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# Combined use of two defensive traits in pupae of *Scymnus posticalis* ladybirds

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## Abstract

Coccinellid pupae have various defensive traits, such as keeping their final-instar larval exuviae and secreting droplets containing defensive chemicals at the tips of glandular hairs. Although each of these traits has been described separately in different species, it is unclear how each defensive trait functions when more than one trait coexists. We found that pupae of the ladybird *Scymnus posticalis* have two different types of traits; they were partially covered with their own final-instar larval exuviae, with thick wax structures, and had many glandular hairs secreting transparent droplets on the exposed parts of the body without waxy exuviae (head and medial parts of thoracic and abdominal dorsum). In this study, the defensive effects of these two traits were evaluated against two ant species, *Tetramorium tsushimae* and *Lasius japonicus*, which are potential predators. When encountering a pupa, neither ant species were able to make frequent physical contact with the exposed parts of the pupa because of the presence of the waxy exuviae. When touching the exposed body part that was covered with droplets, both ant species immediately stepped backward and then swept their antennae. The droplets suppressed ant feeding when mixed with sucrose solution, suggesting that the droplets contain repellent chemicals. These results indicate that the two traits of *S. posticalis* pupae (the larval waxy exuviae and the droplets) effectively play defensive roles against ants, functioning as a physical obstruction and a chemical repellent, respectively.

KEYWORDS
coccinellidae, glandular hairs, pupation site, wax structure

## 1 | INTRODUCTION

The immobile pupal stage of a holometabolous insect is vulnerable to attack by natural enemies. In place of escape, the pupae of coccinellid beetles have a variety of defensive traits to avoid predation and parasitism. For example, coccinellid pupae of the subfamilies Chilocorinae, Scymninae and Ortaliinae maintain their final-instar larval exuviae; the exuviae split lengthwise on the dorsal side during pupation so that they cover most parts of the pupal bodies (Nedvěd & Honěk, 2012; Richards, 1980). In Epilachninae, only the posterior part of the abdomen (fourth, fifth and sixth abdominal segments) of pupae is covered with the larval exuviae (Nedvěd & Honěk, 2012; Richards, 1980). Nedvěd and Honěk (2012) hypothesised that the exuviae physically protect the pupae from predators, especially when the larvae are spiny (Chilocorinae, Epilachninae) or covered with waxy structures (Scymninae). A recent study clarified that the spiny structure in the larval stage functions as a defence against predatory ladybirds (Hautier, San Martin, Jansen, Branquart, & Grégoire, 2017), which would support the hypothesis. Additionally, in some species of Epilachninae, Microweisinae and Scymninae, transparent droplets are secreted from the tips of glandular hairs distributed on their pupal body surfaces (Attygalle, McCormick, Blankespoor, Eisner, & Meinwald, 1993; Attygalle, Smedley, Eisner, & Meinwald, 1996; Deyrup et al., 2014; Lu, Souphanya, & Montgomery, 2002; Montgomery et al., 2002; Smedley et al., 2002). These droplets often contain harmful chemicals such as azamacrolides, polyazamacrolides and sesquiterpenoids (Attygalle et al., 1993, 1996; Deyrup et al., 2014; Schröder, Farmer, Attygalle et al., 1998, Schröder, Farmer, Smedley et al., 1998, Schröder, Smedley, Gibbons et al., 1998, Schröder et al., 2000; Smedley et al., 2002), which likely act as a defence against enemies. Predatory ladybirds and ants were reportedly observed to back away and clean themselves after touching the droplets (Attygalle et al., 1993; Deyrup et al., 2014; Schröder, Smedley, Gibbons et al., 1998; Smedley et al., 2002). Furthermore, a characteristic structure known as the “gin trap”, which lies between the abdominal tergites of a pupa, is found to act as jaws in the tribe Coccinellini (Escalona et al., 2017), suggesting that it contributes to defence (Eisner & Eisner, 1992). Although each trait of coccinellid pupae has been described separately to function as protection from natural enemies, pupae do not necessarily depend on only one defensive trait. In fact, it is known that a number of coccinellid pupae have plural defensive traits (Richards, 1980). To understand the antipredation strategies of coccinellid pupae, it is important to delineate how each defensive trait of a pupa functions to avoid predation when it has more than one defensive trait.

