

Associative Learning in Response to Color in the Parasitoid Wasp *Nasonia vitripennis* (Hymenoptera: Pteromalidae)

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*A parasitoid that can learn cues associated with the host microenvironment should have an increased chance of future host location and thereby increase its reproductive success. This study examines associative learning in response to simultaneous exposure to the colors yellow and blue in mated females of the parasitoid wasp *Nasonia vitripennis*. Preference was measured as the proportion of time spent on a color. When trained with one color rewarded with hosts and honey and the other unrewarded, females showed an increase in preference for the rewarded color with increasing number of training days (1, 3, and 7 days). Hosts and honey together produced a slightly greater preference toward the rewarded color than just hosts, which produced a greater preference than just honey. When trained with a variable reward on one color and a constant reward on the other, females preferred the color associated with the variable reward when it was yellow, but not when it was blue. Thus, relative to no reward, the presence of a variable reward decreased the strength of preference toward the constantly rewarded color. Finally, females trained with regular hosts on one color and used hosts on the other preferred the color associated with the regular hosts when that color was blue but showed no preference in the reverse situation. The presence of used hosts instead of no reward did not increase the strength of preference for the color associated with the regular hosts.*

KEY WORDS: associative learning; color learning; parasitoid wasp; Pteromalidae; *Nasonia*.

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INTRODUCTION

There may be natural selection for parasitoids to learn the characteristics associated with their hosts' environment, as a means of improving the chances of future host location and hence reproductive success (Arthur, 1966). Learning can be defined as a change in behavior based on past experience (Wardle, 1990), and when a change in behavior results from the association of a stimulus with a reward or punishment, this type of learning is associative learning. Here, using the parasitoid wasp *Nasonia vitripennis* (Hymenoptera: Pteromalidae), we establish a simple protocol for studying associative learning in response to visual cues. We look specifically at associations of food and hosts with color by females.

Much of what is known about insect learning comes from the well-studied honey bee (Hymenoptera: Apidae) (reviewed in Gould, 1993; Menzel *et al.*, 1993). The information available on the learning capabilities of parasitoid wasps is limited, and much of that research has focused on chemical cues, with less work on visual cues (e.g., Turlings *et al.*, 1993; Wackers and Lewis, 1994; Takasu and Lewis, 1996).

N. vitripennis parasitizes the pupae of several fly species commonly found in carrion, nests, and refuse (Rueda and Axtell, 1985). *N. vitripennis* females oviposit on hosts and feed on hosts and honey in the laboratory, and presumably on nectar in the wild as observed in other parasitoid wasps (Godfray, 1994). Females have eggs available for oviposition upon emergence but must feed on hosts to produce additional eggs. Females that do not host feed will begin to resorb eggs within three days and will continue to do so until host feeding commences (Whiting, 1967).

N. vitripennis has been studied behaviorally, ecologically, physiologically and morphologically (e.g., Velthuis *et al.*, 1965; Davies and King, 1975; Beukeboom, 1994; King *et al.*, 1995). Yet very little is known about the learning capabilities of *N. vitripennis*. *N. vitripennis* appears to be able to discriminate between colors (R. F. Madej, unpublished). Therefore, it seemed likely that females could learn to associate hosts with some aspects of color.

We used yellow and blue in our experiments to maximize the chance of detecting learning: the peak wavelengths of yellow and blue are at opposite ends of the color spectrum. *N. vitripennis* is likely to encounter yellow and blue in its natural environment, at least while foraging for nectar. In fact, yellow is the most common flower color (Weevers, 1952).

Females were exposed to both colors simultaneously. First we tested females for an innate color preference for the yellow versus the blue. Then we examined their ability to learn using hosts and/or honey as a rewarded experience and their absence as an unrewarded experience. We determined

the effect of number of training days on the strength of learning and determined whether the effect depended on which color was rewarded. We determined whether hosts, honey, or both is the most effective reward. We also determined whether females still learn the color associated with a constant reward when the alternative is a color associated with a variable reward rather than no reward. Finally, we determined whether females still learn to prefer the color that was associated with regular hosts when the alternative is a color associated with used hosts.

METHODS

General Methods

An inbred line of the scarlet eye strain of *N. vitripennis* was obtained commercially from Carolina Biological Supply Company and was maintained on pupae of *Calliphora vomitoria*. The *C. vomitoria* were obtained from Grubco Inc. as larvae, allowed to pupate, and then stored at 4°C. Adult wasps were fed clover honey ad libitum. The wasp colony was kept in an incubator at 23–26°C with a 12:12 light:dark cycle.

