

Host resistance and the evolution of kin recognition in polyembryonic wasps

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Recognition of relatives is considered a key factor in the evolution of sociality as it ensures that the benefits of altruism flow to those who share the altruist's genes. However, theory predicts that genetically based recognition systems will not persist if the only selection maintaining them derives from the recognition system itself. Kin-recognition systems, therefore, are hypothesized to involve genetic variation maintained by other functions. Polyembryonic wasps are parasites of moth larvae that clonally produce large numbers of offspring and two morphologically distinct castes. Some embryos develop into reproductive larvae that mature into adult wasps, whereas others develop into soldier larvae whose function is defence. Soldiers from *Copidosoma floridanum* distinguish relatives from non-relatives on the basis of relatedness. Here, we report that the recognition cues used by soldiers derive from the extraembryonic membrane, which also protects these parasites from the host's immune response. This suggests that the kin-recognition system used by *C. floridanum* may be maintained in part by selection for resistance against the host.

Keywords: *Copidosoma floridanum*; parasitoid; caste formation; sociality; recognition; immunity

1. INTRODUCTION

Social insects are characterized by having caste systems in which some individuals of the group reproduce while others function as altruistic helpers. The best-known caste-forming insects are ants, bees and termites, but caste systems have also evolved in certain taxa of aphids, thrips and polyembryonic wasps (Strand 2003; Zabloutny 2003).

Unlike other caste-forming species, polyembryonic wasps are parasitoids that form clonal social groups inside their insect hosts. The best-studied species is *Copidosoma floridanum*, which parasitizes eggs of the moth *Trichoplusia ni* (Strand & Grbic' 1997). After parasitism, the host egg hatches and the larva develops to its final instar. During this period, the *C. floridanum* egg initially forms a single morula stage embryo surrounded by a polar-body-derived extraembryonic membrane. The primary morula then gives rise to additional embryos called secondary morulae. Up to 24% of these embryos develop during the host's first to fourth instar into soldier (precocious) larvae with fighting mandibles and elongate bodies whose function is

defence (Grbic' *et al.* 1992; Harvey *et al.* 2000; figure 1*a,b*). The remaining embryos develop during the host's fifth (final) instar into reproductive larvae with rounded bodies that consume the host, pupate and emerge as adult wasps (figure 1*c,d*). By contrast, soldier larvae always die after the host is consumed. Notably, both soldiers and reproductive larvae remain enveloped by the extra-embryonic membrane until death or pupation (figure 1*b,d*).

The features that are important in the evolution of soldiers by polyembryonic wasps probably include clonal development, a relatively long life cycle and the need for defence from competitors (Strand 2003). In a purely clonal brood, the reproductive altruism and self-sacrificial behaviour of soldiers reflect clone level allocation to defence over reproduction, with no conflict between group members. However, if more than one egg is laid per host and clones mix, genetic conflicts of interest arise. *Copidosoma floridanum* soldiers recognize clone-mates on the basis of relatedness and aggressively attack non-relatives (Grbic' *et al.* 1992; Giron *et al.* 2004).

Other clonally developing animals, like marine colonial invertebrates (tunicates, cnidarians, bryozoans, sponges), distinguish close kin from non-relatives on the basis of polymorphic allorecognition loci (Buss 1987; Stoner & Weissman 1996). However, theory predicts that recognition systems based on variable genetic loci will not persist if the only selection involved in maintenance of the polymorphism derives from the recognition system itself (Crozier 1986; Grosberg 1988). This is because common alleles at such loci are favoured since individuals possessing them will find more cooperative partners and fewer aggressive antagonists. Kin-recognition systems, therefore, are hypothesized to involve traits maintained by other selective mechanisms (Grosberg & Hart 2000; Queller 2000). Here, we report that kin-recognition cues used by *C. floridanum* derive from the extraembryonic membrane, which also protects this parasite from the host's immune system.