In Scymninae, including the genus *Scymnus*, pupae of some species are covered with larval exuviae, with wax structures that were secreted during their larval stages (Nedvěd & Honěk, 2012; Richards, 1980). The wax structures prevent predation from syrphid larvae (Agarwala & Yasuda, 2001) and attacks by ants not only physically (Völkl & Vohland, 1996), but also chemically (Hayashi, Nomura, & Nakamuta, 2016; Schwartzberg, Haynes, Johnson, & Brown, 2010), during their larval stage. Larvae of some *Scymnus* species are thus able to prey on aphids that are tended by ants without being attacked by the ants (Kaneko, 2002, 2007; Schwartzberg et al., 2010). The pupae also may protect themselves by covering themselves with waxy exuviae. In contrast, the pupae of two *Scymnus* species—*S. sinuanodulus* and *S. ningshanensis*—are naked and have glandular hairs, with droplets at the tips, over the entire body surface (Lu et al., 2002; Montgomery et al., 2002). However, how such pupal traits of *Scymnus* act as defence remains poorly understood.

In this study, we investigate the defensive tactics of pupae of the ladybird *Scymnus posticalis* (Coleoptera: Coccinellidae). Larvae of *S. posticalis* typically live near and prey on colonies of aphids that are attended by ants (Kaneko, 2002, 2007). The larvae cover their body surfaces with secreted waxes, which provide physical and chemical protection from ants and other predators (Agarwala & Yasuda, 2001; Hayashi et al., 2016). We found that the ladybird pupae are partially covered with the larval waxy exuviae. Furthermore, the pupae were found to have many droplets at the tips of glandular hairs on the exposed body surfaces without waxy exuviae. The droplets, as with the waxy exuviae, may contribute to protection from enemies. To examine this hypothesis, we first investigated pupation sites of the ladybirds in the field to estimate which animals are potential predators of the pupae. Second, we described the two described traits of the ladybird pupae in detail using an electron microscope. Third, we presented the ladybird pupae to two ant species that are potential predators of the pupae, to investigate how each pupal trait functions as defence. We further tested whether substances included in the droplets were aversive and unpalatable for ants.

## 2 | MATERIALS AND METHODS

### 2.1 | Study insects

Adult *S. posticalis* were collected from the campus of Chiba University, Matsudo, Japan. The ladybirds were maintained in plastic cases (100 mm diameter, 40 mm depth) and fed cowpea aphids (*Aphis craccivora*) every 2 days. The aphid colony was established from an adult female collected at Chiba University and was maintained on broad bean plants (*Vicia faba*) grown from seed in soil in plastic pots (90 mm diameter, 75 mm depth). Eggs laid by the ladybirds were transferred into additional plastic cases, and the larvae were similarly fed on aphids. Pupae were removed for the experiments.

Two colonies of *Tetramorium tsushimae* (Hymenoptera: Formicidae), including their queens, were collected in the field at the Center for Bioresource Field Science (CBFS), Kyoto Institute of Technology, Kyoto, Japan. The colonies were maintained in plastic cases (510 × 700 × 70 mm) in an insectary and were given water, sucrose solution and chopped house crickets (*Acheta domestica*) every 2 days. Foraging worker ants of *Lasius japonicus* from two colonies were also collected from the field. Behavioural bioassays (described below) were conducted within one hour of separation from natural or maintained colonies in both ant species. The insects and plants were maintained in climate-controlled rooms (24 ± 3°C, 16:8 light/dark ratio).