To generate wasps for experiments, individual females were removed from the colony and given fresh hosts for 24 h. Several days before expected emergence of the offspring, the host puparia were cracked open to facilitate aging of the wasp pupae. Wasps were allowed to mass mate and females 0–1 day old from adulthood were removed and used in experiments. Prior to use in experiments, the females had not been given honey or hosts and had been kept in an environmental chamber lined in white, so they had not been exposed to yellow or blue.

In all experiments, during both training and testing, each female was placed in a 100 × 15 mm (diameter × height) petri dish with a 10 mm high clear plastic divider down the center. The divider did not restrict the female's access to either side of the dish. A yellow and a blue unruled index card had been placed under the petri dish so that half the dish was yellow and half was blue. Petri dishes were separated from each other by corrugated cardboard partitions so that the female could see only her own dish. Overhead fluorescent lights were used as a light source. Spectral reflectance curves were generated for the yellow and blue cards following King *et al.* (1994) (Fig. 1).

For each day of training a clean petri dish and fresh reward were provided. To minimize learning of location cues rather than color during training, if on day 1 the rewarded color was on the right, on day 2 it would

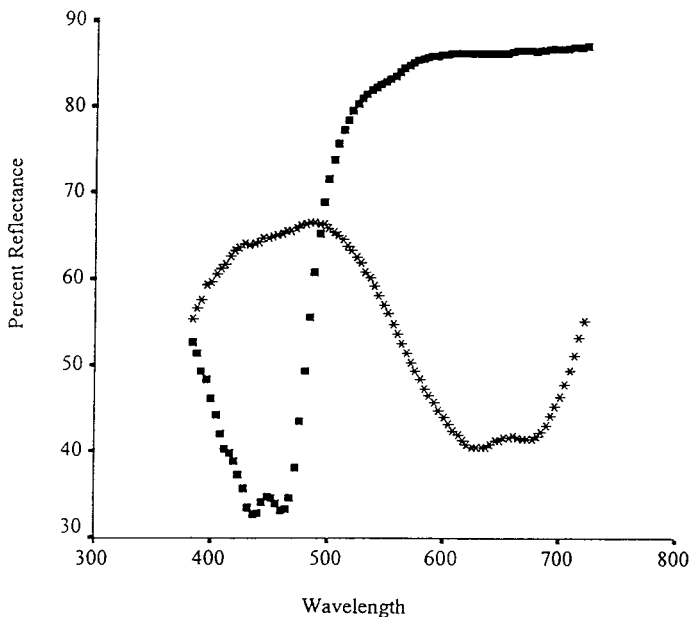


Fig. 1. Percentage reflectance at different wavelengths (nm) for the yellow (■) and the blue (*) backgrounds.

be on the left and vice versa. Also which side the rewarded color started on was alternated between females. Host and honey rewards were not reused.

During testing the colors were perpendicular with respect to training orientation to avoid the confounding of color and location cues. Neither color was associated with a reward during testing. Each female was tested only once. Testing was within 10 min of the end of training. The female was placed on the dividing line between yellow and blue in the test dish; behavior was monitored for 10 min. The first color chosen along with the cumulative time spent on each color was noted. First color chosen was the first color onto which the female moved at least half of her body. Preference was calculated as the cumulative time spent on a given color divided by the total time spent on any color. Total time spent on any color, that is total time not on the dividing line, was usually close to 10 min, e.g., in the Innate Color Preference experiment, mean 9.8 ± 0.13 s.e. min, range 3.6–10 min. Preference, i.e., proportion of time on a given color, is presented as mean \pm standard error. The color on which more time was spent is referred to as the preferred color.

All results were analyzed using the statistical software package SPSS

6.1 for Windows. Parametric tests were used because the assumptions of normality were met as determined by testing for skewness and kurtosis at $\alpha = 0.001$. $\alpha = 0.001$ was chosen because t -tests and ANOVA are robust to deviations from normality (Scheffé, 1959). 1tP indicates a one-tailed test and 2tP a two-tailed test. Statistical significance of preference was tested by comparison to 0.5 by t -test. The comparisons between experiments should be viewed with greater caution than comparisons within experiments since different experiments were not conducted simultaneously, although basic protocol was consistent.

