## **Current Biology**

## **Lycaenid Caterpillar Secretions Manipulate Attendant Ant Behavior**

#### **Highlights**

- Lycaenid butterfly secretions modify the locomotory activity of attending ants
- Lycaenid butterfly secretions modify the aggressive behavior of attending ants
- Lycaenid butterfly secretions alter the brain dopamine levels of attending ants
- The secretions are manipulative drugs that can enforce the ants' cooperative behavior

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#### In Brief

Nutritious secretions are among the most common mediators of associations between ants and their apparently mutualistic symbionts. Hojo et al. find that exocrine secretions from a myrmecophilous lycaenid butterfly are not simply nutritious rewards, but also act as manipulative drugs enforcing ants' cooperative behavior via dopaminergic regulation.





# Lycaenid Caterpillar Secretions Manipulate Attendant Ant Behavior

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#### **SUMMARY**

Mutualistic interactions typically involve the exchange of different commodities between species [1]. Nutritious secretions are produced by a number of insects and plants in exchange for services such as defense [2, 3]. These rewards are valuable metabolically and can be used to reinforce the behavior of symbiotic partners that can learn and remember them effectively [4, 5]. We show here novel effects of insect exocrine secretions produced by caterpillars in modulating the behavior of attendant ants in the food-for-defense interaction between lycaenid butterflies and ants [6]. Reward secretions from the dorsal nectary organ (DNO) of Narathura japonica caterpillars function to reduce the locomotory activities of their attendant ants, Pristomyrmex punctatus workers. Moreover, workers that feed from caterpillar secretions are significantly more likely to show aggressive responses to eversion of the tentacle organs of the caterpillars. Analysis of the neurogenic amines in the brains of workers that consumed caterpillar secretions showed a significant decrease in levels of dopamine compared with controls. Experimental treatments in which reserpine, a known inhibitor of dopamine in *Drosophila*, was fed to workers similarly reduced their locomotory activity. We conclude that DNO secretions of lycaenid caterpillars can manipulate attendant ant behavior by altering dopaminergic regulation and increasing partner fidelity. Unless manipulated ants also receive a net nutritional benefit from DNO secretions, this suggests that similar reward-for-defense interactions that have been traditionally considered to be mutualisms may in fact be parasitic in nature.

#### **RESULTS AND DISCUSSION**

Some ant-associated insects such as myrmecophilous lycaenid butterflies or beetles have specialized exocrine glands that produce secretions that can be modified or modulated by their host ants [6, 7]. Like other ant-associated lycaenid butterflies,

Narathura japonica caterpillars have a dorsal nectary organ (DNO) on the seventh abdominal segment, flanked by tentacle organs (TOs) on the eighth abdominal segment [8]. The DNO secretes nutritious droplets containing sugars and amino acids [6, 8, 9], and the TOs are thought to secrete volatile substances that both attract and alert ants when the larva is alarmed or the DNO is depleted [6, 10]. In contrast with parasitic lycaenid butterflies, whose chemical mimicry of cuticular hydrocarbons is sufficient to modify host worker behavior [11-13], DNO secretions or cuticular hydocarbons on their own do not elicit myrmecophily, but together they act synergistically to promote ant fidelity in mutualistic N. japonica [8]. Attendant ants spend little time soliciting DNO secretions; typically, they are relatively immobile and simply stand around the caterpillars (Figure 1) [8]. Once the ants have foraged on DNO secretions, they are more likely to tend caterpillars again, even those whose DNO has been experimentally occluded [8]. These observations suggest that the DNO secretions may be more than simply nutritious reward, but may also contain substances that manipulate attendant ant behavior.