### 2.2 | Pupation site and pupal defensive traits of *Scymnus posticalis*

To investigate the pupation sites of *S. posticalis* in the field, we monitored the ladybird larvae at the campus of CBFS, in May 2017. At the campus, Thunberg’s spiraea plants (*Spiraea thunbergii*) have been planted, and their shoots are sometimes colonised by the aphid *Aphis spiraecola*. Ladybird larvae were observed in the aphid colonies and preying upon them. We labelled 34 shoots of the plants that were colonised by aphids and also had the final-instar *S. posticalis* larvae. The presence or absence of ladybird larvae on the shoots was checked every day until the larvae pupated, or left. Fallen leaves and dead branches below the plants were also checked to confirm the presence or absence of *S. posticalis* pupae.

We observed the external traits of *S. posticalis* pupae through a microscope and scanning electron microscope at 20 kV (SM-200, Topcon Co., Tokyo, Japan).

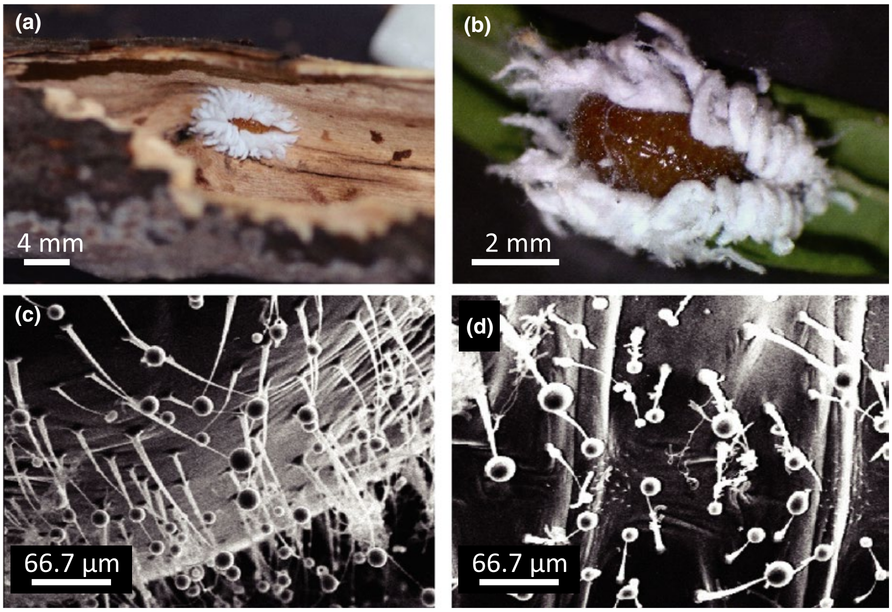

**FIGURE 1** Pupae of *Scymnus posticalis* with two kinds of defensive traits. (a) Pupa inside dead branch. We partly broke the branch to take the picture. (b) The pupa partially covered with wax structures. (c) Droplets at the tips of glandular hairs on head parts. (d) Droplets at the tips of glandular hairs on the medial part of the abdominal dorsum. [Colour figure can be viewed at wileyonlinelibrary.com]

## 2.3 | Behavioural bioassays

### 2.3.1 | Contact-blocking effect of waxes and repellent effect of droplets on ants

An untreated pupa of *S. posticalis* was presented to a worker of the two ant species, *T. tsushimae* or *L. japonicus*, in a Petri dish (3.5 cm diameter). We counted the frequency of antennal contacts with the wax structures and exposed body for 2 min. The observations started at the first contact. We also simultaneously recorded the ant's response to each of the body parts on first contact. When the ant made contact with only one of either the wax structures or the exposed body within the 2 min, we continued to observe until the ant touched the other body part. We also presented pupae without droplets to an ant in the same manner. We blotted the droplets with Kimwipes (Nippon Paper Crecia Co., Ltd.) and removed waxes around the exposed body under a microscope so that contact with the exposed body was easier. We recorded the ant's responses to the exposed body without droplets on first contact. Fifteen pupae for each treatment were presented to two species of ants, and all study insects were used only once. We categorised ants' responses as neutral (ignoring or antennating), repellent (quickly stepping backward or cleaning antennae) or attack reactions (mandible-opening, biting or abdominal bending).

