

Buying time for colony mates: the anti-predatory function of soldiers in the eusocial aphid *Ceratovacuna japonica* (Homoptera, Hormaphidinae)

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Received: 8 May 2012 / Revised: 31 August 2012 / Accepted: 3 September 2012 / Published online: 23 September 2012
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Abstract Eusocial aphids produce sterile individuals (soldiers) that specialize in colony protection. Killing predators is often considered the main function of soldiers. In this study, we tested the effect of harassment by soldiers of a eusocial aphid, *Ceratovacuna japonica* (Homoptera, Hormaphidinae), on predation by this species' natural enemy, the larvae of *Atkinsonia ignipicta* (Lepidoptera, Stathmopodidae). We experimentally introduced some aphids and a predator to petri dishes and compared the survivorship of first-instar reproductives in the presence and absence of soldiers. We showed that soldiers can reduce the rate of predation on their colony mates without killing the predators. When predators encountered soldiers, they did not attempt to prey on them. Instead, they evaded them and often started to make a nest by spinning silken threads. The soldiers, in contrast, waved their forelegs and attacked the predator, and they sometimes succeeded in grasping the predator's body. Because the predator used its mandibles to

remove any soldier that succeeded in grasping its body, the soldier did not kill the predator. The reduction of predation was apparently caused by the delay of predation resulting from the harassment behavior of the soldiers. In eusocial aphids, a defensive strategy that delays predation may buy the soldiers' colony mates time to reproduce or to escape from the predator.

Keywords Anti-predator behavior · Defensive strategy · Eusociality · Predation · Predator–prey interaction

Introduction

Predator–prey interaction has a significant impact on trait evolution in prey, and prey species have adapted to cope with diverse predation pressures (Tollrian and Harvell, 1999; Abrams, 2000; Thompson, 2004). As one anti-predator strategy, some animals have evolved eusociality (Hölldobler and Wilson, 1990), which is characterized by production of sterile individuals with various specialized traits that allow them to kill or repel predators (Hölldobler and Wilson, 1990; Ono et al., 1995; Dornhaus and Powell, 2010). The defensive functions of sterile individuals have been intensively investigated in hymenopterans (Hölldobler and Wilson, 1990; Ono et al., 1995; Strohm and Liebig, 2008; Dornhaus and Powell, 2010), but rarely in other eusocial animals such as eusocial aphids (but see Foster, 1990; Foster and Rhoden, 1998).

Aphids are preyed on by various kinds of predators (lady beetles, anthocorid bugs, lacewings, hoverflies, etc.; Dixon, 1998), and have also evolved various anti-predator strategies (escape by flight, defensive mutualism with ants, etc.; Stadler and Dixon, 2005; Weisser et al., 1999). Several aphid species belonging to the Pemphiginae and the Hormaphidinae produce sterile soldiers that specialize in colony protection

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(eusociality; Pike and Foster, 2008). Soldiers attack other insects, including predators, that invade their colonies, and soldiers typically display defensive morphologies and behavior: for example, a piercing stylet and thick hind legs (*Pemphigus spyrothecae*, Foster, 1990), a piercing stylet and thick fore- and middle legs (*Colophina clematis*, Aoki, 1977), piercing spiny horns and thick forelegs (*Pseudoregma alexanderi*, Aoki et al., 1981), and venom injection through a stylet (*Tuberaphis styraci*, Kutsukake et al., 2004).

Killing predators has been considered to be the main function of soldiers (Stern and Foster, 1996). In fact, many studies have shown that aphid soldiers can kill insects invading their colony (Aoki, 1977, 1980; Ôhara, 1985; Kurosu and Aoki, 1988; Foster, 1990; Kurosu et al., 1990; for a review see Stern and Foster, 1996). However, these studies often used not only predators but also non-predatory insects as victims and reported their observations anecdotally (Aoki, 1977, 1980; Kurosu and Aoki, 1988; Kurosu et al., 1990; reviewed by Stern and Foster, 1996). Therefore, the effectiveness with which soldiers defend their nest mates against natural enemies needs to be clarified quantitatively (Foster, 1990). At present, we do not have quantitative information as to whether aphid colonies are effectively protected by soldiers, whether the colony's natural enemies are actually killed by the soldiers, whether the soldiers can protect their colony without killing the predators, or whether the soldiers can survive after attacking predators.

