

**Research article**

**Behavior and morphology of monomorphic soldiers from the aphid genus *Pseudoregma* (Cerataphidini, Hormaphididae): implications for the evolution of morphological castes in social aphids**

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**Summary**

Soldier-producing aphids have evolved at least nine separate times. The larvae of soldier-producing species can be organized into three general categories: monomorphic larvae, dimorphic larvae with a reproductive soldier caste, and dimorphic larvae with a sterile soldier caste. Here we report the discovery of a novel soldier type in an undescribed species of *Pseudoregma* that is morphologically similar to *P. bambucicola*. A colony of this species produced morphologically monomorphic first-instar larvae with a defensive behavioral dimorphism. These larvae attacked natural predators, and larval response to a simple assay, placing the tips of forceps in front of larvae, was correlated with this attacking behavior. Approximately one third of the first-instar larvae in the colony attacked and this proportion was uncorrelated with the time of day, the ambient temperature, or the diel migratory behavior of the aphids. Migrating larvae rarely attacked. Attacking behavior was correlated with another defensive behavior, hind-leg waving. Attackers were more likely to possess the next-instar skin, suggesting that they were older than non-attackers. This is the first example of a possible within-instar age polyethism in soldier-producing aphids. Canonical variates analysis of seven morphological measurements failed to discriminate between attacking and non-attacking larvae. The monomorphic larvae share some morphometric characteristics in common with the soldiers of *P. bambucicola* and other characteristics in common with normal larvae. We discuss these results with respect to the evolution and loss of soldier castes in the tribe Cerataphidini.

**Introduction**

The soldier-producing aphids of the Pemphigidae and Hormaphididae exhibit extensive morphological and behavioral diversity of soldier types (Aoki, 1987; Aoki and Kurosu, 1989; Stern and Foster, 1996). This diversity can be organized into

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three general types of soldiers (Stern and Foster, 1996, 1997): (1) monomorphic larvae, all of which are capable of attacking during a particular larval instar, and which then molt and reproduce; (2) specialized larvae that are different from normal larvae, which can molt and reproduce (leading to a larval dimorphism); and (3) specialized larvae that do not molt and are therefore reproductively sterile. This diversity of soldier types is puzzling and the relative advantages of the different types are unclear, even though the different types can be produced within the same taxonomic groups, and even within the same species at different times in the life cycle (for review see Stern and Foster, 1996).

It has been suggested (Aoki, 1987; Stern and Foster, 1997) that these three types represent an evolutionary progression in a more complicated scenario entailing at least five steps: (1) behavioral specialization of an instar for defense, (2) morphological specialization of the instar for defense, (3) prolongation of the duration of the defensive instar, (4) dimorphism between soldiers and non-soldiers of the same instar, and (5) sterility of the soldier caste. (Steps 2 and 3 are thought to be interchangeable). If species do transform between these states (and presumably they can transform in both directions depending on selective pressures), then species or populations that show intermediate states might exist. If the transformations are slow relative to speciation, then an evolutionary transformation between the states might be observed along a phylogeny.

