $\chi^2_1$=20.88**** | 0.450 (±0.039) (55) | $\chi^2_1$=3.38 |
| a+c+d | 0.385 (±0.036) (57) | $\chi^2_1$=9.69*** | 0.272 (±0.032) (55) | $\chi^2_1$=17.74**** | 0.652 (±0.049) (57) | $\chi^2_1$=10.09*** | 0.369 (±0.040) (55) | $\chi^2_1$=9.71*** |
| a+b+c+d | 0.414 (±0.036) (53) | $\chi^2_1$=9.72*** | 0.288 (±0.033) (52) | $\chi^2_1$=15.53**** | 0.701 (±0.047) (53) | $\chi^2_1$=10.09*** | 0.390 (±0.040) (52) | $\chi^2_1$=9.71*** |

*$P$<0.05, **$P$< 0.025, ***$P$<0.01, ****$P$<0.001

[a]For the proportion of wasps emerging from control treatments see Table 3

[b]The mean proportion of wasps emerging from control treatments under this assumption are (±SE, *n*): 0.834 (±0.041, 44) AG from AG only; 0.588 (±0.043, 42) AP from AP only

developmental failures (though later reclassified to test the effect of unidentified wasps on the results).

The proportion of pupae producing *A. genevensis* (in the multiparasitism treatment) was tested against the proportion of pupae producing wasps in the control treatments (all of which are *A. genevensis*). There was a significant reduction in the proportion of hosts producing *A. genevensis* wasps in the experimental treatment (AG1+AP2) compared to the control treatment (Table 4). In the AP only versus AP1+AG2 comparison (calculating the proportion of pupae producing *A. pallipes* in the same way) there was also a significant reduction in the proportion of hosts producing *A. pallipes* in the experimental treatment (Table 4).

The previous analysis assumes that no other confounding variable is present. In the AG only versus AG1+AP2 comparison, the proportion of developmental failures did not significantly increase in the multiparasitism treatment, but it did approach significance: $\chi^2_1=3.57$, $P<0.1$. In the AP only versus AP1+AG2 comparison, in the experimental treatment there was a significant increase in the proportion of developmental failures: $\chi^2_1=15.43$, $P<0.001$. Whilst this is biologically interesting, it is problematic in terms of directly comparing the treatments. An alternative method of measuring the success of *A. genevensis* or *A. pallipes* in emerging is to consider their relative success per replicate rather than their absolute success. Relative success was measured by calculating the proportion of wasps of a given identity that had emerged from those pupae that had successfully pupated (i.e. produced an organism; either wasps or flies). This relative proportion was calculated for both the control treatments and experimental treatments. Statistical comparisons between this measure exclude developmental failures in the analysis. Using this relative measure there was still a significant reduction in the proportion of pupae producing *A. genevensis* in the AG1+AP2 treatment compared with the control treatment. In the AP only versus AP1+AG2 comparison there was no significant difference in the proportion of pupae producing *A. pallipes* (Table 4).

In order to determine how robust the initial results were, for both measures of developmental success, we systematically reclassified or removed certain classes of datapoints and analysed what differences the alterations made both individually and in certain combinations (see Table 4). For example, individual wasps that could not be identified because they had not initially survived upon eclosion were reclassed as *A. genevensis*. The identification of males that had emerged singly might (in a minority of cases) be prone to some misidentification. Reclassifying all single males identified as *A. pallipes* in both experimental treatments as *A. genevensis* allowed us to test that the results were not dependent upon singly emerging males because although there is a broad statistical trend in mating preferences, it is conceivable that any particular male may act atypically (for unknown reasons). Reclassing all singly emerging *A. pallipes* males and females as *A. genevensis* is an extremely conservative measure, so that only those *A. pallipes* wasps that had emerged from multiple broods were, in effect, counted as *A. pallipes*. Removal of replicates that failed to produce any organisms was done because conditions particular to those replicates (such as the humidity in the petri dish or some other aspect of the microenvironment) may have made it unlikely that any successful pupation could subsequently occur and consequently, if this was the case, artificially lower the average proportion of wasps emerging from the treatments.