Innate Color Preference

This experiment was to determine whether females have an innate preference for yellow or blue. Females were not trained prior to testing. Which color was on the right versus the left was alternated between females. A total of 50 females were tested. Comparison of preference for the yellow versus for the blue was by a paired t -test. In hopes of shortening the testing, we also tested whether females spent most (greater than 50%) of their time on the first color they chose; this was tested within each color by independent t -tests.

Number of Training Days

This experiment was to determine the effect of the number of training days and the rewarded color on preference. The number of training days tested were one, three, and seven. The reward was five hosts and a small droplet of honey about 4 mm in diameter. No reward was offered on the other side of the dish. A total of 160 females were trained—with blue rewarded: 29 for one day, 24 for 3 days, 27 for 7 days; with yellow rewarded: 28 for 1 day, 29 for 3 days, and 23 for 7 days. The first color chosen along with the total time spent active and inactive on each color were noted. Active and inactive times were combined to calculate preference.

Effect of training color and of the number of training days on preference were examined by an analysis of covariance (ANCOVA). The ANCOVA assumption of no interaction between the training color (independent factor) and number of days trained (covariate) was met ($F_{1,156} = 0.70$, $P = 0.41$).

For the seven-day treatment, we examined whether the first color chosen was dependent on the color which had been rewarded (analyzed by chi-square test). This information was collected for methodological reasons:

collecting just first color would reduce testing time considerably, but would be meaningful only if the first color chosen was an indicator of preference. We also examined whether females spent more time active on the rewarded color than on the unrewarded color.

Hosts Versus Honey

This experiment was to determine whether hosts or honey is a more effective reward for associative learning. Although there was no significant effect of color in the previous experiment, the trend was for a stronger preference by females trained on blue (see Results). Consequently, during training, blue was rewarded for all females. Training was for 7 days, again based on the results of the previous experiment. During training, the reward was either five fresh hosts daily ($n = 39$ females) or a fresh spot of honey daily ($n = 42$ females). Independent t -tests were used to test for a preference for the rewarded color within each treatment as well as to test for a difference in the strength of preference between the two rewards. We also compared strength of preference when the reward was hosts (this experiment) versus when the reward was hosts and honey (the part of the previous experiment in which females were likewise trained for seven days with blue rewarded). This comparison was by independent t -test.

Variable Versus Constant Reward

This experiment was to determine whether females still prefer the color that is associated with a constant reward when the alternative color is associated with a variable reward. The constant reward was five hosts daily for eight days, for a total of 40 hosts. The variable reward was on days one through eight 0, 10, 0, 10, 0, 10, 0, 10 hosts, respectively, for a total of 40 hosts. A total of 56 females were trained, 26 with a constant reward on blue and 30 with a constant reward on yellow. Independent t -tests were used to test for a preference within each of these two sets of females. We also compared the strength of preference toward the constantly rewarded color blue when the other color, yellow, was associated with a variable reward (this experiment) versus no reward (previous experiment).

Regular Versus Used Hosts

This experiment was to determine whether females still prefer the color that is associated with regular hosts when the alternative color is

associated with used hosts. Used hosts were hosts from which wasps had already emerged. Used hosts (but not regular hosts) had been frozen and then thawed prior to use, to ensure the absence of any live wasps within. Each experimental female was given five regular hosts on one color and five used hosts on the other. There were two treatments, females with regular hosts on blue and females with regular hosts on yellow ($n = 40$ females per treatment). The females were trained for a total of 7 days. Whether females preferred the color that had been associated with regular hosts was tested by an independent t -test within each of the two treatments.

To understand better the response of females to regular versus used hosts, each of 10 females was videotaped with a regular host and a used host for 3 h. Behavior was recorded with a color video camera and a s-VHS digital recorder on s-VHS extended play tapes. Tapes were reviewed on a 48-cm color monitor. The following information was collected: the first host contacted, antennated, and successfully drilled into; and the total time spent on each host [for behavior definitions see King (1998)]. Since females can oviposit or feed from regular hosts but not from used hosts, we expected females to preferentially go to and explore the regular hosts and the used host to be no reward or a negative reward (but see Results and Discussion). Since the sample size was small, only descriptive statistics are provided.