2. MATERIAL AND METHODS

Copidosoma floridanum is haplo-diploid with unfertilized eggs developing into males and fertilized eggs developing into females. Female wasps produce all-male or all-female broods by laying one egg per host and mixed broods by laying two (one male and one female) (Strand 1989). The number of reproductive progeny per host are similar between brood types, but female eggs produce many soldiers whereas male eggs produce almost none (Ode & Strand 1995). This asymmetry in soldier development probably evolved because of the mating structure of *C. floridanum* populations. Females preferentially produce mixed broods in the field, which have strongly female-biased offspring sex ratios because female soldiers kill most embryos of the male clone (Grbic' *et al.* 1992). Surviving males, however, mate with sisters before dispersal. For the current study, two *C. floridanum* cultures were established from hosts collected at field sites in southern (Georgia) and northern USA (Wisconsin). Each laboratory population was reared separately as large randomly mating populations, as previously outlined (Strand 1989). Soldiers and reproductive larvae used in experiments were from all-female or all-male broods of known genetic background. Sisters that were used in experiments were either clone-mates from the same host or had the same parents, but were collected from a different host (Georgia population). Brothers had the same mother but were also collected from a different host. Unrelated males and females were from hosts parasitized by females from the Wisconsin population. All soldiers used in experiments were female.

(a) Experiment 1: kin recognition with and without an extraembryonic membrane

We first compared soldier attacks towards reproductive larvae with and without an extraembryonic membrane (figure 1*e*). Hosts were reared until 72 hours old in the fifth instar and then dissected in

