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Soldier behaviour and division of labour in the aphid genus *Pemphigus* (Hemiptera, Aphididae)

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Summary. We devised a test for quantitatively measuring the defensive behaviour of individual aphids of the gall generations of the genus *Pemphigus.* Five species (*P. spyrothecae*, *P. gairi*, *P. bursarius*, *P. phenax*, and *P. protospirae*) were shown to have soldiers, but the early instars of *Pemphigus populi* showed no aggressive behaviour. A higher proportion of the first instars of *P. spyrothecae* showed aggressive behaviour, compared with the other four soldier-producing species. Although the first instars were always the most likely to be aggressive, later instars also showed significant levels of aggression in all the soldier-producing species. Soldier behaviour was elicited by a range of stimuli. A live, restrained, predator was the most effective stimulus tested, but physical stimulation (with a paintbrush) enhanced the attack rate. The aphids could distinguish between lures soaked in water and those soaked in aphid haemolymph, which elicited a higher level of response. First instars of the early and late gall generations of *P. spyrothecae* were equally likely to attack predators. Although the species with the most specialized soldiers (*P. spyrothecae*) also had the most prolonged gall phase, this is clearly not the only factor that is important in the evolution of soldiers in this genus.

Key words: Soldier aphids, *Pemphigus*, eusocial behaviour.

Introduction

Soldiers have evolved independently at least six times within the aphid subfamilies Hormaphidinae and Pemphiginae (Aoki, 1977; Stern and Foster, 1996; Stern, 1998). These soldiers show a wide range of defensive behaviours and associated weaponry, but what unites them all is that they represent a significant cost to the clone that produces them. The central evolutionary question about soldier aphids is how to explain their very restricted distribution within the family Aphididae. There are approximately 4,400 species of aphids in twenty five subfamilies (Remaudière and Remaudière, 1997), and soldiers are restricted to about 60 of the 475 species within the two subfamilies Hormaphidinae and Pemphiginae. Since aphids are in general exceptionally vulnerable to predation, it is perhaps remarkable that so few of them have invested in a specialized defender caste.

There are essentially only two key issues we need to understand in order to study social evolution in aphids: the ecological factors that might favour soldier production and the extent of clonal mixing within the aphid colonies. These two issues must be set in a historical context, so we need also to know the phylogeny of the aphids we are studying. But before we can do any of this, we need to be certain which species produce soldiers and which do not. We have enough data to know that the broad pattern of soldier-producing taxa, at the level of the subfamily, has something to do with gall-living: the various origins of soldiers have all occurred within lineages that live in galls on their primary host (Foster and Northcott, 1994; Stern and Foster 1996, 1997). We need to consider now what has determined the distribution of soldier-production within the two soldier-producing subfamilies, and in this paper we look in detail at the Pemphiginae.

The best understood soldier aphids are those of the tribe Cerataphidini (Hormaphidinae). We have extensive observations and experiments on the behaviour and ecology of a range of species (e.g. Aoki, 1977, 1978; Shibao, 1998, 1999), and Stern (1994, 1998) has provided a well-supported phylogeny of the tribe, which shows that there has probably been a single origin of soldiers on the primary host, and a single origin, together with either one or two losses, of horned soldiers on the secondary host. We are now in a position to ask focussed questions about the genetic and ecological factors that are relevant to these origins and, perhaps even more importantly, losses of soldiers.

This paper seeks to apply a similar approach to the aphids of the tribe Pemphigini, in particular the genus *Pemphigus.*

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These aphids provide a useful contrast with the horned aphids. They are much more widespread geographically, having a world-wide distribution, and are usually very abundant and easy to find where they do occur. Soldier behaviour has been reliably reported in three species of *Pemphigus*: *P. dorocola* (Aoki, 1978), *P. spyrothecae* (Aoki and Kurosu, 1986; Foster 1990), and *P. obesinymphae* (Moran, 1993). In addition, there is a fourth species, *P. monophagus*, which has morphologically distinct first instars that look as though they might be soldiers (Aoki and Kurosu 1988). All four of these species have galls that persist for a long time on the primary host, either because they do not host alternate (*spyrothecae* and *monophagus*) or for other ecological reasons. It has been argued that this long gall duration is the reason why soldiers have evolved in these species (e.g. Moran, 1993; Foster and Northcott, 1994; Stern and Foster, 1997): either soldiers might have enabled galls to persist longer than predation would otherwise have allowed, or ecological factors during other parts of the life-cycle might have constrained aphids to persist in the gall for an extended period which is only made possible by investing in defenders.

