Male soldier caste larvae are non-aggressive in the polyembryonic wasp *Copidosoma floridanum*

David Giron¹, Jeffrey A. Harvey², Jena Anne Johnson³ and Michael D. Strand⁴,*

¹Institut de Recherche sur la Biologie de l'Insecte, UMR-CNRS 6035, Faculté des Sciences et Techniques, 37200 Tours, France
²Institute for Ecology, 6666 ZG Heteren, The Netherlands
³Department of Entomology, University of Georgia, Athens, GA 30602, USA
⁴Author for correspondence (mstrand@bugs.ent.uga.edu).

*Polymbryonic wasps are the only parasitoids in which sociality has evolved. Theory implicates both competition and sex ratio manipulation in the evolution of a sterile soldier caste. However, investment in soldiers by males and females is predicted to differ depending upon how offspring are allocated to hosts and the mating system. Here, we compared male and female soldiers in the polyembryonic wasp *Copidosoma floridanum*. We found that male and female soldiers are morphologically identical. Unlike females, however, male soldiers were non-aggressive towards all competitors. We discuss these results in relation to theory and polyembryonic wasp biology.*

**Keywords:** caste; altruism; haplodiploidy; social evolution; competition

1. **INTRODUCTION**

A key feature of eusocial animals is the evolution of reproductive altruism, whereby some members of the social group reproduce while others develop into non-reproductive helper castes (Queller 2000). Eusociality has evolved multiple times, but its greatest diversity occurs among insects in the order Hymenoptera (bees, wasps and ants) that exhibit haplodiploid sex determination and form colonies of related individuals (Alexander et al. 1991; Crozier & Pamilo 1996; Queller & Strassmann 1998; Foster et al. 2006). Polyembryonic wasps in the family Encyrtidae exhibit fascinating biology for the study of sociality owing to their patterns of progeny allocation and unique form of clonal development in parasitized hosts (Harvey et al. 2000; Giron et al. 2004, 2007).

*Copidosoma floridanum* is a polyembryonic encyrtid that parasitizes the egg stage of *Trichoplusia ni* and related moth species (Strand & Gribic 1997; Guerrier & Noyes 2005). After hatching, the host larva develops to its final (fifth) instar over 14 days. During this period, the *C. floridanum* egg clonally proliferates to produce as many as 3000 embryos that form an assemblage called a polymorula (Ode & Strand 1995; Strand & Gribic 1997). Up to 24% of embryos develop into soldier larvae. During the host’s final instar, the remaining embryos develop into reproductive larvae, which consume the host, pupate within the remnant host cuticle and emerge as adults, 12–14 days later. Soldier larvae, in contrast, die of desiccation after consumption of the host by their reproductive siblings (Grbic et al. 1992; Harvey et al. 2000; Giron et al. 2004).

*Copidosoma floridanum* produces clonal broods by laying either a fertilized (diploid) egg into a host that produces all female offspring or an unfertilized (haploid) egg that produces all males (single-sex broods). Hosts containing more than one egg also occur by wasps laying one fertilized and one unfertilized egg during the same oviposition event (mixed-sex broods) or by wasps ovipositing into hosts previously parasitized by another female (superparasitism) (Giron et al. 2007). Female and male eggs produce similar numbers of adult wasps but soldier development distinctly differs, with female eggs producing many soldiers throughout host development and male eggs producing only a few soldiers late in host development (Grbic et al. 1992; Ode & Strand 1995). This asymmetry results in all soldiers in mixed-sex broods being females that kill most brothers to produce a strongly female-biased sex ratio in the emerging adult wasps (Grbic et al. 1992; Ode & Strand 1995). In superparasitized hosts, resident female soldiers almost always eliminate the intruder, whereas offspring from different wasps often coexist in hosts first occupied by a male clone (Giron et al. 2007). Female soldiers also readily distinguish kin from non-kin and other parasitoid species (Harvey et al. 2000; Giron & Strand 2004; Giron et al. 2004).

Two factors have been implicated in the evolution of sterile soldiers by polyembryonic wasps: (i) defence against heterospecific competitors (Cruz 1981; Harvey et al. 2000; Giron et al. 2007) and (ii) resolution of sex ratio conflict in mixed-sex broods (Grbic et al. 1992; Gardner et al. 2007; Giron et al. 2007). Theory predicts that soldier production will be favoured in both sexes if their primary function is defence, whereas soldier production will be biased towards females if their primary function is sex ratio manipulation. Here we report that although *C. floridanum* males produce soldiers, they are non-aggressive towards all competitors.