Natural selection favors predators that are less likely to be killed by soldiers, because predator–prey interaction results in reciprocal adaptation (Benkman et al., 2003; Kishida et al., 2006; Toju and Sota, 2006; reviewed by Thompson, 2004). In fact, specialized predators of eusocial aphids have traits that appear to be effective against soldier attacks: the flower bug *Anthocoris minki* can paralyze soldiers (Stern and Foster, 1996), and some predatory caterpillars (*Assara formosana*, Aoki and Kurosu, 1992; *Dipha aphidivora*, Arakaki and Yoshiyasu, 1988; *Taraka hamada*, Aoki and Kurosu, 2011; *Atkinsonia ignipicta*, Aoki and Kurosu, 2011) make nests of silken threads to escape attacks by soldiers. Foster (1990) experimentally showed that soldiers of *Pemphigus spyrothecae* often cannot kill a single predator introduced to their colony. Moreover, an aphid colony is often attacked by several predator individuals (Shibao, 1998; Ijichi et al., 2005; Hattori et al., in review). These facts suggest that it is not an easy task for soldiers to kill predators in the field. Rather, soldiers may use subtle, non-suicidal defenses such as harassment of predators to defend their colony (Stern and Foster, 1996), although observational support for such harassment is rare (Shingleton and Foster, 2000; Shibao and Fukatsu, 2003). Here, we hypothesized that soldiers can mitigate predation pressure without killing predators.

In this study, we examined whether the eusocial aphid *Ceratovacuna japonica* (Homoptera, Hormaphidinae) uses a defensive strategy that can mitigate predation pressure without killing predators. We introduced first-instar reproductives, soldiers, and a specialist predator to petri dishes, and compared the predation rate on the first-instar reproductives in either the absence or presence of soldiers. We revealed that soldiers were unable to kill predators, but found that soldier–predator interaction significantly reduced the predation rate on first-instar reproductives. These results suggest that soldiers can improve the survival of colony mates by buying time, without killing the predator.

Materials and methods

The eusocial aphid

The eusocial aphid *Ceratovacuna japonica* produces obligately sterile, pseudoscorpion-like soldiers on secondary host plants. Although the soldiers are first instars that do not develop into second-instar nymphs (Aoki et al., 1981), they inherently have a pair of longer frontal horns, longer forelegs, and larger body size than first-instar reproductives (Hattori and Itino, 2008). The sole role of soldiers appears to be protection of the colony against predators. If a soldier encounters a predator, it instantly grasps the predator with its forelegs and attempts to pierce the predator with its frontal horns.

The main predators of these aphids on their secondary hosts are larvae of the lepidopterans *Atkinsonia ignipicta* (Moriuti, 1982) and *Taraka hamada* (Banno, 1997). Although these predators sometimes feed on other aphids (Morimoto and Shibao, 1993), they feed mainly on *C. japonica* (Aoki and Kurosu, 2011). At our study sites, *A. ignipicta* larvae were the dominant predator (see “Results”).

Ceratovacuna japonica is widely distributed in Japan. It has one primary host plant, *Styrax japonica* (Ebenales: Styracaceae), and several secondary host plants (Poaceae spp.) (Aoki and Kurosu, 1991, 2011). Although it has a complete life cycle and sometimes produces winged sexuales on its secondary hosts for host alternation, it has rarely been observed on its primary host plant in central Japan (Carlin et al., 1994). Therefore, other than in midwinter, when it sometimes produces sexuales, this species reproduces almost exclusively parthenogenetically on the secondary hosts. Here, we define a colony as an aggregation of aphid individuals on a single leaf of the secondary host *Sasa senanensis* (Poaceae: Bambuseae). An aphid colony on a leaf persists for up to several months.