To explore the effect of DNO secretions on ant behavior, we created experimental treatments for potential ant associates: (1) "experienced" ants could freely access both caterpillars and their DNO secretions, (2) "inexperienced" ants had no access to caterpillars, and (3) "unrewarded by DNO secretions" ants could access only caterpillars that had previously had their DNO experimentally occluded. In all treatments, cotton soaked in 10% sugar solution (food for the ants), young leaves of Quercus glauca (food for the caterpillars), and water were supplied in the foraging arena. After 3 days, we compared locomotory activity (number of times the ants walked across the center line of a Petri dish) among the three types of workers (for more details, see the Experimental Procedures). The experienced Pristomyrmex punctatus workers showed significantly less locomotory activity than the inexperienced and unrewarded workers (Figure 2A and Movie S1; n = 12, generalized linear mixed model [GLMM]: df = 2,  $\chi^2$  = 520.8, p < 0.00001; Tukey's honest significant difference [HSD] test, p < 0.01). These results suggest that provisioning by DNO secretions contributes to the maintenance of a "standing guard" of ants for the caterpillars.

During our behavioral observations, the caterpillars often everted their TOs. We found that experienced workers responded aggressively toward everted TOs after 3 and 6 days of exposure (Figure 2B; n = 12, GLMM: df = 1,  $\chi^2$  = 5.9647, p = 0.01459). However, inexperienced and unrewarded workers



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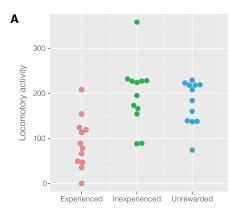
Figure 1. Attendant Workers of Pristomyrmex punctatus Standing on and around Narathura japonica Caterpillars

The DNO is located on the seventh abdominal segment of the caterpillar, and the retractable TOs (not visible) flank it on the eigth abdominal segment.

did not respond aggressively and simply ignored the eversions of the TOs (Figure 2B; n = 12 for each treatment, GLMM: inexperienced, df = 1,  $\chi^2$  = 0.3401, p = 0.5598; unrewarded, df = 1,  $\chi^2$  = 2.4077, p = 0.1207). Thus, the aggressive responses of P. punctatus workers toward TOs does not appear to be an innate behavior, but one that is induced and/or greatly enhanced following DNO provisioning. Lycaenid caterpillars are more likely to evert their TOs when they are attacked by predators, and aggressive ants can act to defend against these predators [6, 10]. Thus, the DNO secretions can help to induce more efficient ant defense for the caterpillars.

Since biogenic amines that function as neurotransmitters, neuromodulators, and/or neurohormones are known to mediate the plasticity of various behaviors in insects [14], we reasoned that DNO secretions, which also appear to evoke behavioral plasticity (Figure 2), may affect levels of biogenic amines in workers of P. punctatus. Using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS), we measured the brain biogenic amine levels of P. punctatus workers in the different treatments. Among four biogenic amines measured (Figure 3A; serotonin, dopamine, octopamine, and tyramine), dopamine levels were significantly decreased in the experienced ants (Figure 3B; n = 15, LMM: df = 2,  $\chi^2$  = 9.1435, p = 0.01034; Tukey's HSD test: p < 0.05), whereas levels of other biogenic amines did not differ statistically among treatments (Figures 3C and 3D; serotonin, df = 2,  $\chi^2$  = 2.8789, p = 0.2371; tyramine, df = 2,  $\chi^2$  = 1.0857, p = 0.5811; octopamine, df = 2,  $\chi^2 = 0.9707$ , p = 0.6155).

Because dopamine signaling is involved in both locomotory activity [15] and aggression [16] in Drosophila melanogaster, it seems likely that inhibition of dopamine signaling directly affects the observed behavioral plasticity in attendant ants. To explore the role of dopamine in the ants' behavior, we treated workers with reserpine and observed their locomotory activity. In D. melanogaster, reserpine depletes synaptic dopamine signaling by inhibiting the vesicular monoamine transporter (VMAT) [17] and is known to reduce dopamine levels, but not se-



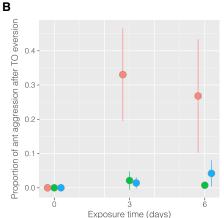


Figure 2. Exocrine Secretions from the DNO of Narathura japonica **Caterpillars Modify Their Locomotory Activity and Aggressiveness** (A) Effect of DNO secretions from the caterpillars on the locomotory activity of associated ants.