However, there are sixty-five species of *Pemphigus* (Remaudière and Remaudière, 1997), and before we embark on further speculations about the correlates of social evolution in this genus, we need to have more extensive data on the occurrence of soldier behaviour. This is the purpose of the present paper. We describe a novel method for quantifying soldier behaviour in these aphids and use it to demonstrate which species have soldiers and how defensive behaviour is distributed within the developmental stages of these species. We also present data on the stimuli that the aphids might use in detecting predators.

Methods

Study organisms

The life-cycles of 17 of the 65 *Pemphigus* species have been described (Blackman and Eastop, 1994). All of these 17 species form galls on the leaves or twigs of *Populus*, the primary host. The winged sexuparae give birth to the sexuals that mate on the bark to produce overwintering eggs and these subsequently hatch into the gall-forming foundress. In 13 of these species, the foundress then produces winged offspring (the winged emigrants) that fly from the gall to produce several generations on the secondary herbaceous host. Winged sexuparae then fly back to the poplar trees. The other four species whose life-cycle is known spend their entire life on the poplar tree. *P. spyrothecae* is one of these non-host alternating species. The foundress gives birth to thick-legged first instars, which have already been shown to be soldiers (Aoki and Kurosu, 1986; Foster, 1990) and which grow up into wingless gall adults. These adults produce two sorts of third-generation offspring: individuals that develop into winged sexuparae that fly from the gall to produce sexuals on poplar bark, and thick-legged individuals that are presumed to act as soldiers and grow up into wingless adults (Lampel, 1968-9).

We studied six species: the non-host alternating *P. spyrothecae* and four host-alternating species, *P. bursarius*, *P. phenax*, *P. gairi*, *P. protospirae*, and *P. populi* (see Blackman and Eastop (1994) for details of life-history). Aphids were obtained, shortly before each experiment, from within galls collected from poplar (*Populus nigra* var *italica*) at sites in and around Cambridge. For comparisons of the second and third generation soldiers of *P. spyrothecae*, which are morphologically indistinguishable by eye, first instars were reared within excised galls in the laboratory either from a foundress (second-generation soldiers) or a wingless gall virgin (third-generation soldiers).

Lure experiments

To test for aggressive behaviour in the aphids, we used lures made by rolling up filter paper fragments into spindle shapes of approximately 0.5 × 2 mm. A new lure was used for each experiment. Treatments used were: dry lure; distilled-water-soaked lure; honeydew-soaked lure; and crushed-aphid-soaked lure. The crushed aphids (of any instar) were of the species whose behaviour was being observed. In addition, lassoed late-instar predatory larvae of *Episyrphus balteatus* (Degeer) [Diptera; Syrphidae] were used as lures. They were restrained with a 0.5 mm diameter twisted tissue-paper "rope" lassoed round their middle. This greatly restricted the predator's movements. It was important to ensure that the fluid-soaked lures were well saturated but not so wet as to interfere with the aphid's movements. We used haemolymph as a lure, since it has been shown that this is an effective stimulus for attacking behaviour in soldiers of the horned aphid *Pseudoregma sundanica* (Schütze and Maschwitz, 1991). We used water and honeydew to see if the aphids could distinguish between mere wetness, honeydew (abundant within the galls), and their own haemolymph as potential attack stimuli.