To analyse whether the frequency of contacts with each pupal body part was different depending on the ant species, the data were analysed using a generalised linear model (GLM) with a logit link function and a binomial error distribution. A model was constructed using the frequency of contact with each body part (i.e., frequency of contact with wax structures versus exposed body) as a response variable and the two ant species as an explanatory variable. The effect of ant species was tested using a likelihood ratio test. Statistical analyses were conducted using R v.3.4.2 software (R Core Team, 2017).

Furthermore, to test whether the presence of the droplets affected the ants' response towards pupae, we analysed ant's responses when they first contacted with the exposed pupal bodies. The ants' responses were compared between when the droplets were present and when they were not using Fisher's exact tests and residual analyses for both ant species.

### 2.3.2 | Deterrent effect of droplets on ant feeding activity

To test whether the pupal droplets deterred ants from feeding, we gave ants a sugar solution with or without droplets. We infiltrated droplets of one pupa equivalent into a filter paper (2 × 2 mm), and then applied 1.5 µl of 20% sucrose solution onto it and onto another untreated filter paper as a control. We simultaneously presented both filter papers, 3 cm apart, to 10 worker ants in a Petri dish (5 cm diameter) and counted the number of ants feeding from each sample every 30 s for 5 min. We starved the colonies of *T. tsushimae* for 3 days before the experiment to increase their sensitivity to sucrose, but we used worker ants of *L. japonicus* immediately after collection from the field because their sensitivity was already high. We repeated the procedure 10 times for each ant species, using new ant individuals.

The probability of ants feeding from a droplet-treated solution, and the upper 95% confidence interval were estimated by a generalised liner mixed model (GLMM) with a logit link function and a binomial distribution. A GLMM model was constructed using the number of ants that fed from each sample (i.e., the number of ants at the droplet-treated solution versus the control solution) as a response variable, and the time from the beginning of the experiment as an explanatory variable with a random factor as each five minutes after the initiation of the procedure. We used the "glmer" function in the program package "lme4" (Bates, Maechler, Bolker, & Walker, 2015) for GLMM analysis, and the "confint" function in the program package "drc" (Ritz, Baty, Streibig, & Gerhard, 2015) for estimating the upper 95% confidence interval (CI) in R software.

## 3 | RESULTS

### 3.1 | Pupation site of *Scymnus posticalis*

In all the 34 monitored shoots, final-instar ladybird larvae left the shoots even though aphid individuals remained on 18 of them. The ladybird pupae were not found on, nor adjacent to, the monitored shoots. In total, 18 final-instar ladybird larvae were observed to descend from stems of the aphid-colonised plants to the ground, and another 14 pupae were observed under deposited materials on the ground near the foot of the plants. All pupae were observed in narrow spaces such as the inner parts of wrinkled fallen leaves or inside dead branches (Figure 1a).

Although we could not observe direct contact between the ladybird pupae and any natural enemies, we observed that some species of ants, including *T. tsushimae*, were foraging around the foot of the plants that the ladybirds developed on. Hence, ants were considered to be potential predators of the ladybird pupae.

### 3.2 | Pupal defensive traits of *Scymnus posticalis*

The final-instar larval exuviae of *S. posticalis* split along the dorsal midline during pupation. Thus, the head and medial parts of the thoracic and abdominal dorsum were exposed, and the other parts of the body were covered with wax structures (Figure 1b). Transparent droplets at the tips of glandular hairs were always present on the exposed parts irrespective of disturbance (Figure 1c,d). The droplets are likely to be produced only during pupation because they were not reproduced after removal. Almost all hairs had droplets, the size of which varied from approximately 10–30 μm in diameter.