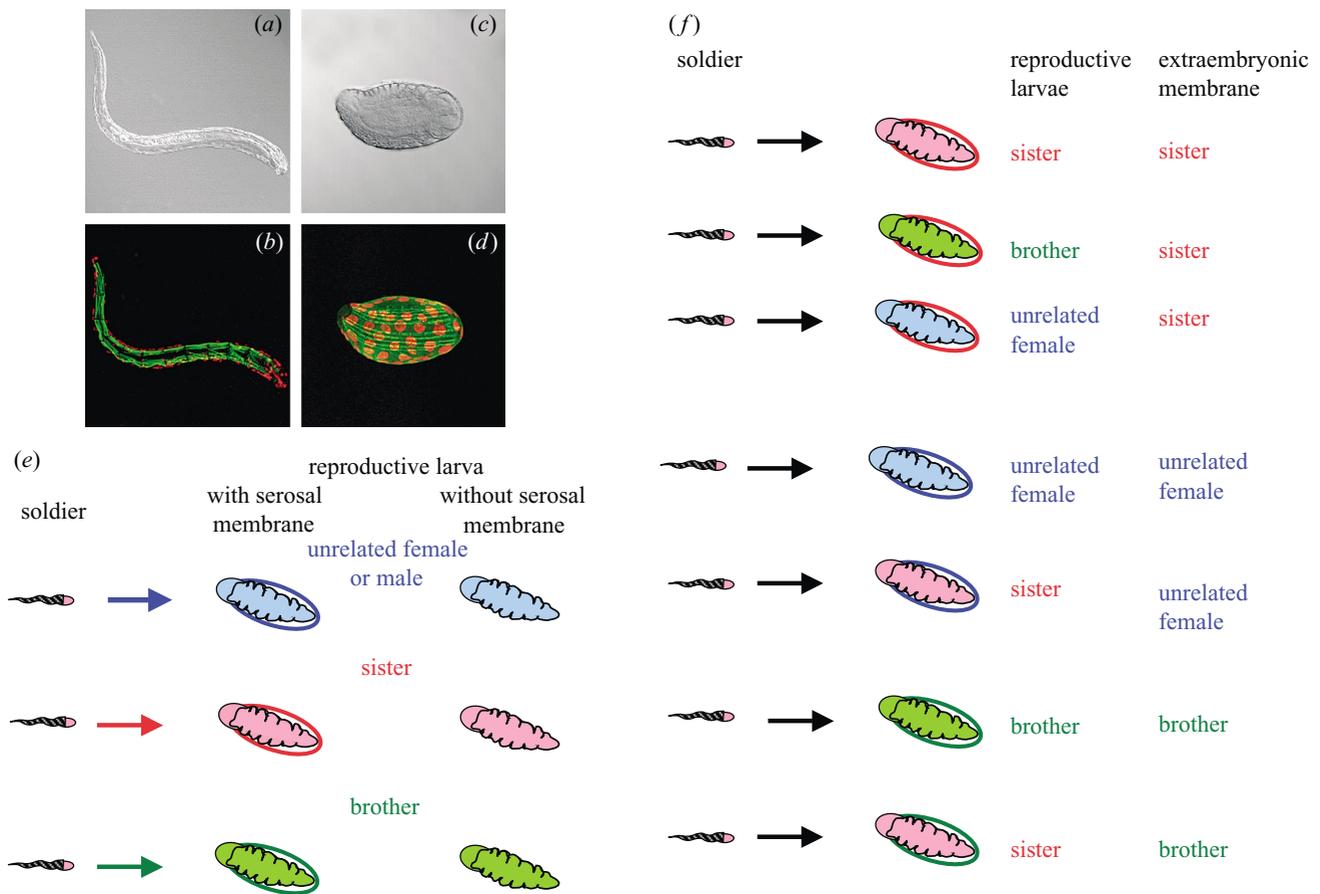


Figure 1. (a–d) *Copidosoma floridanum* larvae, and (e–f) diagrams of experiments 1 and 2. The presence of the extraembryonic membrane that envelopes (a) soldier and (c) reproductive larvae is difficult to see under differential contrast optics but is readily visible using confocal microscopy after staining (b,d) with Alexa 488 phalloidin (1 : 500) (Molecular Probes), and an anti-H1 histone antibody (1 : 200) (Santa Cruz Biotechnology) plus secondary antibody conjugated to Texas Red (1 : 1000) (Jackson Laboratories). The outline of the larvae is seen by phalloidin staining which labels F-actin (green). H1 staining labels nuclei (red) of the extraembryonic membrane. Larvae in (a–d) are oriented with the head to the right. (e) Experiment 1: soldiers were bioassayed with unrelated females or males, sisters or brothers (with or without an extraembryonic membrane). (f) Experiment 2: soldiers were bioassayed with sisters, brothers and unrelated females in membranes from another larva (sister, brother or unrelated female).

phosphate buffered saline (pH 7.2) to collect larvae. The extraembryonic membrane was removed either enzymatically using Dispase (Gibco) or mechanically using tungsten needles. Dispase selectively digests extracellular matrix proteins and quickly removed the extraembryonic membrane with no effect on the larva itself, which was protected by its cuticle. Bioassays were conducted in 1 ml glass dishes containing 100 μ l of TC-100 medium (Sigma). One soldier from a Georgia brood was placed with a reproductive larva that was a sister, brother, unrelated female or unrelated male. Soldiers were continuously observed for 2 h during bioassays. An attack was recorded if the soldier gripped the reproductive larva with its mouthparts for more than 1 min.

(b) Experiment 2: kin recognition when the extraembryonic membrane is exchanged

We next enzymatically removed the extraembryonic membranes from reproductive larvae and then individually placed larvae into intact membranes mechanically removed from other individuals (figure 1f). We first compared soldier attacks towards sisters, brothers and unrelated females enveloped by their own membrane (unmanipulated) to sisters, brothers and unrelated females placed into membranes from other sisters, brothers or unrelated females, respectively (manipulated). We then compared soldier attacks towards: (i) sisters, brothers and unrelated females in membranes from other sisters; (ii) unrelated females and sisters in membranes from other unrelated females; and (iii) brothers and sisters in membranes from other brothers. Bioassays were conducted as described in experiment 1.

(c) Experiment 3: the role of the extraembryonic membrane in evasion of host defences

The primary immune response by insects towards parasites is encapsulation (Lavine & Strand 2002). During an encapsulation response, host haemocytes recognize and bind to the parasite forming a multicellular sheath. Here, we assessed the encapsulation response of *T. ni* towards larvae (Georgia and Wisconsin populations) with and without an extraembryonic membrane. Each treatment consisted of five larvae collected from 20 randomly selected broods. The five larvae were injected into recipient hosts as previously described (Harvey *et al.* 2000). Hosts were then dissected 24 h later and the proportion of larvae encapsulated was determined by visual inspection.

(d) Analysis

Statistical analyses were performed using JMP, v. 3.0 software. In experiments 1 and 2, the association between soldier aggression, relatedness and the extraembryonic membrane was analysed by likelihood ratio χ^2 -tests. In experiment 3, encapsulation data were analysed by logistic regression.

3. RESULTS

(a) Kin recognition by soldier larvae requires the extraembryonic membrane

To evaluate the role of the extraembryonic membrane in kin recognition, we compared soldier attacks towards

Table 1. Soldier aggression towards a competing larva with and without an extraembryonic membrane.