First, the test aphid was picked up as gently as possible by allowing it to walk on to the lure, which was held with forceps. The aphid was then observed for one minute, moving the lure to keep the soldier in view and on top of the lure. If the aphid was observed to attack for longer than five seconds the experiment was scored as a spontaneous attack. If not, the observation was continued for one further minute, this time agitating the soldier with a paintbrush. If an attack was observed during this second minute, the experiment was scored as a provoked attack. Provoked attacks are reported in detail here only for the comparison of the second and third generation soldiers of *P. spyrothecae.*

Establishing that we are observing true attacking behaviour is very important. The most likely confusion results from possible feeding behaviour. A true attack may involve mainly the stylets, but is almost always accompanied by distinctive movements of the body and legs, a characteristic general body stance, and/or a downwards pointing of the antennae (Rhoden, 1997). In addition, during an attack, if an aphid is nudged it will cling on and continue or intensify its behaviour, whereas a non-soldier exhibiting a feeding response will walk away. If any doubt was present, this stimulus was applied to confirm or deny.

The test using the syrphid larva was carried out on all the gall-living instars of each of six species of *Pemphigus.* The tests comparing the different stimuli were all performed on first instars (*P. phenax* was not used in these experiments). In all tests with first instars on a live syrphid and in all tests with first instar *P. spyrothecae*, a sample size of 50 was used. In all other tests a sample size of 30 was aimed for, but occasionally a lack of suitable material forced us to use a smaller sample (see Figs. 1 and 2). For any particular treatment, no more than six individuals from the same gall were used.

Arena experiments

The experiments were carried out in approximately hemispherical arenas just under 1 cm in diameter moulded in "Plasticine", using a round-ended tool inserted to a constant depth. Each experiment involved a first-instar two-spot ladybird larva (*Adalia bipunctata*) hatched from the egg within the previous 24 h. Ten first-instar aphids from the same gall of the species under test were placed inside the arena. The ladybird larva was then introduced, the arena was covered with a coverslip, and whether or not aphids attacked the larva was observed over a period of one hour. This technique did not produce very reliable quantitative data, but was useful for confirming whether or not the 1st instars showed attacking behaviour. A sample size of 20 was used for each species, each ladybird larva was used only once.

In a second set of experiments to test for the efficacy of different numbers of aphids in overcoming a predator, the same protocol was used but with a second instar of the syrphid *Episyrphus balteatus* as the preda-

