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RESEARCH ARTICLE

TRAIL FOLLOWING BEHAVIOR IN THE AMERICAN PAINTED LADY CATERPILLAR *VANESSA VIRGINIENSIS* (HEXAPODA: LEPIDOPTERA)

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ABSTRACT

The objective of this study was to determine whether painted lady caterpillars, *Vanessa virginiensis*, exhibit trail following behavior via a pheromone. We conducted 450 trials with caterpillars in a T-maze. For a control treatment, we did not expose caterpillars to pheromones in either arm of the maze. For two experimental treatments (trail treatment and extract treatment), we left one arm untreated, and we treated the other arm with natural pheromones (trail treatment) or pheromone extract (extract treatment). We recorded the arm choice of each caterpillar. The choices made by both experimental treatments were significantly different than those of the control treatment, indicating that *V. virginiensis* does exhibit trail following behavior involving a pheromone. Future research should be conducted on *V. virginiensis* to determine the function of trail following, the chemical composition of the pheromone, whether a hierarchy of signals is used in communication, and whether degree of relatedness impacts pheromone reception.

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INTRODUCTION

The caterpillars of many butterfly and moth species (Lepidoptera) are social insects that frequently communicate with conspecifics. Social caterpillars can either be patch-restricted, nomadic, or central place foragers (Costa, 1997). Patch-restricted social caterpillars feed within their own home webs, nomadic caterpillars travel between different locations of resources, and central place foragers periodically leave a communal nesting site, such as silk webbing, to find resources (Fitzgerald and Peterson, 1998). Pheromones play a role in social caterpillar communication, and social caterpillars use pheromones to recruit conspecifics to pheromone pathways (Costa, 1997). Social caterpillars without processionary behaviors (following a trail together single file in a head-to-tail fashion) follow pheromone trails that conspecifics secrete from the ventral surface of their abdomen (Ruf and Fiedler, 2000). Insect pheromones can be extracted from the body and then exposed to conspecifics to examine their effects. For example, sex pheromones from the Simao pine caterpillar moth, *Dendrolimus kikuchii*, were successfully extracted from female sex pheromone glands (Kong *et al*., 2011). As adult females, moths and butterflies must be able to find food as well as oviposition sites.

These insects have two options to the conflict that arises when looking for both food site and an oviposition site. The first option is searching for both sitessimultaneously. If a female performs these tasks simultaneously, the outcome is reduced search efficiency for both a food source and a location to lay eggs (Bernays, 2001). The second option involves primarily searching for one resource. This decreases the chance of finding the resource not being focused on (Scheirs *et al*., 2000). Both options result in costs that can lead to reduced fitness. The painted lady butterfly, *Vanessa cardui*, searches for food sources and oviposition sites simultaneously without the costs because *V. cardui* can use some of its larval food plants as nectar sources. By utilizing a nectar-producing plant as a habitat for caterpillars, *V. cardui* adults and larvaeboth have a food source at the same location (Janz, 2005). The location of egg placement in relation to larval food sources is a critical component of fitness in many lepidopteran species (Dethier, 1959). If caterpillars cannot find food sources after hatching, they will not survive. While some species of butterflies, such as *V. cardui*, are precise at laying eggs on or near a caterpillar food source, others such asthe American painted lady (*Vanessa virginiensis*) are not precise at laying eggs in the vicinity of caterpillar food sources, thus requiringlarvaeto search for food sources (Dethier, 1959). Some caterpillar species exhibit trail following behavior via the production of pheromones; however, this has not been examined in *V. virginiensis*. Since adult *V. virginiensis* are not

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precise about depositing eggs near caterpillar food sources, it is possible that *V. virginiensis* caterpillars use pheromones to communicate the location of food to conspecifics. We performed extractions to determine whether *V. virginiensis* produce pheromones, and we tested whether caterpillars would exhibit trail following behavior when these extractions were placed on a substrate.