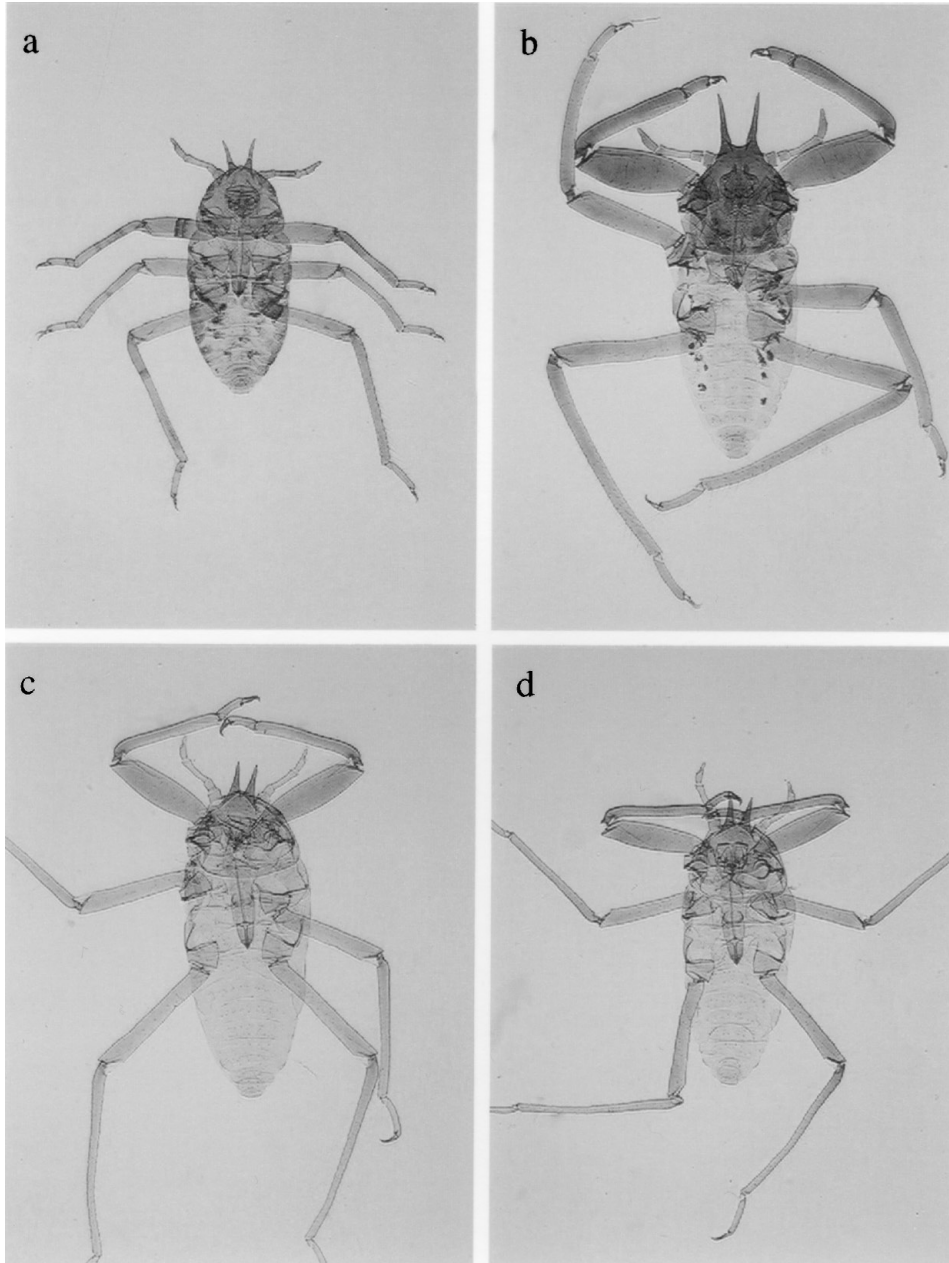
Here we present a behavioral and morphological analysis of an unusual species of *Pseudoregma* found to produce monomorphic larvae with an apparent temporal polyethism within an instar. All previous reports have shown that species of this genus produce morphologically dimorphic larvae with sterile soldiers (Fig. 1: Aoki and Miyazaki, 1978; Aoki et al., 1981; Aoki and Kurosu, 1989; Schütze and Maschwitz, 1991; Stern, et al., 1996; Stern and Foster, 1996). The existence of this type of aphid soldier suggests that the current model for the evolution of soldier-aphid castes may be inadequate.

## Materials and methods

### *Identification of attackers*

The focal colony was found on a shoot of *Schizostachyum zollingeri* (Bambusae) in Kepong, Malaysia during January of 1993. The adult and larval morphology were very similar to *Pseudoregma bambucicola*, except that the adults had a more heavily sclerotized dorsum than is normally found in *P. bambucicola* (Noordam, 1991). We therefore call this colony *Pseudoregma* sp. near *bambucicola* and assume that it is phylogenetically closely related to *P. bambucicola*.

The colony contained at least tens of thousands of individuals and apparently lacked morphologically specialized soldiers, which are easy to identify on living colonies (Fig. 1; also see Aoki and Miyazaki, 1978; Aoki et al. 1981; Stern et al., 1996). However, some of the first-instar larvae would attack forceps placed within approximately 0.5 cm of the front of the aphid. The aphids would quickly grab the forceps with their fore-legs; some would then release the forceps and others persistently held on.