We collected aphids, including soldiers and first-instar reproductives, and the specialist predator (larvae of *A. ignipicta*) on *S. senanensis* in midsummer and brought

them to the laboratory to test the defensive capability of soldiers against the specialist predator *A. ignipicta*.

The proportion of soldiers in the field

To assess the proportion of soldiers in the field, we conducted a field survey in the foothills of Mt. Jyonen, Nagano, central Japan (1,261 m above sea level; 36°19'N, 137°47'E). The *C. japonica* population inhabits the deciduous forest edge [dominated by *Castanea crenata* (Fagales: Fagaceae) and *Quercus crispula* (Fagales: Fagaceae)], where *S. senanensis* is abundant.

On 26 July 2011, we randomly selected ten *S. senanensis* leaves, each with an aphid colony, collected all aphids and predators on the selected leaves, fixed them in 70 % ethanol, and counted the number of aphids of each type (soldiers, adult reproductives, and young reproductives) and the number of predators under a binocular microscope. Then, we calculated the average percentage of soldiers in each colony relative to the total number of aphids in the colony. We used this estimated percentage of soldiers when designing our indoor experiment, described in the next section.

The defensive capability of soldiers against the specialist predator *A. ignipicta*: indoor experiment

We collected fifth-instar larvae of *A. ignipicta* from the wild aphid population at Mt. Jyonen on 26 July 2011. We introduced the *A. ignipicta* larvae individually into plastic petri dishes (3.5 cm in diameter) and fasted them for 24 h in an environmental chamber, which was maintained at 20 °C and a relative humidity of over 65 % on a 16-/8-h day/night cycle.

We collected 30 aphid colonies from the same population on 27 July 2011. The numbers of soldiers and first-instar reproductives varied among the colonies collected. To use as many as possible of the collected aphids in the experiment, we mixed aphid individuals of the 30 colonies immediately after bringing them back to our laboratory, and then later for the indoor experiment we randomly assigned soldiers and first-instar reproductives to the experimental arenas [i.e., petri dishes (3.5 cm in diameter)] of the following three treatments (Fig. 1). We introduced 50 and 47 first-instar reproductives into the arenas of treatments 1 and 3, respectively, and we introduced 47 first-instar reproductives and 3 soldiers into the arena of treatment 2. Thus, the total number of individual aphids (i.e., 50) was the same in treatments 1 and 3, and the total number of first-instar reproductives (i.e., 47) was the same in treatments 2 and 3. We performed 7, 11, and 7 replications of treatments 1, 2, and 3, respectively. We set the total number of aphids per arena and the number of soldiers per arena in treatment 2, so that the percentage of soldiers

would be similar to that observed in our field survey (i.e., $3/50 = 6\%$; see “Results”). We did not expect that the slight difference in the initial number of first-instar reproductives between treatments 1 and 3 would affect the consumption rate of the predators, because first-instar reproductives do not have defensive role. Therefore, we hypothesized that there would be no difference in either the number of preyed-upon first-instar reproductives or the survivorship of the predators between treatments 1 and 3. In the course of these experimental manipulations, we observed no confounding events such as a soldier attack on conspecifics.

After introducing the aphids into the treatment arenas, we next introduced an individual starved predator into each aphid-containing petri dish. We recorded the survival rates of first-instar reproductives and soldiers and the condition of the predator every 30 min over a 150-min period (i.e., five times). Then, at the end of the experiment, we recorded whether the predator had survived.

To understand how soldiers reduce predation on first-instar reproductives, we observed behavior of aphids and predators for a 5-min period soon after the introduction of the predator. We paid special attention to predators' behavior when they encountered soldiers or first-instar reproductives, soldiers' behavior when they encountered predators, and predators' behavior when they were attacked by soldiers.