(n , the number of replicates bioassayed for each treatment (membrane present or membrane absent) and competitor type (sister (clone-mate), sister (different host), brother, unrelated female or unrelated male) tested. Soldier attacks towards reproductive larvae enveloped by their extraembryonic membrane varied significantly with relatedness ($G^2 = 31.50$; d.f. = 4; $p < 0.0001$), whereas soldier attacks towards reproductive larvae lacking an extraembryonic membrane did not ($G^2 = 0.57$; d.f. = 4; $p > 0.90$).

relatedness of the competing larva	proportion of larvae attacked by soldiers	
	membrane present	membrane absent
sister (clone-mate)	0.16 ($n = 30$)	0.03 ($n = 30$)
sister (different host)	0.13 ($n = 30$)	0.03 ($n = 30$)
brother	0.50 ($n = 30$)	0.07 ($n = 30$)
unrelated female	0.77 ($n = 30$)	0.03 ($n = 30$)
unrelated male	0.73 ($n = 30$)	0.03 ($n = 30$)

reproductive larvae enveloped by an extraembryonic membrane, and reproductive larvae in which we removed the extraembryonic membrane. These experiments revealed a strong association between soldier aggression and relatedness of the potential competitor when the extraembryonic membrane was present (table 1). Soldier attack rates were highest towards non-relatives, intermediate towards brothers and lowest towards sisters. Cues for recognition of kin and non-kin could derive from either the individual or the surrounding environment, which for *C. floridanum* is the host haemocoel. However, the similarly low attack rates towards clone-mates and sisters obtained from another host (table 1) strongly suggested recognition cues originated from the individual rather than the host in which the individual develops. When the same experiments were conducted with competitors in which the extraembryonic membrane was removed, we found that soldier larvae rarely attacked any larvae regardless of relatedness (table 1). Membranes were removed enzymatically from reproductive larvae for the data presented in table 1 but the same results were obtained when membranes were removed mechanically (data not presented). Soldiers were able to contact the potential competitor during these bioassays. Soldiers became increasingly active after contacting non-relatives and brothers with a membrane and then attacked. This usually resulted in death of the competitor. By contrast, soldiers contacting sisters or larvae without a membrane usually remained sedentary and exhibited no aggressive behaviour.

(b) *Soldier aggression correlates with relatedness of the extraembryonic membrane*

We next conducted experiments in which we removed reproductive larvae from their own extraembryonic membrane and placed them into the extraembryonic membrane from another individual. In preliminary studies, we found no difference in soldier attacks when we compared sisters surrounded by their own membrane (unmanipulated) to sisters placed in a membrane from another sister (manipulated) ($n = 5$ replicates for each

treatment, $G^2 = 0.09$, d.f. = 1, $p > 0.77$). We also found no difference between unmanipulated and manipulated brothers ($n = 15$ replicates for each treatment, $G^2 = 0.04$, d.f. = 1, $p > 0.83$), or unmanipulated and manipulated unrelated females ($n = 15$ replicates for each treatment, $G^2 = 0.06$, d.f. = 1, $p > 0.81$). This indicated that the transfer process itself had no effect on the frequency of soldier attacks.

We then removed reproductive larvae from their own extraembryonic membranes and placed them individually into extraembryonic membranes of different relatedness. This experiment revealed that soldier attack rates differed significantly among all larva by membrane combinations we tested (table 2). Closer inspection of these data suggested that soldier attack rates did not differ among larvae of different relatedness if they were placed into extraembryonic membranes of the same relatedness. To test this hypothesis, likelihood ratio χ^2 -tests were calculated for subset models that compared soldier attack rates towards (i) sisters, brothers and unrelated females placed into extraembryonic membranes from other sisters; (ii) brothers and sisters placed into extraembryonic membranes from other brothers; and (iii) sisters and unrelated females placed into extraembryonic membranes from other unrelated females (table 2). These analyses indicated that soldier attacks were low and did not significantly differ between sisters, brothers or unrelated females in membranes from other sisters (table 2). Correspondingly, soldier attack rates were intermediate and did not differ between sisters and brothers in membranes from other brothers, whereas attack rates were high and did not differ between unrelated females and sisters in membranes from unrelated females (table 2). Collectively these results indicated that soldier attack rates correlate with relatedness of the extraembryonic membrane that surrounds a reproductive larva, rather than the relatedness of the reproductive larva itself.