### 3.3 | Contact-blocking effect of waxes and repellent effect of droplets on ants

Average rates of contact with pupae by the ants were 56.4 ± 4.3% and 73.3 ± 7.0% with wax structures and 43.6 ± 4.3% and 26.3 ± 7.0% with exposed bodies, by *L. japonicus* and *T. tsushimae*, respectively (Figure 2). The ant species slightly and marginally significantly affected the rate of contact (likelihood ratio test, $\chi^2 = 3.61$, df = 1, $p = .0576$). When the ants touched the pupal wax, neutral responses were shown by all 15 workers of *T. tsushimae* (nine ignoring, six antennating) and by 11 of 15 workers of *L. japonicus* (four ignoring, seven antennating). The other four *L. japonicus* workers showed attack (three abdominal bending) or repellent (one quickly stepping backward and cleaning antennae) responses to the pupal wax structures.

In both ant species, behaviours differed significantly between pupal exposed bodies with and without droplets (Figure 3; Fisher's exact test: $p < .01$). In *L. japonicus*, theresponses of all 15 workers to exposed bodies with droplets were repellent (three quickly stepping backward, two cleaning their antennae and 10 doing both). Six workers presented with pupae without droplets showed neutral behaviour (five ignoring, one antennating), seven showed attack behaviour (four mandible-opening, two biting, one abdominal bending) and two were repelled (quickly stepping backward). The rates of each behaviour were significantly different between pupae with and without droplets (residual analysis: $p < .01$). In *T. tsushimae*, the workers showed no attack responses in either the presence or absence of droplets. The rates of neutral and repellent behaviours differed significantly between pupae with and without droplets (Fisher's exact test: $p < .01$). Two workers showed neutral behaviour (ignoring), and 13 were repelled by pupae with droplets (all 13 quickly stepping backward, and eight then cleaning their antennae). In contrast, 14 workers showed neutral behaviour (13 ignoring, one antennating), and one was repelled by pupae without droplets (quickly stepping backward).

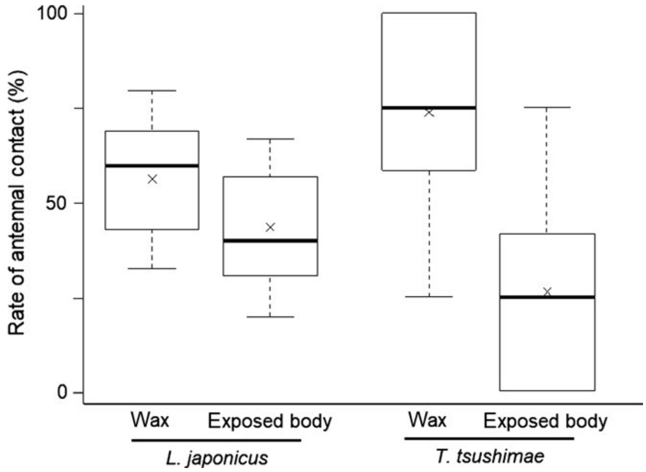

**FIGURE 2** Rate of ant antennal contact with wax structures and exposed pupal body of *Scymnus posticalis*. The box plots show medians, quartiles and ranges and "×" show averages

### 3.4 | Deterrent effect of droplets on ant feeding activity

In both ant species, fewer ants aggregated at the filter paper treated with pupal droplets than at the control paper and the upper 95% CI of the rate that ants fed from treated paper partially fell below 50%. In *L. japonicus*, the number of ants fed from the control paper decreased in the later experimental period (Figure 4a). Thus, the rate of ants fed from the treated paper relatively increased, which was reflected by an increase in the CI over time (Figure 4c). However, in *T. tsushimae*, the number of ants fed from the treated paper decreased over time (Figure 4b), which was reflected by a decrease in the estimated probability and the CI over time (Figure 4d).