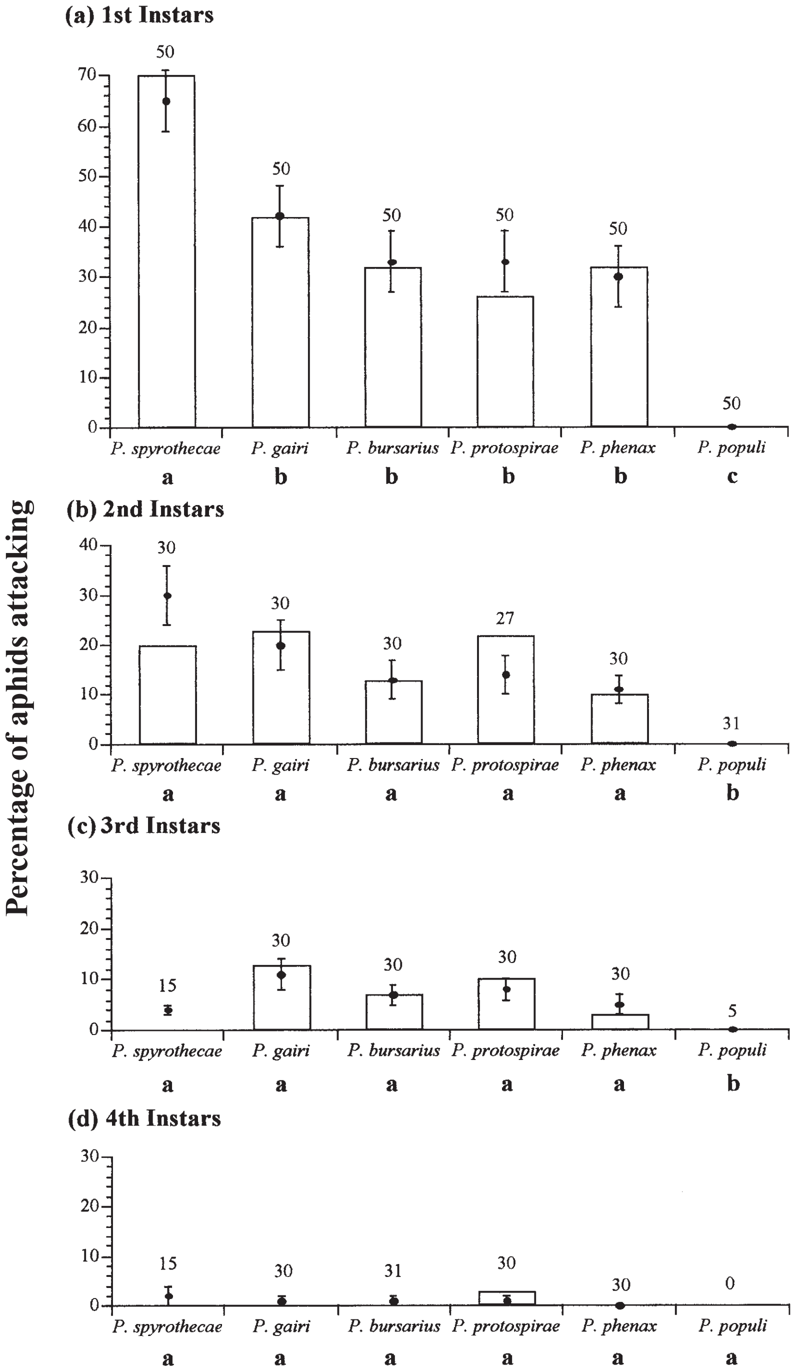

Figure 1. The attack rate of individual aphids of four instars of six species of *Pemphigus* when placed on a restrained late-instar of *Episyrphus balteatus.* The histograms (a–d, one per instar) show the raw data. The points and standard-error bars show the binomial GLIM fitted means and SE. Sample size is shown above each histogram. Bars with the same letter underneath were not significantly different ($P < 0.05$). Binomial GLIM: attack rate = instar + species; Instar – deviance = 119.54, d.f. = 3, $P < 0.0001$; species – deviance = 77.97, d.f. = 5, $P < 0.0001$; error – deviance = 537.2, d.f. 745). Interaction term not significant

tor and a varying number of soldier and non-soldier first-instar *P. spyrothecae.* Each experiment was run for four hours and the mortality of the predators was recorded. Twenty six experiments were done in total.

In all the above experiments, observations were made on the method of attack used by the soldiers, in particular, whether they used their stylets and which legs were used.

Statistical procedures

Data were analysed using the general linear modelling program GLIM (Crawley, 1993) assuming binomial errors.

Results

Soldier behaviour

The first instars of five of the *Pemphigus* species (*P. spyrothecae*, *P. bursarius*, *P. gairi*, *P. phenax*, and *P. protospirae*) all showed some propensity to attack the syrphid predator in the lure experiment and can thus be said to be soldiers (Fig. 1). The only exception was *Pemphigus populi*, which was never observed to attack the syrphid. The first-instar sexuparae (n = 50) of *P. spyrothecae* also did not attack the predator. These observations were backed up by the arena experiments: first instars of all the five species were observed to attack the ladybird predator, but no attacks were ever observed by first instars of *P. populi* or by the first-instar sexuparae of *P. spyrothecae* (20 experiments for each species). There were clear differences in the propensity to attack of the first instars of the six species (Fig. 1). The *P. spyrothecae* soldiers were more likely to attack than the soldiers of any of the other species. There was a second group, consisting of *P. gairi*, *P. bursarius*, *P. protospirae*, and *P. phenax*, whose first instars had a similar level of attacking behaviour.

The later instars of all the soldier-producing species showed some propensity to attack the syrphid lure (Fig. 1). In all five species, the first instars were significantly more likely to attack than the later instars. This effect was particularly marked in *P. spyrothecae*: the second instars showed some soldier behaviour, but there was no obvious attacking behaviour in later instars. In the other species, the third instars showed some attack behaviour, and in one species, *P. protospirae*, even some of the fourth instars (the final instar) attacked the predator.