RESULTS

Innate Color Preference

The females did not demonstrate an innate color preference for blue versus yellow (proportion of time on blue: 0.46 ± 0.05 ; $t = 0.67$, $df = 49$, $2tP = 0.51$). The first color chosen was an indication of the color on which the females would spend the majority of time, but only significantly so when the first color chosen was yellow. Females that chose yellow first spent 0.71 ± 0.08 of their time on yellow ($t = 2.64$, $df = 21$, $1tP = 0.01$). Females that choose blue first spent 0.60 ± 0.06 of their time on blue ($t = 1.48$, $df = 27$, $1tP = 0.08$).

Number of Training Days

Preference did not differ significantly in response to which color was rewarded ($F_{1,157} = 1.80$, $P = 0.18$), but preference increased with increasing

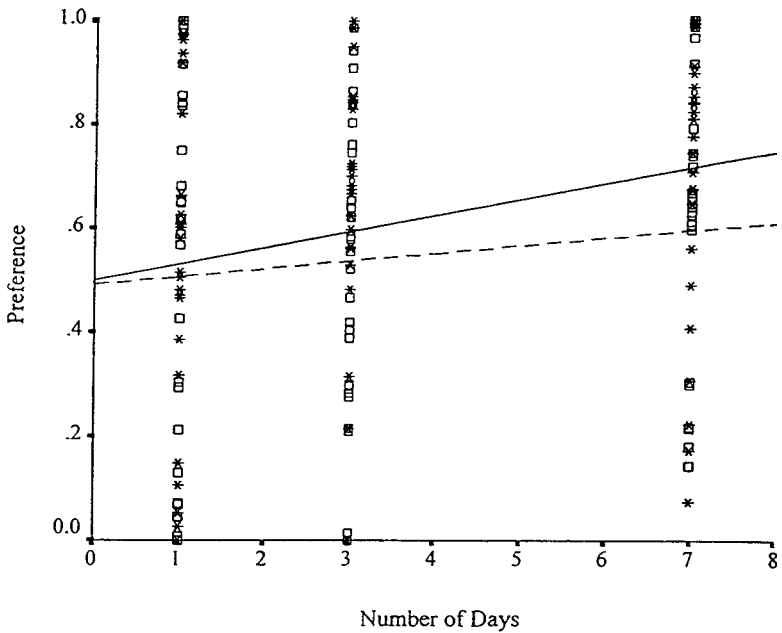


Fig. 2. Preference of *N. vitripennis* females for (= proportion of time spent on) the color rewarded with hosts and honey when there were 1, 3, or 7 training days, yellow (---) and blue (*—).

number of training days (Fig. 2; $F_{1,157} = 5.78$, $P = 0.02$). The first color chosen was independent of the color which had been rewarded (Table I). After 7 days of training, regardless of which color had been rewarded, seventy percent of females were active their entire time on each color during testing. Of the remaining females, only 5 of 14 spent a greater proportion of their time active on the rewarded color than on the unrewarded color (sign test, no ties, $2tP = 0.42$).

Table I. First Color Chosen by *N. vitripennis* Females When Trained 7 Days with Hosts and Honey Versus No Reward

Color	First color chosen		χ^2	P
	Blue	Yellow		
Rewarded	Blue	Yellow		
Blue	15	7	2.90	>0.05
Yellow	12	16	0.57	>0.25

Hosts Versus Honey

Females preferred the rewarded color regardless of whether the reward was hosts (0.68 ± 0.05 ; $t = 3.86$, $df = 38$, $1tP < 0.0005$) or honey (0.57 ± 0.04 ; $t = 1.68$, $df = 41$, $1tP = 0.05$). However, females rewarded with hosts had a significantly stronger preference for the rewarded color than did females rewarded with honey ($t = 1.80$, $df = 79$, $1tP = 0.03$). Comparison with the previous experiment showed a slight, but significantly stronger, preference for the rewarded color when the reward was hosts and honey versus just hosts ($t = 7.34$, $df = 64$, $2tP < 0.001$).

Variable Versus Constant Reward

Females that had been trained with the constant reward on blue, preferred the variably rewarded color (0.67 ± 0.03 ; $t = 4.50$, $df = 25$, $2tP < 0.001$). Yet females trained with the constant reward on yellow, showed no significant preference for either color (preference for variably rewarded color = 0.48 ± 0.07 , $t = 0.32$, $df = 29$, $2tP = 0.75$). Thus, relative to no reward in the previous experiment, under otherwise similar conditions the variable reward in this experiment decreased the preference toward the constantly rewarded color from 0.68 ± 0.05 to 0.33 ± 0.03 ($t = 5.30$, $df = 63$, $2tP < 0.001$).