(B) Effect of DNO secretions from the caterpillars on ant responses toward the eversion of TOs. The mean ± 95% confidence intervals are shown. Red, green, and blue circles indicate experienced, inexperienced, and unrewarded treatments, respectively (see the main text for further descriptions). See also Movie S1.

rotonin levels, in the brain [18]. Reserpine treatments similarly affected the ants' locomotory activity (Figure 4; n = 9, GLMM: df = 2,  $\chi^2$  = 846.68, p < 0.00001): ants fed with 100  $\mu$ M reserpine significantly decreased locomotory activity of P. punctatus workers compared with those fed with 0 and 10 μM reserpine (Figure 4 and Movie S1; Tukey's HSD test: p = 0.00075 and p = 0.01475, respectively). However, subsequent analysis of the brains of ants treated with reserpine showed that in contrast to what has been reported in D. melanogaster, reserpine significantly increased levels of both dopamine and serotonin in the brains of P. punctatus (Figure S1; t test: dopamine, n = 4-6, t = -2.7299, df = 8, p = 0.02585; serotonin, n = 6, t = -3.4075, df = 10, p = 0.006687). Thus, manipulation of the dopaminergic pathway in P. punctatus, first by the action of lycaenid DNO secretions and second by VMAT inhibition following treatment with reserpine, acts to decrease ant locomotory behavior, albeit in different ways.

Overall, these results support the hypothesis that DNO secretions from caterpillars manipulate the behavior of their attendant ants via dopaminergic regulation. Although research has shown

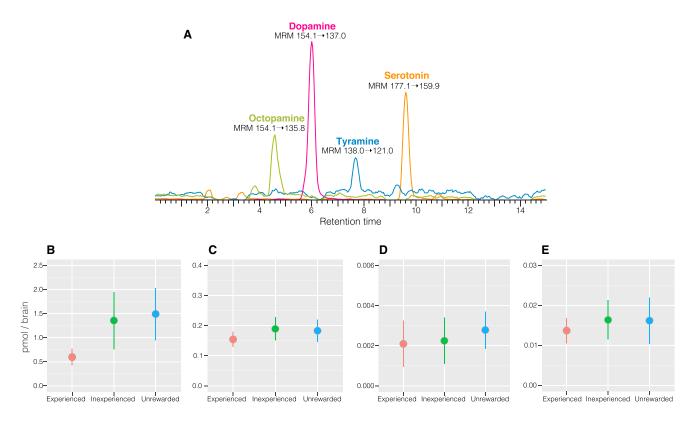


Figure 3. Exocrine Secretions from the DNO of Narathura japonica Caterpillars Reduce Levels of Dopamine in the Brains of Their Attendant Ants, Pristomyrmex punctatus

(A) Representative LC-ESI-MS/MS chromatograms of biogenic amines extracted from brains of five workers of *P. punctatus*. (B–E) Effect of DNO secretions from the caterpillars on the levels of dopamine (B), serotonin (C), tyramine (D), and octopamine (E) in the brains of attendant ants. Red, green, and blue circles indicate experienced, inexperienced, and unrewarded treatments, respectively. The mean ± 95% confidence intervals are shown.

biogenic amines to be correlated with age, task, and sensory sensitivities in social insects [19], it remains to be seen exactly how the DNO secretions can modulate the levels of biogenic amines in the attendant ants. Consumption of sucrose is known to decrease levels of dopamine in the brains of workers of Formica japonica [20], and the DNO secretions from several species of lycaenids have been shown to secrete sucrose as well as other sugars and amino acids [6, 8, 9]. However, it's unlikely that the effect of the DNO secretions is mediated simply through sucrose since the ants in all three treatments were also provisioned with unlimited 10% sucrose solution and water. Ant-associated lycaenid butterflies are also known to feed on nitrogen-rich host plants [21], and although this has been thought to be selected for due to the additional nutritional demands placed on ant associated caterpillars, nitrogen-related compounds such as amino acids, alkaloids, and peptides might also be needed as precursors for producing manipulative secretions.

Mutualistic relationships often show asymmetric dependences between interacting partners [22], and in this case, ant protection is vital for the survival of lycaenid caterpillars, but the reward that lycaenids secrete may not be as necessary for their associated ants, which typically have additional food resources [6, 23]. Our results show that the DNO secretions from lycaenids may provide not only nourishment, but also manipulative drugs that could function to enforce cooperative behavior (partner fidelity and aggressive defense) from attendant ants.