In the five soldier-producing species other than *P. spyrothecae*, there was no significant difference in the attack rate of the second and third instars, but both were more aggressive than the fourth instars (Fig. 1). None of the juvenile stages of *P. populi* or of the sexuparae of *P. spyrothecae* ever attacked the predator. The wingless adult gall virgins of *P. spyrothecae* (daughters of the foundress) were never observed to attack predators (n = 30). In the five other species, the adult daughters of the foundress are winged migrants which fly from the gall; their attacking behaviour was not tested.

Comparison of second and third generation soldiers of P. spyrothecae

In *P. spyrothecae*, soldiers are produced by both the foundress (second-generation soldiers) and by the wingless gall virgins (third-generation soldiers). They are indistinguishable from each other morphologically (see Whitfield, 1998). Soldiers of both generations attacked the syrphid lure with equal propensity. In samples of 50 soldiers of each generation, 23 second-generation and 26 third-generation soldiers attacked spontaneously, and a further 12 second-generation and 14 third-generation soldiers attacked when provoked.

Proximate cues releasing attack behaviour

The lures used to stimulate attack behaviour were ranked in the same sequence for each of the four species that showed any response (Fig. 2). (*P. phenax* was not tested and *P. populi* showed no response). The dry lure never elicited a spontaneous attack. The syrphid was always the most effective in eliciting an attack, followed by aphid haemolymph, followed by water. In each of the four species that showed a response, the attack rate was significantly different for each type of lure. The only exception to this was *P. spyrothecae* for which an additional type of lure was tested – filter-paper soaked in aphid honeydew. The attack rate elicited by this lure was not significantly different from that elicited by a water-soaked lure.

Effectiveness of soldiers against predators

First instars of all the species that attacked predators were observed to be able to rupture the cuticle of the syrphid larvae with their hind legs. It is assumed that the aphids, provided that there were enough of them, would be able to kill the predators. This has already been recorded for *P. spyrothecae* (Foster, 1990), and it was directly observed in preliminary experiments in which second-instars of the syrphid *Episyrphus balteatus* were imprisoned within natural galls (n = 2 for each species) of *P. gairi*, and *P. bursarius*.

We decided to look in more detail at the effect of soldier number on predator mortality by carrying out arena experiments with *P. spyrothecae.* With densities of between 5 and 20 first-instar soliders of *P. spyropthecae* per predator, 38% (n = 11 replicates) of the predators (second-instar syrphid larvae) died; with between 25–40 soldiers, 52% (n = 8) of the predators died; and with between 45 and 90 soldiers, all the predators died (n = 7). This shows a clear effect of soldier density on predator mortality (binomial GLIM: percentage mortality of predator = no. of soldiers. No. of soldiers – deviance = 9.21, d.f. = 1, $P < 0.005$; error – deviance = 27.23, d.f. = 25). In equivalent experiments with first-instar sexuparae of *P. spyrothecae*, none of the predators was killed.

Observations on aphid attack behaviour

The aphids of each of the species attack predators in a similar manner. They almost invariably pierce the predator with their stylets in a sustained thrust: repeated stabbing was observed only rarely. In many, but not all, attacks the aphids also squeeze the predator with their legs, sometimes using all three pairs, but usually the hind pair is used solely or more vigorously than the other pairs. This leg squeezing can tear the predator's cuticle and lead to rupture of internal structures (Foster, 1990; Rhoden, 1997). *P. spyrothecae* and *P. protospirae* used their legs more vigorously than did the other species observed in detail (*P. gairi* and *P. bursarius*). Soldiers usually project their antennae down towards the surface of the predator during an attack.

This attacking behaviour is costly to the soldiers (see Foster, 1990), although no detailed quantitative observations were made in these experiments. In experiments with syrphid larvae, dead 1st instars were observed on the surface of the predator in the five soldier-producing species, but this was not observed in *P. populi*, where the aphids were killed either by being eaten or squashed by the predator.