## 4 | DISCUSSION

The pupae were partially covered with final-instar larval waxy exuviae and secreted transparent droplets from the tips of glandular hairs on the exposed head and medial parts of the thoracic and abdominal dorsum (Figure 1). On encountering the pupae, both ant species often made contact with the waxy exuviae and frequently had neutral responses and short interactions with them, suggesting that the exuviae decrease the frequency of ant contact with

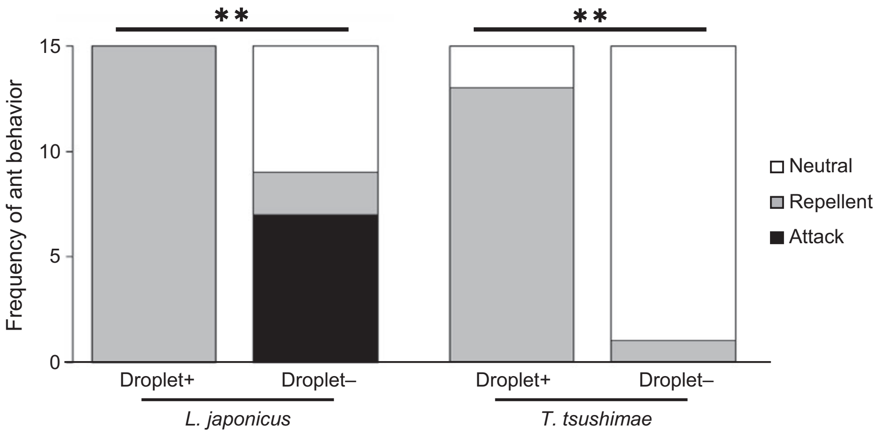

**FIGURE 3** Frequency of ant responses to pupal body of *Scymnus posticalis* with ("droplet+") and without ("droplet−") droplets at the tips of glandular hairs. $^{**}p < .01$ by Fisher's exact test

exposed pupal bodies (Figure 2). The droplets repelled the ants when they made contact with the exposed pupal bodies (Figure 3), and ants tended to avoid feeding on the sucrose solution laced with the droplets (Figure 4a,b). The estimated probability of the rate that ants fed from the treated paper fell much below 50% in both ant species (Figure 4c,d). Although the upper 95% CI of *T. tsushimae* surpassed 50% at the early experimental period, this decreased with time, probably because the ants stopped feeding from the treated paper earlier than from control paper. The CI of *L. japonicus* surpassed 50% at the later experimental period because the ants left the control paper in the later experimental period due to exhausting the sucrose solution applied to it. These suggest that the droplets provide a chemical defence against ants. We conclude that pupae of *S. posticalis* protect themselves from ants physically and chemically using these two defensive traits. To our knowledge, this is the first study showing the simultaneous actions of two kinds of defensive traits in coccinellid pupae.

We found that final-instar larvae of *S. posticalis* did not pupate near the colonies of ant-tended aphids, but in the inner parts of deposited materials on the ground near the foot of the trees colonised by the aphids. As many ladybird species usually pupate on the vegetation where the larvae developed, the pupae are often exposed to cannibalism or intraguild predation (Nedvěd & Honěk, 2012). On the other hand, a few species of aphidophagous coccinellids are known to pupate apart from plants colonised by their aphid prey, resulting in a decrease in the risk of predation by intraguild predators (Lucas, Coderre, & Brodeur, 2000; Osawa, 1992). The pupation site choice of *S. posticalis* may be beneficial for reducing the chance of encounter by foraging enemies at aphid colonies, such as intraguild predators and ants that are travelling between aphid colonies and their nests.