This finding has important implications for the evolution of DNO-mediated defensive associations and highlights the need for a better understanding of the nature of the exocrine secretions of myrmecophilous lycaenids and other ant-associated insects. It's possible that these common food-for-defense interactions, which are typically assumed to be mutualistic, may in fact be maintained primarily through parasitic manipulation of ant behavior.

#### **EXPERIMENTAL PROCEDURES**

#### **Study Organisms**

We collected *N. japonica* eggs and early-instar caterpillars feeding on *Quercus glauca* in Kyoto city from 2007 to 2009 and in Okinawa in 2011 and reared them on young leaves of *Q. glauca*. We collected three colonies of *P. punctatus* in Kyoto in 2007 and three colonies in Okinawa in 2011. The ants were reared in plastic nest boxes ( $70 \times 50 \times 40$  cm) with nest material (humus and wood). Mealworms, maple syrup solution, and Bhatkar-Whitcomb diet [24] were provided as food in the foraging area twice each week.

#### **Ant Treatments**

Three treatments (experienced, inexperienced, and unrewarded ants) were prepared as described previously [8]. In brief, for the experienced treatment, 50 workers from the foraging area of each colony were collected and kept in a plastic arena (17  $\times$  12  $\times$  5 cm) with a final instar caterpillar of *N. japonica*. For the treatment unrewarded by DNO secretions (unrewarded treatment), the ants were kept with a "reward-less" caterpillar whose DNO had been previously occluded by applying a small amount of clear nail polish on and around

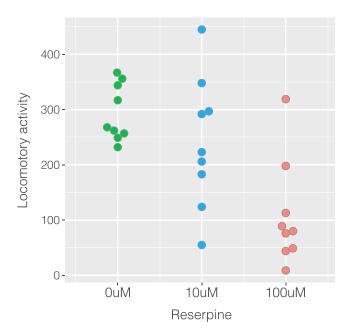


Figure 4. Reserpine Treatment Decreases the Locomotory Activity of Workers of the Ant Pristomyrmex punctatus

Effect of reserpine, a known inhibitor of dopamine in Drosophila, on the locomotory activity of ants. Green, blue, and red circles indicate feeding treatments with 0, 10, and 100  $\mu M$  reserpine diluted in 10% sucrose solution, respectively. See also Figure S1 and Movie S1.

the DNO of the fifth instar. Earlier studies of caterpillars treated with and without clear nail polish in locations separate from the DNO demonstrated that ant and caterpillar behaviors are not disrupted by nail polish alone [8]. In the inexperienced treatment, the ants were kept without caterpillars. In all treatments, cotton soaked in 10% sugar solution (food for the ants), young leaves of Q. glauca (food for the caterpillars), and water were supplied in the foraging arena.

#### **Locomotory Activity**

3 days after each treatment was set up, ten workers were randomly chosen from a foraging arena and moved to a plastic Petri dish (4.5-cm internal diameter × 1-cm height). The dish was placed on a piece of white paper over a line that bisected the dish. The ants were allowed to become familiar with their environment for 15 min. Their behavior was then recorded for 15 min using a video camera (HDR-CX560V, Sony), and the number of times each ant crossed the center line was recorded (n = 12 using six colonies in each treatment).

#### Aggression toward Eversion of TOs

Soon after the introduction on day 0 (when workers from both treatments had not yet contacted the caterpillar), on day 3 and day 6, we observed the aggressive behavior of ants toward the TO eversions. In this assay, ten workers were randomly chosen from a foraging arena and moved to a plastic Petri dish (4.5-cm internal diameter × 1-cm height), where they were allowed to become familiar with their environment for 15 min. A fresh, intact caterpillar was introduced to the Petri dish, and behavioral interactions were recorded for 15 min using a video camera (HDR-CX560V, Sony) (n = 12 using six colonies in each treatment). We measured two behavioral responses: (1) the number of the TO eversions produced by the caterpillars and (2) the ant behavior after eversions of the TOs. After each assay, the workers were not returned to the original colony to prevent social learning. We used different individual caterpillars for each tending assay.