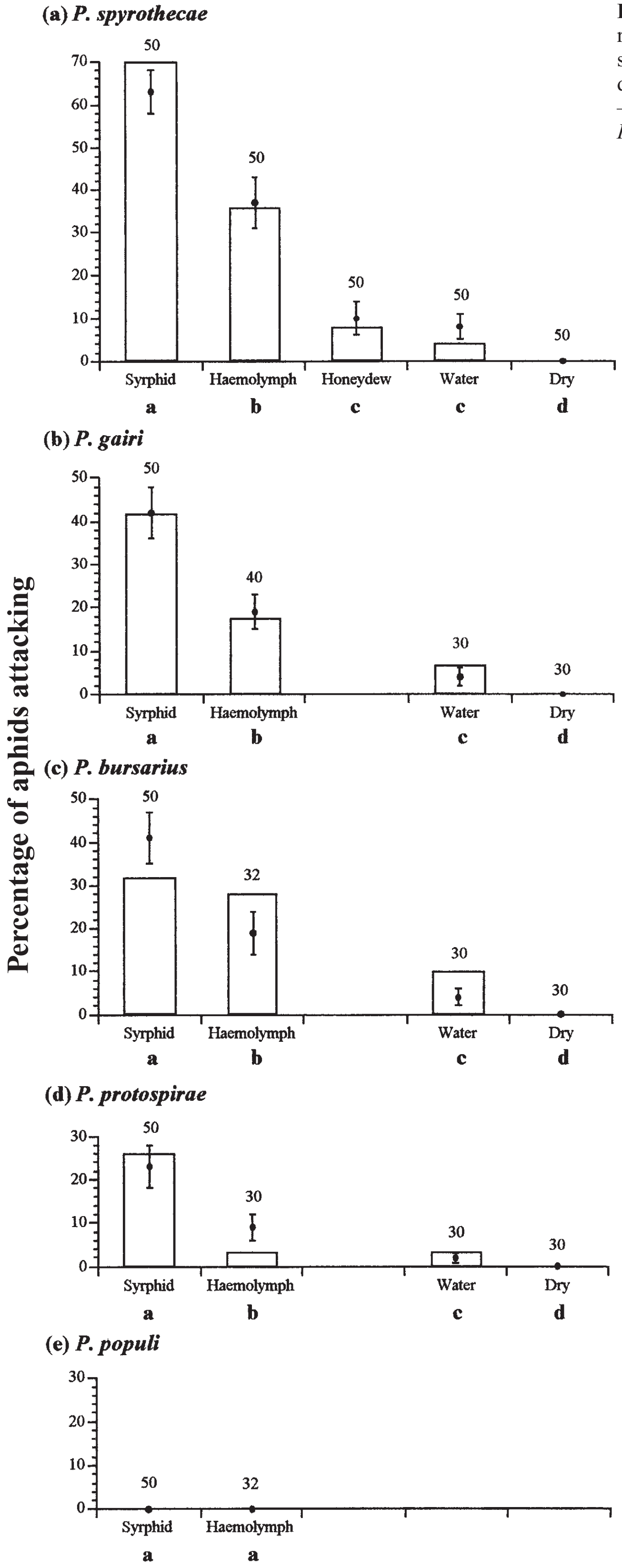

Figure 2. The response of the first instars of five *Pemphigus* species to a range of lure types: restrained late-instar of *Episyrphus balteatus*; filter paper soaked in aphid haemolymph, honeydew (*P. spyrothecae* only), or water; and dry filter paper (see Fig.1]). Binomial GLIM: attack rate = lure + species. Lure – deviance = 162.6 , d.f. = 4, $P < 0.0001$; species – deviance = 91.7, d.f. = 4, $P < 0.0001$; error – deviance = 497.1, d.f. 755). Interaction term not significant