2. **MATERIAL AND METHODS**

(a) **Bearing and soldier larva morphology**

Two populations of *C. floridanum* were established from field material collected in the southern (Georgia) and northern (Wisconsin) US (Giron & Strand 2004). Twenty hosts containing full brothers or sisters were produced as previously described (Giron et al. 2004) and dissected in the fifth stadium in physiological saline. Hosts containing males and females were near identical in mass at the time of dissection (475–525 mg). The length and head capsule width of five randomly selected soldiers per host were measured using an ocular micrometer mounted on a compound microscope.

(b) **Experiment 1: male and female soldier behaviour towards intraspecific competitors**

We compared male and female soldier behaviour towards intraspecific competitors using a previously described ex-vivo assay (Giron et al. 2004). Thirty sister, brother, non-relative female (Wisconsin population) and non-relative male (Wisconsin population) polymorulæ were collected from hosts, labelled with carboxy-fluorescein diacetate succinimidyl ester (CFSE) and injected into fifth instar hosts containing a male or female brood from the Georgia population. As a negative control, CFSE-labelled testes from fourth instar hosts were injected into hosts. Each host was then dissected 18 h later and the soldiers examined using a Leica epifluorescent microscope. Soldiers that attacked CFSE-labelled polymorulæ

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contained the fluorescent tracer in their gut, whereas non-attacking soldiers did not. We also directly observed soldier behaviour in vitro (Giron et al. 2004). One soldier was placed with a full sister, brother, non-relative female or non-relative male polymorula in a culture well of a 96-well culture plate (Corning) containing 100 μl of TC-100 medium (Sigma). Thirty replicates per treatment were conducted with the number of soldiers that attacked a polymorula during a 2 h observation period recorded.

(c) Experiment 2: outcome of heterospecific competition
The most abundant heterospecific competitors of C. floridanum are solitary larval endoparasitoids that include the solitary braconid, Microplitis demolitor (Giron et al. 2007). We determined the survival to adulthood of C. floridanum and M. demolitor in singly parasitized hosts (n = 36 per treatment). We then determined the outcome of competition in multiparasitized hosts by allowing M. demolitor to oviposit into 40 fourth stadium hosts already occupied by a male or female C. floridanum brood. We observed the behavioural response of 30 male and female soldiers towards M. demolitor in vitro as described in §2b.

3. RESULTS
(a) Male and female soldiers are morphologically identical
Prior studies describe the morphology of C. floridanum soldiers (Grbic et al. 1992; Giron et al. 2007). In the current investigation, visual inspection indicated that female and male soldiers from fifth instar hosts were morphologically indistinguishable. Correspondingly, measurements revealed no differences in length (t-test; t = 1.95; d.f. = 98; p > 0.1) or head capsule width (t = 1.60; d.f. = 98; p > 0.1).

(b) Experiment 1: male soldiers are non-aggressive towards intraspecific competitors
As previously reported (Giron et al. 2004), female attack rates in vitro varied with relatedness of the competitor both in terms of the proportion of hosts that contained at least one soldier with fluorescent tracer in its gut (figure 1a) and the mean proportion of labelled soldiers per host (figure 1b). Attack rates were highest towards non-relatives and lowest towards clonemates and host gonads. In contrast, male soldiers exhibited low attack rates towards all competitors (figure 1a,b). We considered the possibility that these results reflected abundance differences, since female broods from fifth instar hosts contained significantly more soldiers (56.3 ± 5.0 s.e.) than male broods (10.4 ± 1.2 s.e.) (t-test; t = 9.0; d.f. = 38; p < 0.0001). In vitro bioassays, however, indicated that this was not the case since individual female soldiers usually attacked non-relatives but rarely attacked sisters, whereas male soldiers rarely attacked any competitor (figure 1c).

(c) Experiment 2: male soldiers are also non-aggressive towards a heterospecific competitor
Survival rates of C. floridanum male broods, female broods and M. demolitor in singly parasitized hosts did not differ (G-test; G² = 0.35; d.f. = 2; p > 0.10; figure 2a). In multiparasitized hosts, however, C. floridanum female broods usually outcompeted M. demolitor, while M. demolitor usually outcompeted C. floridanum male broods (figure 2b,c). Direct observations during in vitro bioassays further indicated a disparity in attack frequencies with 60 and 3% of female and male soldiers, respectively, attacking M. demolitor (two-tailed binomial test; p = 0.032).