**Figure 1.** Photos of (a) a normal and (b) a soldier larva of *Pseudoregma bambucicola* and (c) a non-attacker and (d) an attacker from the focal colony of *P. sp.* near *bambucicola*

We performed the following experiments to determine whether these aggressive larvae would attack real predators and whether the probability of a larva attacking the forceps (henceforth termed the forceps assay) indicated the likelihood that the larva would attack a real predator. Larvae that either attacked or did not attack the forceps were placed in a small plastic container in groups of four or five larvae and then a predator, either a small unidentified lepidopteran larva or a small unidentified coccinellid larva, was placed in the container. The aphids were then observed for at least 10 minutes and attacking behavior was scored if the aphids either grasped the predator with their fore-legs or grasped the predator with their fore-legs and stuck their frontal horns into it. These are the two major defensive behaviors performed by soldiers of *Pseudoregma* and *Ceratovacuna* species (reviewed in Stern and Foster, 1996).

#### *Analysis of behavior*

*Leg waving and attackers* – Individuals of many species of *Pseudoregma* wave their hind legs, apparently as a defensive effort to ward off parasitoids and predators (Sakata and Itô, 1991; Schütze and Maschwitz, 1992). We observed that when predatory wasps passed near and walked over the surface of the colony many individuals waved their hind legs. Physical stimulation of the aphids or bamboo shoot also elicited this behavior. We examined whether first-instar larvae that attacked were more likely to leg wave than non-attackers. We stimulated leg waving by tapping the bamboo shoot near a focal individual. The focal individual was then assayed for attacking behavior using the forceps assay.

*Diel migratory cycle and attackers* – We discovered that individuals within this colony showed a diel pattern of migratory activity. After 14:00 hours many of the early instar larvae would begin walking around the colony and leave the colony by walking down the bamboo shoot. We examined whether larval aggression was correlated with the migratory behavior by assaying aggressive behavior throughout the day and comparing this with the migratory activity of first-instar larvae. Thirty stationary larvae were assayed using the forceps assay at one hour intervals from approximately 07:30 to 19:30 on 24 Jan. 1993. Each larva was collected in 95% ethanol after being assayed to ensure that different individuals were assayed throughout the day. The ambient temperature was measured to the nearest 0.5 °C during each measurement period. Migratory behavior was measured by counting the number of aphids that passed a pre-determined point near the base of the bamboo shoot during three three-minute periods during the same time interval and on the same day that larval aggression was assayed. To further examine whether attacking is related to migratory behavior, we tested whether migrating individuals attacked with the same frequency as stationary individuals.

*Aphid/predator interactions* – We observed two kinds of predators at the aphid colony: an unidentified lepidopteran larva and an unidentified aculeate wasp. The two predators hunted in different ways. Several lepidopteran larvae lived underneath each bamboo culm sheath and had produced silken webs that extended out from beneath the culm sheath. An individual caterpillar hunted by slowly crawling out from under the culm sheath to the edge of the silken web and waiting for an

aphid to walk nearby. It then quickly grabbed the aphid with its mandibles and retreated back under the culm sheath where it fed on the aphid. These caterpillars were never found outside the culm sheaths or silken webs. The wasps hunted by landing on the colony and wandering over the top of the colony. Just before the wasp landed, aphids in the vicinity began to leg wave. After the wasp landed, larvae began grabbing at the wasp's legs and the wasp was noticeably hampered by the attackers. The wasp would then grab an aphid in its mandibles and fly away from the colony with its prey.

To investigate how attackers would interact with a caterpillar if the caterpillar was not protected by silk we removed individual caterpillars from beneath a culm sheath and introduced them onto an area of the bamboo devoid of aphids but with many aphids nearby. In three experiments, we scored the behavior of every aphid that came into contact with the predator as one of the following: leg wave, grasp, run away, or no response.

#### *Are attackers morphologically different from non-attackers?*

We performed a morphometric analysis of first-instar larvae from the focal colony to determine whether the larvae possessed a dimorphism that eluded casual inspection. Larvae assigned to the attacker or non-attacker category based on the forceps assay were collected in 95% EtOH. In addition, first-instar larvae (soldiers and non-soldiers) from a large colony of *Pseudoregma bambucicola* collected from *Bambusa magica* on 3 Nov. 1992 in the Cameron Highlands, Malaysia (collection number DS92-164) were measured for comparison with the focal colony. Aphids were cleared, stained and mounted as described in Blackman and Eastop (1994). Digitized images of aphids were captured and analyzed using NIH Image (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). The following lengths were measured: body length from tip of head to base of last abdominal segment, last rostral segment length, fore femur, fore tibia, hind femur, hind tibia, and horn length (see Stern et al., 1996 for a diagram of the lengths measured). Twenty-two individuals were remeasured to estimate the measurement error.