Discussion

Distribution of soldiers in the genus Pemphigus

The results clearly establish that aggressive anti-predator behaviour is widespread within the genus *Pemphigus.* Eight species have now been studied in some detail, and all but one (*P. populi*) have been shown to have soldiers (Table 1). A ninth species, *P. monophagus*, has morphologically distinct first instars that are almost certainly soldiers, but there is no direct observational or experimental evidence (Aoki and Kurosu, 1988). The discovery that host-alternating species with relatively short-lived galls (i.e. *P. bursarius*, *P. phenax*, *P. gairi* and *P. protospirae*) actually have soldiers is, in fact, unexpected. It had been assumed (e.g. Moran 1993; Foster and Northcott, 1994) that a prolonged gall phase might have been a significant, even necessary, factor in the evolution of soldiers in this genus, since the first three species that were shown to have soldiers (*P. dorocola*, *P. spyrothecae* and *P. obesinymphae*) all share this particular trait (Table 1). It is now clear that the explanation cannot be so straightforward, although it is the case in the current study that the species which shows the most extreme specialization in soldiers (*P. spyrothecae*) is the one with the most prolonged gall phase.

It is perhaps surprising that there is no difference in the likelihood of attacking behaviour between the second- and third-generation soldiers of *P. spyrothecae.* The third-generation soldiers are much less likely ultimately to give rise to sexuals and those produced towards the end of the season have zero chance of any personal fitness (Foster, 1990; Rhoden, 1997): they might therefore be selected to be more aggressive.

The experimental protocol developed here would seem to have the power not only to test for the existence of soldiers but also to quantify the degree of soldier aggression in a robust, if still relatively crude, fashion. The chief requirement is that it should be a standard procedure that can be extensively replicated on a range of individuals and on a range of species: different soldier-producing taxa might require different tests, tailored to particular aspects of their ecology. To avoid all ambiguity, it also needs to be shown that presumptive soldiers *can* kill predators, whereas presumptive non-soldiers cannot (as in the syrphid experiments here).

These methods have enabled us to establish that *Pemphigus* is a useful model system in which to test for the evolution of social behaviour: there is at least one species (*populi*) that does not have soldiers and at least one (*spyrothecae*) that has particularly aggressive soldiers. The spotlight now falls on *P. populi* : it would be interesting to know if it has lost sol-

Table 1. Occurrence of soldiers in the genus *Pemphigus*.

Species	Possession of soldiers	Locality	Host-alternating	Approx duration of gall phase (mon.)	Reference
P. bursarius (L.)	Yes	World-wide	Yes	2–3	Current study
P. dorocola Matsumura*	Yes	Japan, Korea Siberia	Yes	5	Aoki 1975, 1978
P. gairi Stroyan	Yes	UK	Yes	2–3	Current study
P. monophagus Maxson	Yes	Western N. America	No	?	Aoki and Kurosu 1988
P. obesinymphae Aoki and Moran	Yes	USA	Yes	5–6	Moran 1993; Aoki and Moran 1994
P. phenax Börner and Blunck	Yes	Europe	Yes	2–3	Current study
P. populi Courchet	No	Europe and Asia	Yes	2	Current study
P. protospirae Lichtenstein	Yes	Europe and central Asia	Yes	2–3	Current study
P. spyrothecae Passerini	Yes	Europe to Western Siberia	No	5–6	Aoki and Kurosu 1986; Foster 1990; Current study

* Possibly a synonym of *Pemphigus borealis* Tullgren (Aoki 1975, Blackman and Eastop 1994).

diers or if it is more ancestral and never acquired them, perhaps because of peculiarities in its ecology.

Temporal polyethism in Pemphigus

The current results show that anti-predator behaviour is widespread not only among the different species of *Pemphigus* but also among the different developmental stages. The first instars were always the most aggressive, but later instars in all the soldier-producing species showed some degree of anti-predator behaviour. It is possible that attacking behaviour in instars other than the chief defender morph is relatively common in soldier aphids (see Stern and Foster, 1996; Aoki et al., 1999: Shingleton and Foster, 2001). It is interesting that *P. spyrothecae* had not only the most aggressive defenders but also the most specialized with respect to instar. First instars of *spyrothecae* were three to four times more likely to attack than the second instars, and later instars did not fight at all. In the other four species, the first instars were only one to three times as likely to attack as the seconds, and the third and, in one species, the fourth instars also attacked the predator. It is possible that defensive behaviour is quite widespread and generalized in the ancestral *Pemphigus* species, but becomes more focussed and specialized, both behaviourally and morphologically, in certain species, such as *spyrothecae* and perhaps *obesinymphae*, as a result of the more extensive risk of predation.