#### **LC-MS/MS** Analyses of Biogenic Amines

3 days after the treatment was set up, the ants and the caterpillars were frozen by liquid nitrogen and kept at  $-80^{\circ}$ C until their brains were dissected (n = 15 using workers from three colonies in each treatment). Brain dissections were conducted within 2 weeks of freezing. The optic lobes were removed from each of five worker brains to prevent contamination with retinal pigments in cold insect saline and the remainder of the brain immediately homogenized in 20 µl 0.05% formic acid solution (Wako Chemicals) with 1 ng of 3,4-dihydroxybenzylamine (DHBA; Sigma-Aldrich) as an internal standard. Samples were centrifuged at 14,000 rpm for 20 min at 4°C, and a 20  $\mu$ l solution of 100:30 chloroform (Wako Chemicals):iso-propanol (Wako Chemicals) was added to the supernatant. After vortex mixing, the samples were centrifuged at 14,000 rpm for 5 min at 4°C, and the upper aqueous layer was injected into a LC-ESI-MS/MS (UFLC; LC-20AD XR, Shimadzu; Quattro Micro API, Waters). The samples were separated by an Allure PFP Propyl Column (RESTEK) at 40°C, with a solution of 10:90 MeCN (Wako Chemicals):0.05% formic acid as an eluent solution at 0.2 ml/min flow rate. Ionization of each amine was obtained by electrospray in the positive ion mode with 3 kV capillary voltage. Multiple reaction monitoring (MRM) transitions for each compound with 10 eV collision energy (parent m/z → daughter m/z) were applied as follows: serotonin, 177.1  $\rightarrow$  159.9; dopamine, 154.1  $\rightarrow$  137.0; tyramine, 138.0  $\rightarrow$ 121.0; octopamine, 154.1  $\rightarrow$  135.8; and DHBA, 140.0  $\rightarrow$  123.0. The amounts of each biogenic amine were calculated from peak areas using calibration curves of external biogenic amine standards (serotonin, dopamine, tyramine, and octopamine; Sigma-Aldrich) with 1 ng of DHBA.

#### **Reserpine Experiments**

Reserpine (Sigma-Aldrich) was first dissolved in DMSO at a concentration of 100 mM. This solution was then titrated in an aqueous 10% sucrose solution to final concentrations of 10 and 100  $\mu M$  with 0.1% DMSO. 10% sucrose solution dissolved in 0.1% DMSO was used as control (0  $\mu$ M reserpine). Fifty workers were randomly chosen from a foraging arena and moved to a plastic Petri dish. The ants were fed with water and 10% sucrose solution with reserpine for 24 hr. Ten workers were then chosen randomly, and their locomotory activities measured for each treatment as described above (n = 9 using three colonies in each treatment). We then dissected the brains of five workers per sample from each treatment, without optic lobes, and measured brain biogenic amine levels (n = 6 samples using three colonies in each treatment).

#### **Statistical Analyses**

For locomotory activity assays, the data were analyzed using a GLMM. A model was constructed using cumulated number of crossings as a response variable with a Poisson error and log-link function, ant colony as a random intercept, and ant treatment (inexperienced, experienced, and unrewarded or 0, 10, and 100  $\mu M$  reserpine) as fixed effects. For the aggression assay, a model was constructed for each treatment independently using tending time as a response variable with binomial errors and logit-link function, ant colony as a random intercept, days after treatment (0 to 6) as fixed effects. For biogenic amine levels, a LMM was constructed using each biogenic amine level as response variables with identity link function, ant colony as a random intercept, and ant treatment (inexperienced, experienced, and unrewarded) as fixed effects. For each analysis, the influence of different factors was tested using the likelihood ratio test. In locomotory assays and biogenic amine analyses, we also applied a post hoc Tukey's HSD test for pairwise comparison among treatments. All statistical analyses were conducted in R version 3.1.1 [25].

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.07.016.

#### **AUTHOR CONTRIBUTIONS**

M.K.H., N.E.P., and K.T. conceived and designed the study, M.K.H. performed experiments, and M.K.H., N.E.P., and K.T wrote the manuscript.

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