To test for morphological castes in *P. sp.* near *bambucicola*, we performed canonical variates analysis (CVA) on the morphometric data using the computer program SAS. Bivariate scatterplots were also used to analyze trait relationships.

#### *Is attacking an age-specific polyethism?*

Attackers and non-attackers were cleared, stained, and mounted as detailed above. Aphids were examined with phase-contrast optics for the presence of the next-instar skin. The claws are the first chitinized morphological features to appear as larvae prepare to moult and therefore the tarsi of all six legs were examined for the next-instar claws.

## Results

### *Identification of attackers*

Of 23 larvae that grasped forceps, 20 attacked a real predator (Table 1). In contrast, zero of 20 larvae that did not grasp the forceps attacked a real predator. Therefore, the forceps assay appears to be a reliable method for assessing the probability that an individual will attack a real predator.

### *Analysis of behavior*

*Leg waving and attackers* – Larvae that performed leg-waving when the colony was disturbed were significantly more likely to attack (84%) than individuals that did not leg wave (44%) (Table 2).

*Diel migratory cycle and attackers* – Averaging over the entire day, 32% of assayed larvae attacked (Fig. 2). There did not appear to be a strong diel cycle to aggressive behavior, whereas there was a strong diel cycle to migratory behavior

**Table 1.** Do attackers identified by the forceps assay attack real predators?

Type of larva <sup>a</sup>	Predator <sup>b</sup>	Attack?	
		yes	no
Attacker	Lepidopteran larva	4	0
Attacker	Beetle larva	3	1
Attacker	Coccinellid larva	4	1
Attacker	Coccinellid larva	5	0
Attacker	Coccinellid larva	4	1
Non-attacker	Coccinellid larva	0	5
Non-attacker	Coccinellid larva	0	5
Non-attacker	Coccinellid larva	0	5
Non-attacker	Coccinellid larva	0	5

<sup>a</sup> Larvae were categorized as attacker or non-attacker based on their response to the forceps assay.

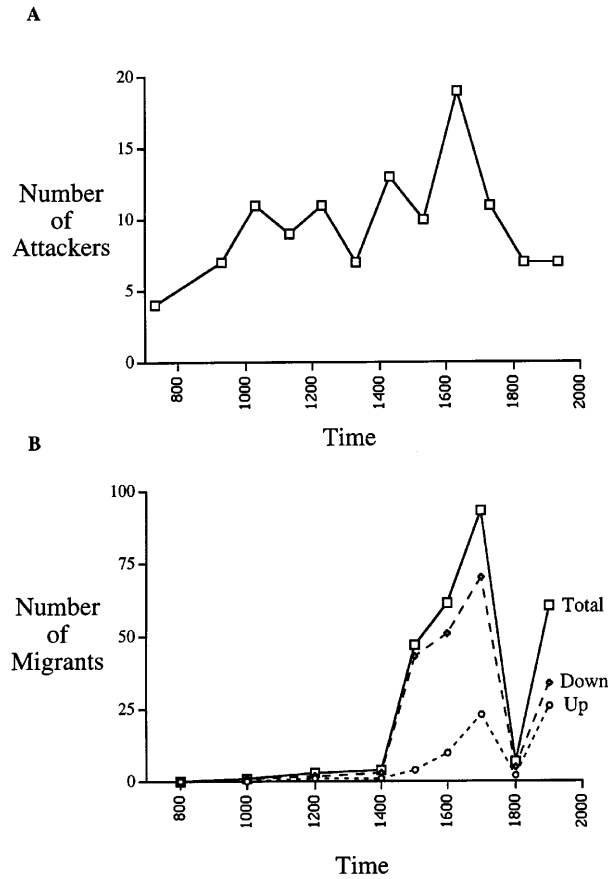
<sup>b</sup> For each experiment, aphid larvae were placed in a small container with an individual predator.

**Table 2.** Are leg wavers more likely to attack than non-wavers?<sup>a</sup>

	Attack		Run away
	grasp and hold	quick grasp	
Non-waver	17	5	28
Waver	35	7	8

Chi-Square (2 df) = 17.675,  $P < 0.0001$ .

<sup>a</sup> Attacking was measured with the forceps assay and leg waving was assayed by tapping the bamboo stem near the aphids.