The average proportion of *A. genevensis* emerging from experimental replicates (using both measures of developmental success) was significantly reduced under all the additional scenarios considered. For *A. pallipes* the situation is more complex. By the measure of developmental success calculated from the total number of pupae present in a replicate, the proportion of hosts producing *A. pallipes* was significantly reduced in the multiparasitism treatment for all of the additional scenarios considered. However, using the number of hosts that successfully pupated to calculate the proportion of hosts that *A. pallipes* had emerged from (in order to exclude developmental failures from the dataset), the only significant decline in the average proportion of hosts producing *A. pallipes* was under the highly conservative assumption that in the experimental replicates all singly emerging wasps identified as *A. pallipes* (of both sexes) were in fact *A. genevensis*. In our view, this is extremely unlikely to be the case. Overall the 'sensitivity' analysis that we have performed demonstrates that our results are not artefacts of the impossibility of identifying all emerging individuals and are insensitive to any possible misidentification of single emerging males as *A. pallipes*.

## Discussion

The primary finding of our study is, that in absolute terms (the number of pupae producing wasps of a given type as a proportion of all pupae), the proportion of hosts from which either *A. genevensis* or *A. pallipes* emerged was significantly reduced in the respective multiparasitism treatments. This is contrary to the prediction of the simple tolerance/intolerance hypothesis (based on the idea of fighting vs non-fighting larval phenotypes), which in the absence of other factors, would predict that under interspecific competition the proportion of hosts producing the solitary species should be robust and that there would always be a large decline in the proportion of hosts producing the gregarious species.

Under the asymmetric mobility hypothesis, a mixed outcome might be expected with reductions in both cases. However, it should be noted that a 50:50 outcome in the Boivin and van Baaren (2000) study was only observed when a single egg from both species had been oviposited into the host. Significantly, the likelihood of a single solitary larva successfully completing development rapidly declined as the number of gregarious larvae increased, until no solitary individuals emerged from hosts in which there had been four or more gregarious

larvae present (Boivin and van Baaren 2000). The degree of any possible reduction, is of course, likely to depend upon the number of different types of larvae present, their levels of movement, searching patterns and the relative fighting ability of larvae.

Recalculation of the relative proportion of pupae producing wasps of a given identity (the number of pupae producing wasps of a given type as a proportion of all pupae that produced an organism) allowed for the reanalysis of the data with developmental failures excluded from the datasets. This analysis indicated that the proportion of hosts producing the gregarious *A. pallipes* was not significantly reduced under multiparasitism. In contrast, even with this relative measure, the proportion of hosts producing *A. genevensis* was still significantly reduced compared to the *A. genevensis* only control treatment.

However, the possibility remains that developmental failures in any of the treatments were, in some unknown respect, systematically biassed. The preceding interpretation of the results must be treated cautiously. Further investigations might reveal other, unaccounted for, variables that could alter the explanation for the observations. There is no prima facie evidence that developmental failures did not occur at random within replicates (and hence within treatments). In addition, there is no evidence that exposure to two sets of wasps resulted in higher host mortality, as the average number of larvae surviving until dissection or until pupation did not significantly differ between the respective control and experimental treatments. Furthermore, Mayhew and van Alphen (1999) using the same species, found that the risk developmental failure did not increase under high rates of intraspecific superparasitism when compared to hosts that had been parasitized by an individual female during a single oviposition sequence. The observed trend was, in fact, that superparasitism marginally reduced developmental failures, therefore multiparasitized hosts are probably not prone to developmental failure simply by virtue of the increased number of parasitization events.

This study also found that first instar larvae of these species had significantly different levels of movement when observed in vitro. The first instar larvae of *A. pallipes* tended to display less movement overall. The difference stemmed from the number of twists recorded whilst the number of folds was not significantly different in these species. These observations are concordant with the phenotypic changes expected under the asymmetric mobility hypothesis, but the functional significance (if any) of these differences in types of movement is not known. In this study, it is also reassuring to note that the levels of movement (for both types of movement and in both species) are seemingly robust across the time of the observational period, hence the difference is not an artefact of larvae from one species rapidly dying and lowering the observed numbers of moves. Whilst in vitro observations are extremely useful and the most practical method for studying endoparasitoid larval behaviour (see Marris and Casperd 1996; Harvey et al. 2000b) they might not reflect actual larval behaviour within hosts, particular with respect to the ability to move through host material.