Statistical analysis

To test how the presence of soldiers affected the survival rate of first-instar reproductives (arcsine transformed data), we conducted repeated-measures analysis of variance (ANOVA) with the presence of soldiers [i.e., treatment 1 ($N = 7$) plus treatment 3 ($N = 7$) vs. treatment 2 ($N = 11$)] as the independent variable and observation time (30, 60, 90, 120, 150 min) as the repeated variable. In this analysis, we pooled the data of treatments 1 and 3, because there were no differences in the survival rate of the first-instar reproductives

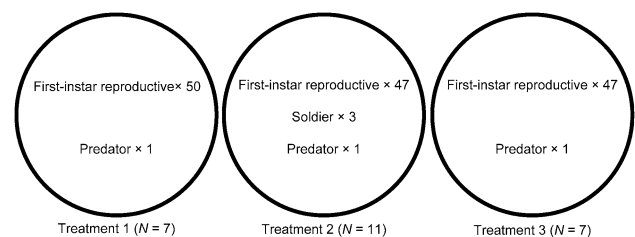


Fig. 1 The design of the predation experiment. Each petri dish was 3.5 cm in diameter. In treatments 1 and 2, the total number of aphids (first-instar reproductives plus soldiers) was equal. In treatments 2 and 3, the number of first-instar reproductives was equal. The proportion of soldiers in treatment 2 was chosen to conform to that in the wild population (5.8 %)

(repeated-measures ANOVA, $F_{2,84\ 25.68} = 0.3, P > 0.05$) or the survivorship of predators (i.e., no mortality of predators in any treatments) between treatments 1 and 3, as we had hypothesized a priori. For the repeated-measures ANOVA result, if Mauchly's test indicated that the sphericity assumption was violated, we show the Greenhouse–Geisser F and P values in the “Results” section. We also used Welch's t test to compare the survival rate of first-instar reproductives at each point of time.

We used the JMP v. 9.0.0 statistical package (SAS Institute) to analyze the data.

Results

The proportion of soldiers in the field

In our field survey, we found that the numbers of aphids, soldiers, and predators per colony were 370.9 ± 82.9 (mean \pm SE), 21.5 ± 7.6 , and 3.0 ± 1.3 , respectively. Thus, the average percentage of soldiers relative to total aphids in the wild population was 5.8 % (21.5 soldiers/370.9 aphids = 5.8 %). Therefore, in treatment 2 of the indoor experiment, we set the ratio of soldiers to total aphids to 6 % (i.e., 3 soldiers/50 aphids in treatment 2).

The maximum number of predators (*A. ignipicta* larvae) observed invading a colony was 10. In this survey, 97 % of encountered predators were identified as *A. ignipicta* larvae.

Survivorship and behavior of predators, soldiers, and first-instar reproductives

All predators survived in all treatments, and about half of the soldiers survived to the end of the experiment (number of surviving soldiers per petri dish: 1.45 ± 0.37 , mean \pm SE).

In every treatment, the survival rate of first-instar reproductives gradually decreased with time, but the pattern of decrease differed between the soldier-present and soldier-absent treatments ($F_{1,96, 45.02} = 20.36, P < 0.001$; Fig. 2). The decrease in the survival rate of first-instar reproductives was less pronounced in the treatment with soldiers than in the pooled treatments without soldiers. In the treatment with soldiers, the survival rate of first-instar reproductives was more than 50 % at 150 min, whereas it was less than 20 % in the pooled treatments without soldiers. These results indicate that soldiers can decrease predation on first-instar reproductives.

The difference in the survival rate of first-instar reproductives between treatments with and without soldiers was marginally significant at 30 min (t test, $t_{23} = 3.39, P = 0.04$; Fig. 2) and clearly significant at 60 min ($t_{23} = 5.58, P < 0.001$), 90 min ($t_{23} = 5.08, P < 0.001$), 120 min ($t_{23} = 5.17, P < 0.001$), and 150 min ($t_{23} = 5.37, P < 0.001$).