The wax structures of *Scymnus* spp. larvae serve as both a physical obstruction (Völkl & Vohland, 1996) and chemical defence to prevent aphid-tending ants from recognising them as aphid enemies (Hayashi et al., 2016; Schwartzberg et al., 2010). The waxy exuviae of *S. posticalis* pupae similarly may inhibit recognition from foraging ants in the same way; almost no ants displayed attack responses when they touched the wax structures in our bioassay. Although the waxy exuviae do not completely cover the pupal body, our results showed that they reduced the frequency of ant contact with the exposed pupal body. The rate of contact with the pupal body was slightly, but not significantly, lower in *T. tsushimae* than in *L. japonicus*. The difference in contact rates is likely to correspond with the body size of the ants as we often observed that the antennae of the larger *L. japonicus* (body length of workers = 3.5 mm) could reach over the wax structures to the exposed dorsal parts of the pupae from the side, whereas those of the smaller *T. tsushimae* (body length of workers = 2.5 mm) could usually only touch the head parts of the pupae. Thus, the physical defensive function of the wax structures of the pupae is likely to be more effective against smaller predators such as *T. tsushimae*.

In contrast, the droplets of *S. posticalis* are likely to be more important for defence against the larger ants *L. japonicus*, considering their higher contact frequency with the exposed body. The droplets may chemically prevent ants from preying on the pupae because their presence deterred ant feeding. Several studies have reported repellent effects of coccinellid pupal droplets against ants, including in *Epilachna varivesti* (Attygalle et al., 1993), *Subcoccinella vigintiquatuorpunctata* (Smedley et al., 2002), *Delphastus catalina* (Deyrup et al., 2014) and against a predaceous coccinellid beetle and a cockroach in *E. varivesti* (Rossini, González, Farmer, Meinwald, & Eisner, 2000). The chemicals present in pupal droplets are azamacrolides in *E. varivestis* (Attygalle et al., 1993), tocopheryl acetates and ployazamacrolides in *E. borealis* (Attygalle et al., 1996; Schröder, Farmer, Smedley, Eisner, & Meinwald, 1998; Schröder, Smedley, Gibbons et al., 1998; Schröder et al., 2000), ployazamacrolides in *S. vigintiquatuorpunctata* (Gronquist & Meinwald, 2001; Schröder, Farmer, Attygalle et al., 1998) and tricyclic pyrones and sesquiterpenoids in *D. catalinae* (Deyrup et al., 2011). As these species belong to the subfamilies Epilachninae and Microweiseinae, there is a possibility that different groups of chemicals are contained in the pupal droplets of *S. posticalis* because they belong to a different subfamily, Coccinellinae.

The two traits of *S. posticalis* pupae, wax structures and droplets, functioned as defences against ants. The use of two defensive traits may allow pupae of *S. posticalis* to avoid attacks by predators. In addition, the combination of traits appears not only to improve defence but also to save energetic costs, as the pupae have to secrete the droplets only on the exposed parts. On the other hand, the two *Scymnus* species, *S. sinuanodulus* and *S. ningshanensis*, completely shed their larval exuviae during pupation, and their whole external