**Figure 2.** Diel patterns of attacking and migratory activity. (A) The number of attackers out of 30 at each census. (B) The number of migrants crossing a mark at the base of the colony at each census

(Fig. 2). There were no significant correlations between the number of migrants and the number of attackers ( $r_s = 0.32$ , 9 df,  $p = 0.36$ ), between temperature and the number of attackers ( $r_s = 0.29$ , 8 df,  $p = 0.34$ ), or between temperature and the number of migrants ( $r_s = 0.28$ , 12 df,  $p = 0.42$ ). However, stationary larvae were significantly more likely to attack (60%) than were migratory larvae (3%) (Table 3).

*Aphid/predator interactions* – Table 4 summarizes the results of three predator introduction experiments where the behavior of aphids was quantified. In all experiments the caterpillar appeared to be attempting to avoid all contact with aphids. Each time a caterpillar touched an aphid it quickly retracted from the spot of contact. In addition, the predator continued moving through the colony until it either found a silken web or a culm sheath, or fell from the colony. Half of the first-instar larvae that encountered the predator attacked, another 15% leg-waved, and the remainder either ran away or did not respond.

**Table 3.** Do migrating larvae attack with the same frequency as stationary larvae?<sup>a</sup>

	Grasp and hold	Quick grasp	Run away
Migrating	0	1	29
Stationary	12	6	12

Chi Square (2 df) = 22.620,  $P < 0.0001$ .

<sup>a</sup> Migrating larvae were assayed below the lowest node of the colony and the stationary individuals were assayed within the colony.

**Table 4.** How do aphids respond to a predatory lepidopteran larva released into the colony?<sup>a</sup>

Experiment	Duration (sec)	Leg wave	Grasp	Run away	No response
1	350	8	23	5	3
2	470	4	15	1	8 <sup>b</sup>
3	1180	4	14	5	18 <sup>b</sup>
Total <sup>c</sup>		16	52	11	25
Percent <sup>d</sup>		15.4	50	10.6	24

<sup>a</sup> All aphids are first instar larvae unless otherwise indicated.

<sup>b</sup> Two individuals from each of these categories were adults.

<sup>c</sup> Total number of first-instar larvae performing each behavior summed over all three experiments.

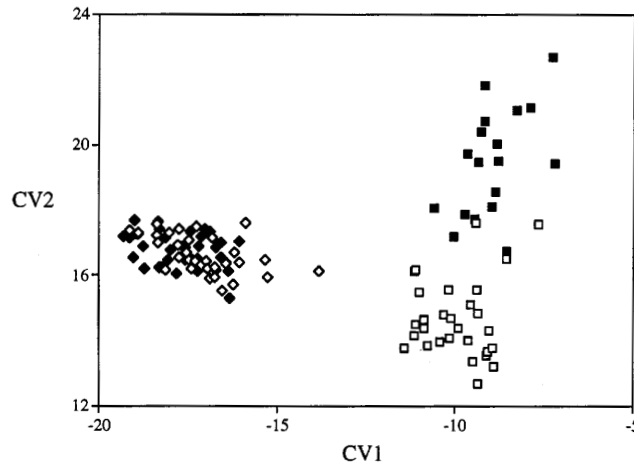
<sup>d</sup> Percent of total first-instar larvae assayed performing a particular behavior.

#### *Are attackers morphologically different from non-attackers?*

The difference between repeated morphometric measurements on the same individual averaged over all organs was 0.00676 mm ( $n = 22$ ). The individual data points in Figures 3 and 4 are larger than the measurement error.

The first canonical variate accounted for 87% of the between class variation and the second canonical variate accounted for a further 13%. The first two canonical variates did not differentiate between attackers and non-attackers of *P. sp.* near *bambucicola* (Fig. 3). In contrast, the first two canonical variates clearly differentiated between soldiers and non-soldiers of *P. bambucicola*. For most morphological traits the larvae of *P. sp.* near *bambucicola* were morphologically intermediate between normal and soldier larvae of *P. bambucicola*. For example, scatter plots of fore-legs versus body size suggest that larvae of *P. sp.* near *bambucicola* (both attackers and non-attackers) are more similar to *P. bambucicola* soldiers (Fig. 4a). Plots for the other leg segments measured were similar to the plot shown for fore-legs (results not shown). However, comparison of horn length versus body length suggests that the *P. sp.* near *bambucicola* larvae follow the horn allometry of *P. bambucicola* normal larvae (Fig. 4b).