(c) *Evasion of the host immune response depends upon the extraembryonic membrane*

The proportion of *C. floridanum* that was encapsulated by *T. ni* varied significantly with the presence or absence of an extraembryonic membrane, larval type (soldier or reproductive) and population (Georgia or Wisconsin) ($G^2 = 208.3$, d.f. = 4, $p < 0.0001$, $n = 20$ replicates for each treatment). Reduced models for each effect indicated that presence or absence of the extraembryonic membrane was the only significant factor ($G^2 = 207.1$, d.f. = 1, $p < 0.0001$). Larvae without an extraembryonic membrane were almost always encapsulated, whereas larvae with a membrane were almost never encapsulated. The type of larva (soldier or reproductive) or population (Wisconsin or Georgia) injected into *T. ni* had no significant effect on encapsulation response.

4. DISCUSSION

Relatedness has long been considered an important factor in the transition from single cells to multicellular individuals, and from individuality to cooperation in social groups (Buss 1987; Queller 2000). Intimately tied to these transitions is the ability to distinguish self from non-self, and kin from distant relatives. Kin-recognition systems are

Table 2. Soldier aggression in response to relatedness of the competing larva and the extraembryonic membrane. (n , the number of replicates for each competitor \times membrane combination tested. The proportion of reproductive larvae attacked by soldiers varied significantly with relatedness of the competing larva and extraembryonic membrane (full model, $G^2 = 25.35$; d.f. = 6; $p < 0.0001$). Calculation of likelihood ratios in subset models revealed that soldier attack rates did not significantly differ between: (i) sisters, brothers and unrelated females enveloped by a sister membrane ($G^2 = 0.33$; d.f. = 2; $p > 0.85$); (ii) brothers and sisters enveloped by a brother membrane ($G^2 = 0.54$; d.f. = 1; $p > 0.46$); or (iii) unrelated females and sisters enveloped by an unrelated female membrane ($G^2 = 0.16$; d.f. = 1; $p > 0.69$.)

relatedness of the competing reproductive larva to the soldier	relatedness of the extraembryonic membrane to the soldier	n	proportion of reproductive larvae attacked by soldiers
sister	sister	15	0.13
brother	sister	15	0.20
unrelated female	sister	15	0.13
brother	brother	15	0.53
sister	brother	15	0.40
unrelated female	unrelated female	15	0.73
sister	unrelated female	15	0.67

often specific and regulated by one or more polymorphic loci (Crozier 1986; Grosberg 1988). The factors maintaining these polymorphisms in social insects are largely unknown, although studies with colonial marine invertebrates suggest roles for indirect selection via mate choice or direct selection for resistance to pathogens, parasitism of the germline or intraspecific competition (Stoner & Weissman 1996; Edwards & Hedrick 1998; Grosberg & Hart 2000).

Clonal development by *C. floridanum* results in broods with a genetic structure that resembles the cooperating cells of a multicellular individual more than the colonies of most social insects. Conflicts of interest in *C. floridanum* should be minimal among individuals from a given egg, but conflicts over resources and other factors clearly arise if progeny from more than one egg are present in a host. Soldiers readily distinguish close relatives from non-relatives (Grbic' *et al.* 1992; Harvey *et al.* 2000; Giron *et al.* 2004), but the source of these recognition cues was previously unknown. Results presented here indicate that *C. floridanum* soldiers distinguish kin from non-kin by their extraembryonic membrane, which is also essential for defence against the host's cellular immune response. Most larvae without the extraembryonic membrane are encapsulated by *T. ni*, whereas almost no larvae are encapsulated when the membrane is present. The extraembryonic membrane does not, however, protect our Wisconsin and Georgia populations from encapsulation in other potential host species (Corley & Strand 2003). This suggests that variation exists among hosts in their ability to recognize *C. floridanum* as foreign, which in turn probably selects for variation in surface features of the parasitoid to resist host defence responses. More striking from the perspective of social evolution is that *C. floridanum* has potentially coopted trait variation maintained to resist the host for kin recognition. Future studies will reveal whether the specific molecules of the extraembryonic membrane that protect *C. floridanum* from the host immune response are also the same molecules used by *C. floridanum* soldiers in kin recognition.

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