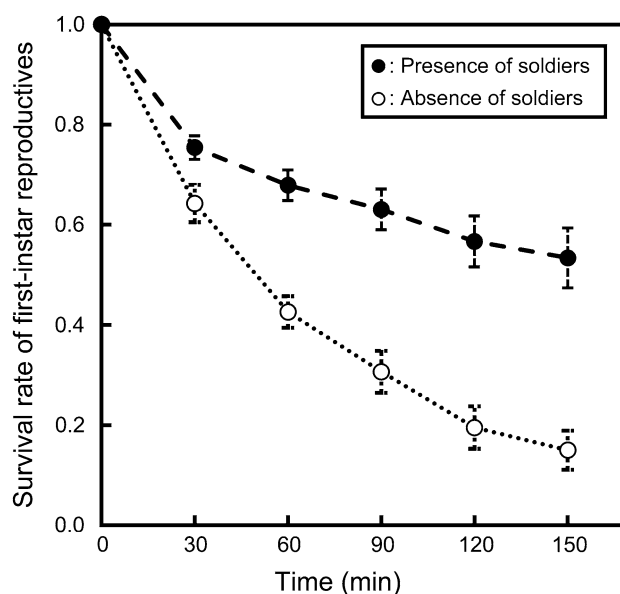


Fig. 2 The effect of the soldiers' defenses against predatory *A. ignipicta* larvae on the survival rate of first-instar reproductives. The presence of soldiers significantly increased the survival of first-instar reproductives (repeated measures ANOVA). Symbols and error bars indicate mean \pm SE

Hence, the predation rate was different between the treatments with and without soldiers soon after beginning of the experiment, and the difference became pronounced over time.

The introduced predator actively preyed on first-instar reproductives in all treatments. In treatment 2, we observed several encounters between the predator and soldiers during the 5-min behavioral observation period. Whenever a predator encountered a soldier, it did not try to prey on the soldier but instead endeavored to escape, often by spinning silken threads to make a nest.

Soldiers and first-instar reproductives actively walked around soon after being introduced into the petri dishes. When a first-instar reproductive encountered the predator, it did not change its behavior. In contrast, when a soldier encountered the predator, it waved its forelegs and attacked and sometimes grasped the predator's body. The predator used its mandibles to remove every soldier that succeeded in grasping its body.

Discussion

We showed that in the eusocial aphid *C. japonica* soldiers reduce predation without killing the predator (Fig. 2). This is empirical evidence of harassment of predators by soldiers as an anti-predator function.

To understand the maintenance of eusociality, it is essential to understand the ecological context in which soldiers are favored. The main role of sterile soldiers in eusocial aphids is

assumed to be to protect colony mates from predators (Queller and Strassmann, 1989; Stern and Foster, 1996; Pike and Foster, 2008). Many studies have anecdotally reported that soldiers kill other insects, including predators, and the kinds of predators killed by soldiers (for reviews see Stern and Foster, 1996; Pike and Foster, 2008). Aoki (1977), for example, observed that soldiers were able to kill unidentified syrphid predatory larvae placed on an aphid colony in each of seven experiments. However, these studies have not demonstrated quantitatively (with an adequate sample size) the effectiveness of the soldiers' protection of colony mates (but see Foster, 1990; Foster and Rhoden, 1998). Foster (1990) reported that about 40 % of individuals of the specialist predator *Anthocoris minki* were dead 24 h after they were introduced to a colony of the eusocial aphid *Pemphigus spyrothecae* inhabiting its primary host (i.e., in the gall). In contrast, wild aphid colonies on secondary hosts (i.e., free-living aphids) usually contain a low proportion of soldiers (0–20 %) (Shibao, 1999; Ijichi et al., 2005; Hattori et al., in review). Furthermore, specialist predators such as larvae of the predatory moth *A. ignipicta* can protect themselves from soldier attacks by making and hiding themselves in silk nests. In such a situation, it may not be easy for soldiers to kill an invading predator. In fact, in our experiments, all of the predators survived.

In our indoor experiment (total duration 150 min), we showed that soldiers of *C. japonica* reduced the rate of predation on colony mates without killing the predators (Fig. 2). Lowered predation on first-instar reproductives in the presence of the soldiers seemed to be caused by harassment of predators by soldiers. During the observation period (duration 5 min), we frequently observed attacks by soldiers on predators, and in each case the attacked predators escaped and they sometimes made a nest instead of foraging on first-instar reproductives. Some aphid species have other defensive strategies, such as the secretion of defensive substances such as wax and droplets from cornicles (Dixon, 1998; Uematsu et al., 2010). Such defensive substances are not likely to be a factor in this model system, because soldiers of *C. japonica* have only thin coats of a wax-like substance and they have no cornicles (Aoki and Kurosu, 2011).