Multiparasitism studies performed with parasitoid wasps provide the wider context for these results (relatively few exist in the context of the evolution of gregarious development). Interactions between competitors can be intricate and complex, with the outcome determined by a number of factors. The order of oviposition and the interval between ovipositions can be important (see Strand and Vinson 1984; Strand 1986; Mackauer 1990; Mackauer et al. 1992). Environmental conditions, such as ambient temperature, may also be important (Kfir and van Hamburg 1988).

However, investigations of interspecific competition between a single larva from both of the solitary braconid *Asobara tabida* and the solitary eucoilid *Leptopilina heterotoma* (in *Drosophila melanogaster* and *D. subobscura* at 20°C) found that the probability of survival was 50:50 irrespective of the order of oviposition so long as the second oviposition had occurred within 6 h of the first one. Elimination of competitors was via physical combat and attack. If the gap in oviposition was around a day, the probability of older individuals surviving increased by around three times compared to the younger individual (van Striern-van Liempt 1983). Given that the genus *Aphaereta* is very closely related (and biologically similar) to *Asobara*, this evidence indicates that the methodological period used in this study (potentially creating a maximum gap of 4 h between ovipositions) should not have resulted in a confounding bias (based on oviposition sequence or the gap between oviposition events) in favour of one species over the other. In addition, the likelihood is that in multiparasitized hosts both species would be first instar larvae at the same time, as *A. genevensis* and *A. pallipes* have very similar developmental trajectories at the temperature at which these experiments were conducted at (see Mayhew and van Alphen 1999).

Interspecific competition between gregarious and solitary endoparasitoids has been examined in a limited number of studies. A recent study using the braconid wasps *Glyptapanteles porthetriae* (solitary) and *G. liparidis* (gregarious) which are larval endoparasitoids of *Lymantrai dispar* (Lepidoptera: Lymantriidae) provides further evidence that gregarious parasitoids can vigorously compete with solitary parasitoids (Marktl et al. 2002). In experiments similar to Boivin and van Baaren's (2000), the gregarious species emerged from 45 to 90% of hosts dependent on the host stage attacked. Wounding was observed in first instar larvae of both species in dissections of multiparasitized hosts. However, Marktl et al. (2002) did not assess the levels of larval mobility in these species. Intriguingly the species used by Marktl et al. (2002) are reclassified species from *Apanteles* (sensu lato Nixon 1965) (see Whitfield 1997), the genus that provided empirical support to the tolerance/intolerance model of Godfray (1987) (see le Masurier 1987).

The outcome of competition between solitary and gregarious larvae can deviate from a pattern of highly asymmetrical competition (Lawton and Hassell 1981)

which always favours solitary competitors. However, the interspecific interaction of the closely related braconid endoparasitoids *Cotesia glomeratus* (gregarious) and *C. rubecula* (solitary) do match the expectation that the solitary species should always have traits associated with competitive superiority with respect to gregarious larvae. Using a common host (*Pieris brassicae*) the solitary species always successfully survived if it had been the first egg oviposited and won (via physical combat) in over 90% of hosts when it had been the secondarily oviposited egg (Laing and Corrigan 1987).