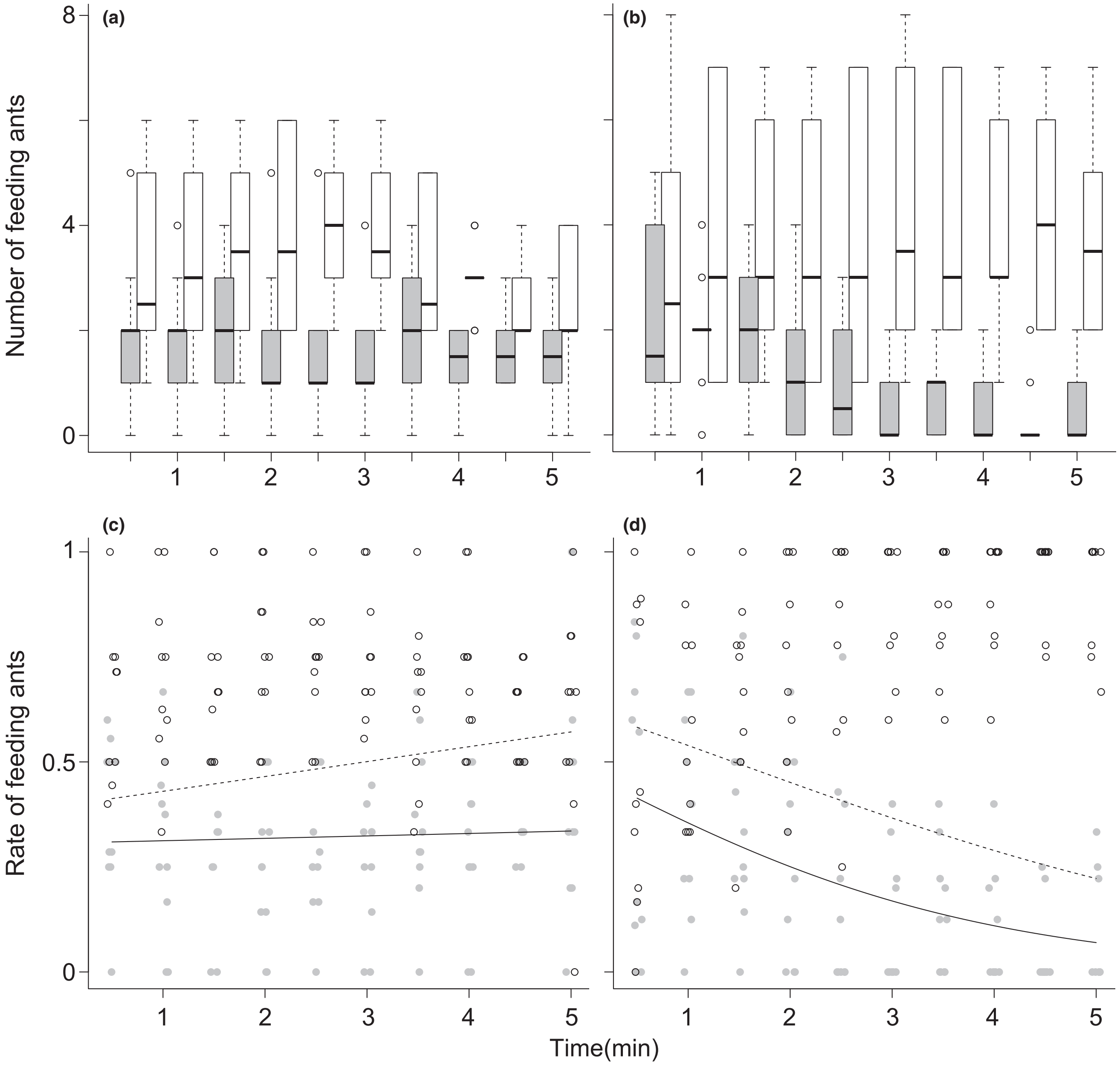

**FIGURE 4** Repellent effect of pupal droplets on foraging by ant workers. The box plots show the number of ants feeding from the droplet-treated paper (grey boxes) and control paper (white boxes) in *Lasius japonicus* (a) and *Tetramorium tsushimae* (b). The scatter plots show the rate of ants feeding from the droplet-treated paper (grey dots) and control paper (white dots) in *L. japonicus* (c) and *T. tsushimae* (d). The solid lines and the dashed lines show the estimated probability of the rate that ants feed on treated paper, and its upper 95% confidence interval, respectively

surface is covered with droplets (Lu et al., 2002; Montgomery et al., 2002). Covering the waxy exuviae may impose some kind of negative fitness in the two *Scymnus* species. Pupal defensive strategies, including defensive traits, appear to vary even within the same genus in *Scymnus*. The cause of the difference is an issue for future research.

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