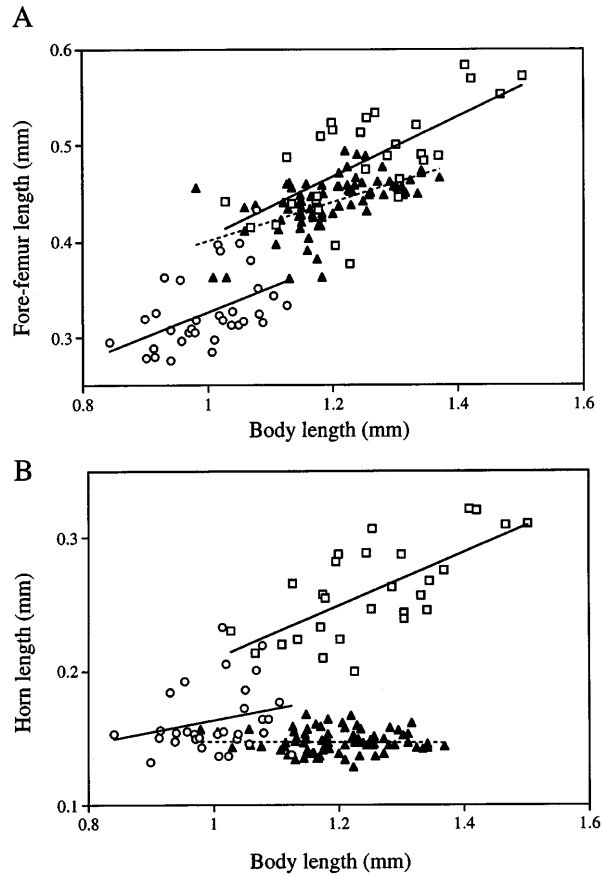
**Figure 3.** Scatterplot of the first two canonical variates for the first-instar larvae of *Pseudoregma bambucicola* and *P. sp. near bambucicola*. Only the first two canonical variates had correlations that were significantly greater than zero by the likelihood ratio test. The open squares (□) are normal larvae of *P. bambucicola*, the black squares (■) are soldier larvae of *P. bambucicola*, the open diamonds are non-attackers of *P. sp. near bambucicola* (◇), and the black diamonds (◆) are attackers of *P. sp. near bambucicola*.  $CV1 = (\text{body length} * 2.3531) + (\text{last rostral segment length} * -93.1386) + (\text{fore-femur length} * -2.7237) + (\text{fore-tibia length} * -27.2796) + (\text{hind-femur length} * -35.5085) + (\text{hind-tibia length} * 18.216) + (\text{horn length} * 66.9732)$ .  $CV2 = (\text{body length} * 4.3266) + (\text{last rostral segment length} * 35.3687) + (\text{fore-femur length} * 12.2096) + (\text{fore-tibia length} * -3.2946) + (\text{hind-femur length} * 2.5738) + (\text{hind-tibia length} * -2.5494) + (\text{horn length} * 26.8343)$

#### *Is attacking an age-specific polyethism?*

Two-hundred and forty-two attackers and 334 non-attackers were examined for the presence of the next-instar skin. Attackers were significantly more likely to possess the next-instar skin than were non-attackers (Table 5). This indicates that attackers were more likely to be older than were non-attackers.

#### Discussion

The evolution of complex traits, such as aphid soldiers, is best understood in the context of their phylogenetic history. Recent work on the phylogenetic systematics of the aphid tribe Cerataphidini has provided a clear view of the evolutionary history of soldier evolution (Stern, 1994). Within the Cerataphidini, many species produce two distinct types of soldiers at different stages in the life cycle, on the primary and secondary hosts. Soldiers produced on the primary host originated before the diversification of the Cerataphidini and can be found in all species currently attacking the primary host plant (Stern, 1994). In contrast, the horned-soldiers, which only occur on the secondary host, arose much later and are only found in the two genera *Ceratovacuna* and *Pseudoregma* (Stern, 1994).