However, recent evolutionary models of the transition between solitary and gregarious development (Pexton et al. 2003) suggest that if a rare allele for reduced mobility (with fighting retained) spreads in a population it subsequently can be easily replaced by a rare allele encoding for full tolerance (non-fighting), particularly if there is a fitness cost to having fully functional weapons (that is well developed mandibles). *C. rubecula* has highly developed mandibles during its first instar stage whilst *C. glomeratus* has comparatively poorly developed mandibles. In addition, observations of *C. rubecula* first instar larvae in host haemocoel demonstrated them to be highly mobile. First instar larvae appeared to actively search or 'hunt' for other larvae (Laing and Corrigan 1987). These observations support the idea that the solitary lifestyle requires high levels of movement and searching, along with the ability to fight, if larvae are initially positioned in a non-contiguous arrangement (Pexton and Mayhew 2001; Pexton et al. 2003), and that conditional tolerance can be a transitional phenotype eventually leading to complete tolerance (Pexton et al. 2003).

This study also found no evidence for mixed broods (with respect to species) under multiparasitism. This is typical of most multiparasitism studies (Miller 1982). The successful sharing of hosts is extremely rare but multiparasitism can sometimes result in both species emerging. This seems to be associated with parasitoid wasps that only partially consume their host before completing development (see Miller 1982; Salt 1961). The parasitoid species used in this study both fully consume their hosts before emerging.

Fighting between larvae may be the most common of method of eliminating competitors in species with contest competition, but physiological suppression may also be important in mediating interactions between parasitoid larvae (Salt 1961; Godfray 1994; Quicke 1997). Endoparasitoid wasps are known to inject a variety of substances into hosts (see Godfray 1994) including some species that inject polydnaviruses (PDV's) (see Fleming 1992) and developing wasps sometimes also secrete substances into the host (see Godfray 1994). In this context, physiological suppression is a term covering a wide range of possible phenomena involving general alterations in host haemolymph or the specific action(s) of toxins and cytolytic chemicals that result in the developmental failure of either conspecifc (intraspecific) or heterospecifc (interspecific) competitors. However, little progress has been made in characterizing any specific toxin or cytolytic substance responsible for physiological suppression per se (see Vinson and Hegazi 1998). It is conceivable that substances either injected by females, or produced by developing wasps, can in some cases, have a secondary effect of harming any other competitors present within hosts. The possible mechanisms that can result in physiological suppression are, in general, poorly understood. In this study, physiological interactions between competitors may play an important role in determining the outcome but this requires further investigation.

In conclusion, our study offers evidence suggesting that the gregarious *A. pallipes* is a robust competitor against the solitary *A. genevensis*. Further investigation could provide evidence of when and how mortality may occur. This study adds to the growing evidence that gregarious development, in parasitoid wasps with small gregarious broods, does not require a lack of active competitive ability. Nonetheless it is also clear that other cases are more generally supportive of changes in tolerance and not in mobility (e.g. Rosenheim 1993, Laing and Corrigan 1987), and there is evidence that a variety of biological traits may facilitate the successful transition to gregarious development (e.g. Roseneheim 1993; Ode and Rosenheim 1998; Harvey et al. 2000a).

Gregarious development based on reduced larval mobility (and less intensive searching) is potentially not only a socially advantageous adaptation within a species (see Pexton et al. 2003), but a gregarious phenotype (of this type) may also provide an additional benefit, as the secondary consequence of such a phenotype could be a competitive superiority during interspecific competitive interactions with a solitary species. The phenomenon of offspring providing services/assistance for each other during development [the offspring facilitation hypothesis (Williams and Williams 1957; Hamilton 1963)] is a well-recorded observation in polyembryonic species of parasitoid wasps. Precocious larvae, whilst primarily an adaptation to local mate competition (see Grbíc et al. 1992), can also form a protective force to defend their "normal" sibs against interspecific competitors by selectively killing heterospecific larvae under multiparasitism (see Cruz 1981, 1986; Strand et al. 1990; Harvey et al. 2000b; Utsunomiya and Iwabuchi 2002). The generation of any such assortative interactions (see Wilson and Dugatkin 1997) is, of course, based on a proposed mechanism that is completely different in gregarious species with 'immobile' larvae (when compared to polyembryonic species, in which precocious larvae are highly mobile). However, the notion that the adaptive benefits of developing in groups may also extend to competitive interactions with interspecific competitors, is an idea that could help explain the widespread evolution and maintenance of gregarious development (in small broods) found throughout the parasitic Hymenoptera.

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