MATERIALS AND METHODS

We conducted research in the Animal Behavior Laboratory at Millikin University in Decatur, IL. We obtained *V. virginiensis* caterpillars of undetermined ages and sexes from the Carolina Biological Supply Company (Burlington, NC) and fed them *Ad libitum* throughout the study. We used a glass T-maze with arms 7.5 cm W x 5.4 cm L and base 4.8 cm W x 5.0 cm L. The T-maze had a glass lid, and we covered the floor of the maze with paper. After every trial, we removed the paper, cleaned the T-maze with hexane, and placed new paper in the maze. For each trial, we recorded the arm on which the caterpillars first chose to walk. If a caterpillar exhibited no movement, we removed it, cleaned the T-maze, and chose a different caterpillar. We conducted a control treatment and two experimental treatments (subsequently referred to as the trail treatment and the extract treatment) with 15 different caterpillars in each treatment. We conducted 10 trials on each of the 45 caterpillars, and we tested each caterpillar individually. For the control treatment (n = 15), we introduced caterpillars into the T-maze with no chemical cue in either arm. When caterpillars reached the T, we recorded whether each one chose to walk down the left or the right arm of the maze. We tested each caterpillar (n = 15) ten times. Between each trial, we cleaned the T-maze and replaced the paper in both arms. For the trail treatment, we randomly placed (via coin flip) a novel caterpillar in one of the arms of the T-maze and blocked the opposite arm with glass. After the caterpillar walked the selected arm of the T-maze, we removed it, lifted the glass barricade, and placed a caterpillar at the base of the T-maze. We recorded each caterpillar's choice of arm. We tested each caterpillar (n = 15) ten times. Between each trial, we cleaned the T-maze, replaced the paper in both arms, and again had a novel caterpillar walk down one arm. For the extract treatment, we excised 5.0 mm of the posterior ends of five caterpillars with a razor. We used the posterior tips because silk moth caterpillars, *Arsenura armada,* secrete pheromones from the posterior tip of the abdomen (Costa *et al*., 2001). We transferred the posterior ends of the caterpillars to a test tube, crushed them, and pipetted 1 mL of hexane into the test tube. We incubated the solution at room temperature for 30 min., and then used a fine- tipped glass syringe to transfer 10 µL of the pheromone extract to a randomly determined (via coin flip) arm of the T-maze. We then placed a caterpillar at the base of the T-maze and recorded its choice of arm. We tested each caterpillar (n = 15) ten times. Between each trial, we cleaned the T-maze, replaced the paper, and then pipetted the extraction into one arm. We made new pheromone extractions daily.

Due to the non-parametric nature of frequency data, we used a series of non-parametric tests to determine if choices differed from random for each of the three treatments, if there were significant differences in the number of"correct choices" (defined as the right arm for control caterpillars and the arm with either the trail or the extract for experimental caterpillars for recording purposes)over successive trials for each treatment and to determine if the total number of correct choices over 10 trials differed among the three treatments. We used chi-square tests to determine if choices differed from random for each treatment at each time point. We then used a chi-square test of association to determine if frequency of choosing the correct route was associated with a treatment affiliation. We used a Cochran's Q test for the control treatment, the trail treatment, and the extract treatment to determine if the ratio of arm choices differed over the 10 trials. We then used a Kruskal-Wallis test to determine if there were differences among the three treatments in the total number of correct choices among the 10 trials (the right arm for control caterpillars). Following a significant Kruskal-Wallis test, we used Mann-Whitney U tests to make each pairwise comparison of total correct choices among the 10 trials for the treatments (trail treatment vs. control, extract treatment vs. control, and trail treatment vs. extract). In each case, we considered a p-value of less than 0.05 to be statistically significant.