Regular Versus Used Hosts

When trained with the regular hosts on blue, females showed a significant preference for that color (0.64 ± 0.031 , $t = 4.61$, $df = 39$, $1tP < 0.0005$). But when trained with the regular hosts on yellow, there was no significant preference (0.50 ± 0.04 , $t = 0.05$, $df = 39$, $1tP = 0.48$).

The degree of preference for the color blue associated with regular hosts was the same regardless of whether the other color, yellow, had been associated with no reward (0.68 ± 0.05 , Honey Versus Hosts experiment) or with used hosts (0.64 ± 0.031 , this experiment) ($t = 0.056$, $df = 65$, $2tP > 0.50$).

The videotapes showed that the used hosts were explored by females. In fact, 8 of 10 females contacted and antennated the used host before the regular host (Table II). Six of the 10 females spent more time on the used host than on the regular host. One of the 10 females crawled into the used host after about 5 min and stayed there through the taping. Some entering

Table II. Video Observations of 10 *N. vitripennis* Females, Each with a Regular Host (H) and a Used Host (E)

First contacted	First antennated	First drilled	Total time on ^a	
			H	E
E	E		122:31	0:05
H	H		1:32	0:00
H	H		7:19	0:00
E	E	E	0:00	0:00
E	E		0:00	1:15
E	E		0:00	3:21
E	E		0:00	119:57
E	E		0:00	0:07
E	E		0:00	174:31
E	E	H	118:59	6:47
			Mean = 24:94	44:88
			SE = 15:94	22:44

^aMinutes:Seconds.

of used hosts also occurred during training in the Regular Versus Used Hosts experiment.

DISCUSSION

The response of *N. vitripennis* females to color in this study could have been a response to wavelength or to brightness. The yellow and blue differed in wavelength. With brightness computed as mean reflectance, they also differed in brightness, yellow being brighter. However, whether the yellow appeared brighter than the blue to the wasps is a more difficult question to address. Brightness as it appears to an animal “cannot be determined from the spectral radiance curves alone, because the sensitivity of the photoreceptors is not uniform across different wavelengths” (Shafir, 1996). A previous study of the visual range of 43 different species of hymenopterans indicated a maximum spectral sensitivity at 340, 430, and 535 nm (see blue curve Fig. 1) and only a minor sensitivity at 600 nm (see yellow curve Fig. 1) (Peitsch *et al.*, 1992). Thus, the females may have even perceived blue as being brighter than yellow. *N. vitripennis* may have been responding to hue and not brightness because other parasitoids have demonstrated an ability to learn color based on wavelength and not brightness (Wardle, 1990; Shafir, 1996).

N. vitripennis females showed no innate color preference for yellow versus blue. Similarly, *Exeristes roborator*, an ichneumonid parasitoid of

hosts in plants, shows no innate preference for orange versus blue (Wardle, 1990). In contrast, innate color preference is found in honey bees (Giurfa *et al.*, 1995), blow flies (Fukushi, 1989) and in many butterflies, though which color is preferred varies among taxa (references in Weiss, 1997).

N. vitripennis females learned to associate color with hosts and/or honey. Some other hymenopterans besides honey bees have also been shown to associate color with hosts or food. For example, the parasitoid wasps *Itoplectis conquisitor* and *Exeristes roborator* (Hymenoptera: Ichneumonidae) learn to associate hosts with color (Arthur, 1966; Wardle, 1990). *Polybia occidentalis* (Hymenoptera: Vespidae) learns to associate sugar with color (Shafir, 1996). The desert ant *Cataglyphis bicolor* (Kretz, 1979) learns to associate a piece of biscuit with color. Color learning in response to food rewards has also been demonstrated in some dipterans, lepidopterans, and orthopterans (references in Wardle 1990; Weiss, 1997).

In *N. vitripennis*, hosts elicited a stronger preference than honey. One explanation is that females can both feed and oviposit on a host; thus, hosts may provide a stronger reward than honey. In addition, in terms of volume the five hosts were a larger reward than the spot of honey. On the other hand, frequently all five hosts were parasitized whereas there was always honey left at the end of a training day (S. E. Oliai, personal observation). The females' preference for the rewarded color was slightly greater with the reward of hosts and honey than with the reward of only hosts. Previously only a few studies with insects have examined the response to different types of rewards (Raubenheimer and Tucker, 1997). Past studies usually have focused on one type of reward, e.g., sugar water, although sometimes the effect of varying concentration has been tested.