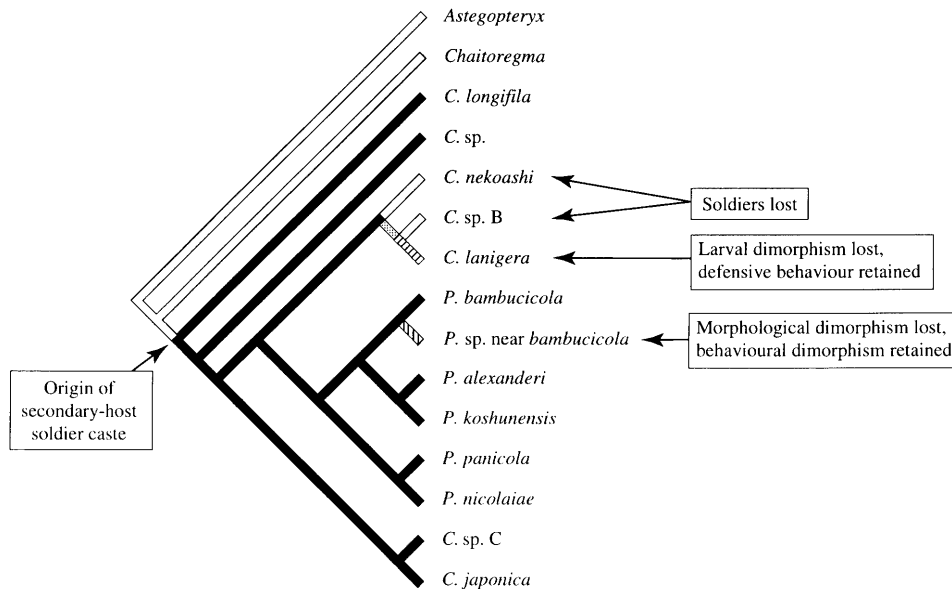


**Figure 4.** Morphometrics of first-instar larvae of *Pseudoregma bambucicola* and *P. sp. near bambucicola*. (A) Body length vs fore-femur length. (B) Body length vs horn length. The open circles (○) are normal larvae of *P. bambucicola*, the open squares (□) are soldier larvae of *P. bambucicola*, and the black triangles (▲) are larvae of *P. sp. near bambucicola*. The least-squares regression equations are as follows: Body length vs fore-femur length: normal *P. bambucicola*,  $y = 0.256x + 0.071$ ; soldier *P. bambucicola*,  $y = 0.312x + 0.092$ ; *P. sp. near bambucicola*,  $y = 0.2x + 0.202$ . Body length vs horn length: normal *P. bambucicola*,  $y = 0.087x + 0.076$ ; soldier *P. bambucicola*,  $y = 0.2x + 0.009$ ; *P. sp. near bambucicola*,  $y = -0.002x + 0.15$

**Table 5.** Is attacking a temporal polyethism?

Type of larva	Next-instar skin	
	yes	no
Attacker	7	235
Non-attacker	1	333

Chi-Square (1 df) = 6.89,  $P = 0.0087$ .



**Figure 5.** Cladogram of the genera *Ceratovacuna* and *Pseudoregma*. *Pseudoregma* sp. near *bambucicola* has been placed as the sister species of *P. bambucicola* because of the extremely similar morphology of these two species, although it was not included in the original phylogenetic analysis. The ancestral states for soldier production have been reconstructed on the phylogeny and are indicated by differently shaded branches: white branches = absence of soldiers; black branches = dimorphic larvae with specialized soldier caste; diagonal striped branch = morphologically monomorphic larvae with soldiers, *C. lanigera*; vertical striped branch = morphologically monomorphic, behaviorally dimorphic larvae with soldiers, *P. sp. near bambucicola*; stippled branch = equivocal ancestral state

The current best estimate of phylogenetic relationships within the genera *Ceratovacuna* and *Pseudoregma*, with a mapping of the evolution of horned soldiers, is presented in Figure 5 (Stern, unpubl. data). Dimorphic larvae with morphologically specialized soldiers originated once and have been lost at least once within *Ceratovacuna*. *Ceratovacuna lanigera*, which is closely related to species that have lost soldiers, produces soldiers that are not morphologically specialized (Aoki et al., 1984). In this species it is assumed that all of the first-instar larvae potentially attack. This species has either retained the defensive behavior and dispensed with the larval dimorphism, or, as its closest relatives do not produce soldiers, it evolved the defensive behavior anew from a non-defensive ancestor.