Figure 1. (a) The proportion of male and female broods that contained at least one soldier with fluorescent tracer in its gut following introduction of a CFSE-labelled intruder. The response of male and female broods to each relatedness class of intruder was compared by two-tailed binomial test with asterisks (*) indicating a significant difference (p < 0.05) between the sexes. (b) The mean proportion of soldiers in male and female broods that contained fluorescent tracer in their gut using the same samples as in (a). Asterisks (*) indicate a significant difference between the sexes (t-test using arcsin transformed data; p < 0.05). (c) Proportion of male and female soldiers that attacked intruders in vitro, with asterisks (*) indicating a significant difference between the sexes (two-tailed binomial tests; p < 0.05; 150 × 227 mm (600 × 600 DPI)).

4. DISCUSSION
As previously noted, both defence against competitors and resolution of sex ratio conflict have been implicated in caste evolution by polyembryonic wasps. Risks from heterospecific competitors are based on the fact that polyembryonic wasps have relatively long development times and their lepidopteran hosts are attacked by several other parasitoid species. Crucial to the latter is the mating system and the degree to which males compete for mates. If all matings take place between brothers and sisters from the same host (e.g. complete

broods are mixed sex, the model framework of Gardner et al. (2007) favours different sex ratios and levels of soldier aggression decrease LMC and lead to increased conflict that have opportunities to obtain mates elsewhere, this will mate daughters would be predicted. However, if males local mate competition, LMC), no sexual conflict exists and is resolution of sex ratio conflict. On the other hand, if females should produce more soldiers if their function is primarily defence, whereas soldiers if their function is dual functions since wasps produce a combination of mixed-sex broods almost exclusively, yet exhibit the same sexual asymmetries in soldier production as C. floridanum (Doutt 1947; Kaeser et al. 2006). Thus, it is also possible that developmental constraints exist that prevent or limit soldier development in males of all polyembryonic encyrtids. Resolving this evolutionary conundrum and the basis for social evolution in these insects will probably require a phylogenetic approach in combination with more detailed functional data across a broader suite of species.

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Figure 2. (a) The proportion of singly parasitized hosts that survived to produce an adult male C. floridanum, an adult female C. floridanum or an adult M. demolitor. (b,c) The proportion of multiparasitized hosts that survived to produce (b) a C. floridanum male brood or M. demolitor or (c) a C. floridanum female brood or M. demolitor. These outcomes differed significantly from the expectation that each competitor had an equal chance of outcompeting the other (two-tailed binomial tests; p<0.05; 151×220 mm (600×600 DPI)).

local mate competition, LMC), no sexual conflict exists and sex ratios yielding the smallest number of sons to mate daughters would be predicted. However, if males have opportunities to obtain mates elsewhere, this will decrease LMC and lead to increased conflict that favours different sex ratios and levels of soldier aggression among parents, sons and daughters. Assuming all broods are mixed sex, the model framework of Gardner et al. (2007) predicts that males should produce more soldiers if their function is primarily defence, whereas females should produce more soldiers if their function is resolution of sex ratio conflict. On the other hand, if all broods are single sex, no sex ratio conflict exists and soldier production for defence could be favoured in both sexes.

In the case of C. floridanum, soldiers could have dual functions since wasps produce a combination of mixed- and single-sex broods in the field (Ode & Strand 1995; Giron et al. 2007). Recent empirical studies support this prediction as the earliest emerging soldiers produced by females are specialized to resolve intraspecific conflict, while later emerging soldiers preferentially defend against heterospecific competitors (Giron et al. 2007). We thus reasoned that male broods have potentially lost early emerging soldiers but continue to produce late emerging soldiers for heterospecific defence. Results of the current study, however, reveal that this is not the case since male soldiers are non-aggressive towards all competitors we tested.

The continued but limited production of soldiers by male C. floridanum could represent a complex trait in the process of being lost due to preferential oviposition of mixed-sex broods by females and concomitant changes in the mating structure of populations. Other species of polyembryonic wasps, however, produce single-sex broods almost exclusively, yet exhibit the same sexual asymmetries in soldier production as C. floridanum (Doutt 1947; Kaeser et al. 2006). Thus, it is also possible that developmental constraints exist that prevent or limit soldier development in males of all polyembryonic encyrtids. Resolving this evolutionary conundrum and the basis for social evolution in these insects will probably require a phylogenetic approach in combination with more detailed functional data across a broader suite of species.