RESULTS

The control treatment choices did not deviate significantly from random in any trial (Table 1; Fig. 1). The caterpillar choices deviated significantly from random in 6 of the 10 trials in the trail group (Table 1; Fig. 1), and choices deviated significantly from random in 9 of the 10 trials in the extract treatment (Table 1; Fig 1.). The chi-square goodness of fit test revealed significant differences in the number of correct choices among the three treatments in 6 of the 10 trials (Table 1; Fig. 1). The Cochran's Q test revealed that the caterpillars in each of the three treatments made statistically consistent choices over the course of 10 trials (control: Q = 10.225, df = 9, p = 0.333; trail treatment: Q = 8.143, df = 9, p = 0.520; extract treatment: Q = 14.077, df = 9, p = 0.120; Fig. 1).

Table 1. Results of statistical analyses of trail following behavior in three treatments: control (no pheromone), pheromone trail, and pheromone extract. For control, trail, and extract, df = 1; for test of association, df = 2; P < 0.05 indicates statistical significance

Control	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10
χ^2	0.600	1.667	0.067	0.600	0.067	3.267	3.267	0.067	0.600	0.067
df	1	1	1	1	1	1	1	1	1	1
p	0.439	0.197	0.796	0.439	0.796	0.071	0.071	0.796	0.439	0.796
Trail	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10
χ^2	7.143	7.143	1.143	4.571	2.571	2.571	2.571	1.143	--------	4.571
df	1	1	1	1	1	1	1	1	1	1
p	0.008	0.008	0.285	0.033	0.109	0.109	0.033	0.285	< 0.001	0.033
Extract	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10
χ^2	--------	8.067	8.067	5.400	5.400	8.067	0.067	8.067	5.400	11.267
df	1	1	1	1	1	1	1	1	1	1
p	< 0.001	0.005	0.005	0.020	0.020	0.005	0.796	0.005	0.020	0.001
Test of Association	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10
χ^2	15.657	2.324	5.907	6.806	2.558	12.266	2.405	3.985	7.124	8.588
df	2	2	2	2	2	2	2	2	2	2
p	< 0.001	0.313	0.048	0.033	0.278	0.002	0.301	0.136	0.028	0.014

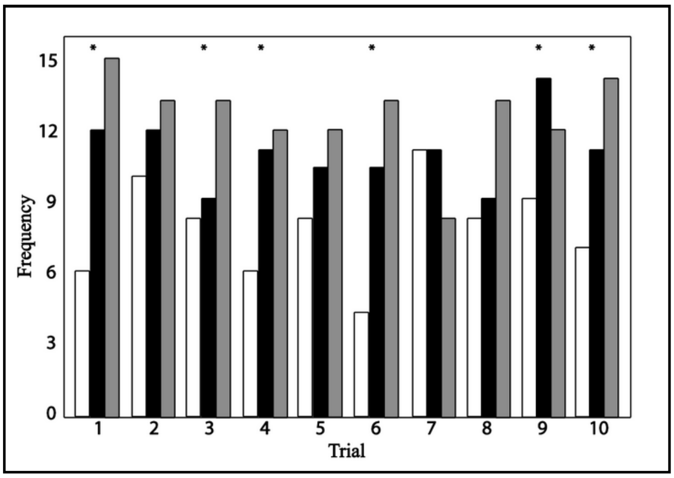

Figure 1. Frequency of correct choices made by Painted Lady caterpillars in a T-maze across 10 trials for each a control group with no chemical cues (white bars), a group with a pheromone trail from a conspecific (black bars), and a group with an experimental extract cue on one side of the T-maze (gray bars). Asterisks denote significant differences within the trial between at least two of the three groups ($p < 0.05$)

Given that none of the three treatments showed changes in preferences over the course of the trials, we used a Kruskal-Wallis test to determine if there were differences in the total number of correct choices among the three treatments for their 10 trials. We found statistically different numbers of correct choices among the three treatments (chi-square = 25.066, df = 2, $p < 0.001$.). To determine pairwise differences among the three treatments, we used Mann-Whitney U tests, which revealed significantly greater correct choices in the trail treatment (mean rank = 21.43) than the control (mean rank = 9.00; Mann-Whitney U = 15.000, $p < 0.001$) and significantly greater correct choices in the extract treatment (mean rank = 22.37) than the control (mean rank = 8.63, Mann-Whitney U = 9.500, $p < 0.001$), but no statistically consistent results between the trail treatment (mean rank = 12.50) and extract treatment (mean rank = 17.33, Mann-Whitney U = 70.000, $p = 0.109$; Fig. 1).