The lowered predation in the presence of soldiers in our indoor experiment shows that soldiers can delay consumption activity by this predator. If the delay of predation allows colony members to reproduce, then the colony can persist for a longer time. Alternatively, if the delay of predation allows colony members to escape, the members may disperse to a safe site (a different leaf) without predators and establish a new colony. Through these hypothetical processes (not mutually exclusive), soldiers may be able to contribute to the fitness of their colony mates (thus increasing their inclusive fitness).

In general, it is not easy to understand natural phenomena by extrapolating results obtained in a highly artificial experiment. It is possible that the artificial environment of our experiment caused overestimation of the effect of the soldiers. Alternatively, the observed effect of the harassment by soldiers might be a rather conservative result, because our study was conducted in a spatially enclosed environment (i.e., small petri dishes). In nature, aphid colonies are open, so predators may escape and leave the colony after being subjected to harassment by soldiers. Therefore, we believe that it is reasonable to infer from our results that harassment by soldiers can effectively contribute to colony fitness in nature. In the future, field studies are needed to confirm whether harassment by soldiers has a similar effect in natural aphid colonies.

The use of an alarm pheromone by aphids to induce escape behavior in the face of predation risk would allow the other aphids to promptly disperse while the soldiers buy time. In fact, many aphid species release an alarm pheromone in the face of predation risk to induce nearby aphid individuals to disperse (Nault et al., 1973; Francis et al., 2005). Although no alarm pheromone has ever been reported in eusocial aphids, the non-eusocial aphid *C. lanigera*, a close relative of *C. japonica*, does produce an alarm pheromone (Arakaki, 1989). In *C. lanigera*, the alarm pheromone induces first-instar nymphs to exhibit attack behavior, and it induces escape behavior in their colony mates (Arakaki, 1989). Similarly, in *C. japonica*, reproductive individuals encountering predators secrete droplets from their cornicles (M. Hattori, pers. obs.). To better understand the adaptive significance of a predation delay by soldiers, the function of this possible "alarm pheromone" in *C. japonica* needs to be clarified. We expect that soldier harassment of predators will be found in other eusocial aphids, and that such harassment may prove to be effective even against specialized or large predators. The results of this study highlight that we should consider not only the benefit of killing predators, but also of harassment against predators when we perform a cost-benefit analysis of soldier production.

In *C. japonica*, the size of the soldiers' weapons (horn and foreleg size) changes plastically in response to seasonal changes in predation risk: reproductive individuals produce soldiers with larger weapons in midsummer, when predators are abundant (Hattori et al., in review). Soldiers with large weapons may be able to grasp predators more easily with their longer forelegs and to effectively injure predators with their longer horns. Therefore, in midsummer, soldiers with large weapons may be able to buy more time for their colony mates to reproduce or escape than soldiers with small weapons. To understand the defensive strategy of eusocial aphids in more detail, we need to know how variation in the size of their weapons influences the ability of the soldiers to buy time for their colony mates.

Most studies of eusocial aphids have focused on the biology of specialized sociality rather than on predator–prey interactions. Larvae of *A. ignipicta* and of *T. hamada*, which are the main predators of *C. japonica* (Moriuti, 1982; Banno, 1990a, b; Morimoto and Shibao, 1993), have specialized traits to protect themselves from soldiers: *A. ignipicta* larvae can use their mandibles to remove any soldiers that succeed in grasping their body (see “Results”), and both *A. ignipicta* (M. Hattori, pers. obs.) and *T. hamada* larvae can make a nest of silken threads to escape from soldier attacks (Pierce, 1995). The evolutionary interplay of specialist predators and eusocial aphids should be another focus of future studies of eusocial aphids.

Acknowledgments This work was supported by grants from the Japan Society for the Promotion of Science to M.H. (no. 216649), O.K. (no. 2277001100), and T.I. (no. 22570015). We thank Ms. Susan Duhon (Rujuke Editorial Service) for her comprehensive English editing of this manuscript.

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