N. vitripennis needed multiple training sessions before exhibiting a preference, and preference increased with number of trainings. This is consistent with other reports on insects. Honey bees, house flies, and butterflies (Hymenoptera, Diptera, Lepidoptera) all demonstrate some increase in the strength of preference when the number of training sessions increases (Fukushi, 1976, 1989; Gould and Marler, 1984; Weiss, 1997). *N. vitripennis* females did not learn to associate color with honey and hosts after just one day of training, in contrast to the ability of honey bees and blow flies to associate color with sugar after only one exposure (reviewed in Gould, 1993; Fukushi, 1989).

The response to colors associated with a variable versus a constant reward is difficult to interpret. Females preferred the color associated with a variable reward, but only when the variable reward was associated with yellow. There was no significant preference when the variable reward was associated with blue. Thus, females never significantly preferred the color associated with a constant reward when the other color was associated with

a variable reward—in contrast to when the other color was associated with no reward.

Preference for a variable reward has been found in some animal species when they are operating under a negative energy budget. Yellow-eyed juncos and starlings prefer variable rewards when on a negative energy budget. On a positive energy budget, the juncos prefer the constant reward, whereas the starlings do not (Bateson and Kacelnik, 1997; Caraco *et al.*, 1990). In contrast, *N. vitripennis* preferred the variable reward even though it was operating on a positive energy budget. The wasps were never in danger of starvation nor were they likely to resorb eggs after such recent exposure to hosts (Whiting, 1967).

Bumble bees (Hymenoptera: Apidae) favor a constant reward over a variable reward when the mean reward between the two choices is the same; but when the mean of the variable reward is greater, the bumble bees switch their preference to the variable reward. Bumble bees appear to forage in a way that maximizes their short-term energy intake (Real, 1991). Thus, one might expect them to prefer an immediate larger payoff. For the *N. vitripennis* in our study, on the last day of training the variable-reward color was associated with ten hosts whereas the constant-reward color was associated with five hosts; consequently the females' last experience with the variable reward was a larger reward and this could explain the preference for the variable-reward color. However, it is then unclear why the females showed preference for the color associated with the variable reward only when that color was yellow, but not when it was blue.

When choosing between colors associated with regular hosts versus used hosts, *N. vitripennis* again showed a significant preference only with one color combination and not the other. There was a preference for the color associated with the regular host only when that color was blue; when that color was yellow, there was no significant preference.

Initially we expected that time spent exploring or drilling into a used host would represent no reward or even a negative reward. However, this may not be the case. Even if used hosts do not provide a place to oviposit, they may still indicate a habitat where regular hosts were found at an earlier time and so may be found later e.g., a bird's nest. In addition, we cannot rule out the possibility that *N. vitripennis* females fed upon the host remains inside the puparium, as has been reported for the solitary confamilial, *S. nigroaenea* Curtis (= *S. muscidarum*), parasitizing stable fly pupae (Pinkus, 1913). This could explain why female *N. vitripennis* were at least as likely to approach, contact, and antennate used hosts as regular hosts.

Information on the effect of different rewards or potentially negative rewards is limited for insects (Papaj *et al.*, 1994). Examples of negative rewards used in previous insect experiments include electric shock and

toxins (review by Bernays, 1993). Neither seems relevant to *N. vitripennis*, whereas *N. vitripennis* females would sometimes encounter unsuitable hosts.

This study demonstrates that *N. vitripennis* is capable of some associative learning in response to visual cues and establishes some basic parameters for this type of learning and for future similar studies. Future experiments on *N. vitripennis*' learning capabilities might explore whether females and males differ in their visual learning capabilities, whether *N. vitripennis* can learn more subtle color differences than yellow versus blue (they can distinguish more subtle differences, a brown versus an orange (R. F. Madej, unpublished)), whether some colors are learned more easily than others, as in blow flies (Fukushi, 1989), and whether they can learn patterns. Future experiments should also address the ecological relevance of learning in this species.

Since learning should increase *N. vitripennis*'s success at locating additional hosts, determining how *N. vitripennis* learns host microenvironment characteristics while foraging may even reveal ways of increasing its effectiveness as a biocontrol agent. *N. vitripennis*'s effectiveness has been questioned (Legner, 1967) but not very well-tested, and it is sold commercially for biological control of pest flies.

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