DISCUSSION

Caterpillars in the control treatment provided with no cues exhibited a random distribution in arm choices indicating that the T-maze was a valid experimental set-up with no biases for one side over the other. In contrast, caterpillars in both experimental treatments exhibited trail following behavior, thus providing evidence for the presence of a pheromone. There was no significant difference in the behaviors of the trail treatment versus the extract treatment, with both showing a significant preference for the marked arm. Our results agree with similar studies of trail following behaviors in other caterpillar species. The pine processionary caterpillar, *Thaumetopoea pityocampa*, will follow both a natural pheromone trail and an artificial pheromone trail as demonstrated using a Y-maze (Fitzgerald, 2003). The tent moth caterpillar, *Eutachyptera psidii*, also displays trail following behavior in the presence of pheromones (Fitzgerald and Pescador-Rubio, 2011). Fitzgerald and Pescador-Rubio (2011) examined the source of the trail marker, the threshold sensitivity to a trail pheromone, and the ability of caterpillars to differentiate pheromone trails of varying ages and potencies.

Social silk moth caterpillars, A. *armada,*use long-lived chemical trail markers and will follow an artificial pheromone extract (Costa *et al*., 2001). It seems likely in *V. virgineinsis* caterpillars follow trail pheromones to food sources since adults do not oviposit near caterpillar food sources (Dethier, 1959). Other species of caterpillars that exhibit trail following behavior, such as larvae of the madrone butterfly (*Eucheira socialis),* use trails for this purpose (Fitzgerald and Underwood, 1997).

Determining the chemical components of the pheromone can aid in elucidating the function of the pheromone. Sex pheromones have been extracted from the coleopteran stem borer, *Sesamia cretica*. and identified using mass spectra comparison and chemical analysis (Avand-Faghih and Frèrot, 2008). Determining the chemical components, volatility, and stability of the pheromone produced by *V. virginiensis* could aid in understanding the adaptive significance of chemical communication in this species. *V. virginiensis* might exhibit modes of communication in addition to pheromones. *Croesus latitarsus* sawfly caterpillars raise their abdomens over their heads and wave them back-and-forth in unison when threatened by predators(Costa, 1997). Larvae of the scarce large bluebutterfly, *Maculinea teleius,* have evolved the behavioral ability to mimic acoustics of the elbowed red ant, *Myrmica scabrinodis,* in order to exploit the ants' resources (Balletto et al., 2010). The coleopteran weevil *Phelypera distigma* exhibits head bobbing behavior in addition to using chemical pheromones for conspecific communication (Costa *et al*., 2004). Relatedness can be associated with pheromone communication. We determined that *V. virginiensis* use a pheromone tocommunicate with conspecifics, but we do not know the degree of relatedness of the caterpillars we used. Caterpillars of the silverspot butterfly, *Dione juno huascuma,* detect chemical trails deposited by siblings and use pheromones specifically to communicate with siblings, as opposed to other caterpillar species that use pheromones to communicate with conspecificsregardless of relatedness (Fitzgerald *et al*., 2011). In conclusion, we determined that *V. virginiensis*caterpillars do produce a chemical pheromone and that they use it to follow the trails of conspecifics. The results of this study lead to many additional questions, and further studies of *V. virginiensis* communication are needed to increase our understanding of the adaptive significance of the complex behaviors exhibited by this species.

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