Given the likely phylogenetic position of *P. sp. near bambucicola* as the sister species of *P. bambucicola*, the production of defensive but monomorphic larvae is probably a derived state. The behavior of these attackers is very similar to the behavior of morphologically-specialized soldiers of other species of *Pseudoregma*. The morphology of these monomorphic defenders is, in general, intermediate between normal and soldier larvae. However, the monomorphic defenders appear to have adopted different traits from each of the two morphs. From the soldier morph they have adopted the allometry of elongated legs relative to body size, and

from the normal larvae they have adopted the allometry of relatively shorter horns (Fig. 4). This illustrates that the specialized morphological characters of soldiers need not be inherited as a single unit. Defenders of *P. sp.* near *bambucicola* have apparently dissociated the soldier morphology, relatively long horns and legs, into its components. This suggests that soldier morphology may be plastic and responsive to selective pressures.

*Pseudoregma sp.* near *bambucicola* is the first aphid species known to display a behavioral dimorphism without a morphological dimorphism, although this type of dimorphism has been hypothesized to exist (Stern and Foster, 1996, 1997). Other possible cases are unlikely to represent true behavioral dimorphism. For example, Kurosu and Aoki (1988, 1991a,b, 1994) have described a behavioural difference in the first-instar larvae of the gall-producing generations of four cerataphidine species, but it is not clear if this is a true dimorphism. In these species, the early-born larvae of the gall foundress enter the subgalls to develop and reproduce whereas the later-born larvae typically cannot enter the closed subgalls and perform defensive behavior. However, this behavioral difference is apparently dictated by the context (aphids outside the gall attack), because when larvae inside the subgalls are released they attack predators (Kurosu and Aoki, 1994).

This is also the first report of a possible within-instar age polyethism in social aphids. Of course, most soldier-producing species have a temporal polyethism since typically only a single instar displays defensive behavior (Stern and Foster, 1996). In all other species studied, soldiers tend to have longer instar durations than non-soldiers (Sakata and Itô, 1991; Akimoto, 1992; Moran, 1993). Therefore, the direction of the age polyethism in these aphids is surprising. Either all aphids attack more frequently towards the end of the instar or the aphids display a strict behavioural dimorphism and attackers have a shorter instar duration. To distinguish between these hypotheses it will be necessary to mark individual larvae and to raise cohorts of larvae of known age.

Our observations suggest that it would be fruitful to investigate other species producing monomorphic larvae for a possible behavioral dimorphism (for example, *Ceratovacuna lanigera*). In addition, it will be worth searching for soldier behavior in other contexts. For example, *P. bambucicola* produces soldiers on the secondary host only during certain times of the year, even though colonies can be found on the secondary host throughout the year. Several investigators have measured the proportions of soldiers in these colonies in attempts to determine the causes of soldier production (Sakata and Itô, 1991; Sunose et al., 1991; Itô et al., 1995; Tanaka and Itô, 1995). However, it is possible that colonies without dimorphic larvae also display defensive behavior and this possibility will obfuscate any possible links between soldiers and environmental factors if only morphology is measured. It is, therefore, important to determine the levels of defensive behavior in colonies that do not produce morphologically-dimorphic larvae.

We found that early-instar larvae of the focal colony underwent mass migrations at specific times of day (Fig. 2) and that migrators had a strong tendency not to attack. Since non-attackers tend to be young, it will be worth testing the hypothesis that migrators tend to be young. Aoki (1979) discovered a specialized migratory morph in *Pachypappa marsupialis*, so the possibility of a behaviorally-specialized migratory caste is not without precedent.

In conclusion, we report the discovery of a dimorphism in defensive behavior without accompanying morphological dimorphism in a genus of aphids previously known to produce only morphologically-dimorphic larvae with specialized soldiers. These findings indicate that behavioral dimorphism may exist without morphological dimorphism, contrary to an earlier proposed model for soldier evolution (Stern and Foster, 1997). This morphological monomorphism evolved from a morphologically dimorphic ancestor and it is possible that the monomorphic attackers of *Ceratovacuna lanigera* also evolved from a dimorphic ancestor. It is not yet clear whether behavioral dimorphism without morphological dimorphism evolved in any species with morphologically-monomorphic ancestors. There are many species of aphids, however, that produce morphologically-monomorphic soldiers (Stern and Foster, 1996), and further work may uncover behavioral dimorphism within these species. Nonetheless, the existence of behavioral dimorphism in *P. sp.* near *bambucicola* suggests that behavioral dimorphism with morphological monomorphism is a viable strategy and therefore may have been the original route to morphological dimorphism.

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