The general pattern of temporal polyethism, where the younger instars are the more specialized in defence, seems to be unique to aphids. In most social insects, individuals become more committed to defence as they get older: this is probably not as a causal result of ageing, but as a result of self-organisation processes that ensure that, as time passes, workers move further from the nest centre (Bourke and Franks, 1995). In addition, life-history theory shows that individuals will be selected to take more risks as they approach the end of their life-span (see also Jeanne, 1986). In aculeates, being endopterygotes, the larval stages are committed to feeding and are probably constrained from having a defensive role. But even in termites, where juvenile instars do contribute to colony labour, defence is usually concentrated in the older instars (e.g. Luykx, 1993; Shellman-Reeve, 1997).

The advantage of using first instars as defenders is perhaps that they are small, which means that they can be manoeuvrable in a gall, that they will feed less (if at all), that they can be produced rapidly and in large numbers in response to predation threats, and that they can be packed at high density onto the surface of a predator (Stern and Foster, 1997). Stern et al. (1997) provided evidence that the attacker first instars in a monomorphic species of *Pseudoregma* were older than non-attacker first instars, perhaps because, as in adult aculeate workers, it is sensible to postpone risky behaviour until as late as possible within an instar, so that useful work is done before death is risked. It is possible that there are two levels of temporal polyethism in aphids: defensive behaviour is focussed in the earlier instars but it occurs at a late stage within any particular instar. To confirm that there is within-instar polyethism, it will be necessary to observe the behaviour of individual aphids of known age.

Stimuli releasing attack behaviour

The lure experiments suggest that the aphids have quite a subtle predator-recognition system. When placed on the syrphid predator, they are able to recognize and attack it in the absence of other cues (Figs. 1, 2). Physical stimulation (using a paintbrush) enhanced the attack rate: this was observed in the laboratory-reared *P. spyrothecae*, but a comparable increase (between 30 and 120%) in the rate of attack was observed in all the other instars that attack spontaneously. Mere surface wetness can also stimulate attack behaviour (Fig. 2). The aphids seem to be able to recognize a chemical component of this surface moisture, since lures soaked in aphid haemolymph always elicited significantly more attacks than did water-soaked lures (Fig. 2). It is especially interesting that, in *P. spyrothecae*, the aphids are able to distinguish between lures soaked in their own haemolymph and those soaked in honeydew. A possible advantage of this is that mere disruption of honeydew droplets, which might be quite common in these crowded galls even in the absence of predators, will not usually elicit an attack.

The use of insect haemolymph as an attacking stimulus was also shown by Schütze and Maschwitz (1991) working on the soldiers of *Pseudoregma sundanica* (Van der Goot) and *P. bambucicola* (Takahashi) (Hormaphididae; Cerataphidini). Contact with haemolymph was apparently the only releasing mechanism identified in *Pseudoregma*, in contrast to the sophisticated combination of surface moisture, physical agitation and chemical stimulus employed by *Pemphigus*.

There is no evidence that pheromones are used for recruitment in these *Pemphigus* species, although it is interesting that all the attacking morphs (the early instars of all the species with soldiers) have cornicles, whereas the non-defensive stages (the sexuparae of *P. spyrothecae* and the gall generations of *P. populi*) lack these structures. Alarm pheromones are well known in aphids (e.g. Pickett and Griffiths, 1980), and the use of cornicle secretions in defence has been shown in the soldiers of *Ceratovacuna lanigera* (Arakaki, 1989) in open colonies on the secondary host. Perhaps in a closed *Pemphigus* type of gall, pheromones would provide little directional information.

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