Color preference and associative color learning in a parasitoid wasp, *Ascogaster reticulata* (Hymenoptera: Braconidae)

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Abstract

Natural enemies of agricultural pests, such as parasitoids and predators, often use chemical and visual cues in search of their hosts and prey, and they can learn the association between the cues and the host and prey presence. The braconid, egg-larval endoparasitoid wasp *Ascogaster reticulata* is a promising biological control agent for tortricid pests, such as *Adoxophyes honmai*, in tea plantations. Although previous studies revealed that *A. reticulata* uses contact chemicals released by tea plants in response to tortricid egg oviposition and that it can learn the associated cues, the diurnal wasp is also expected to use visual cues, especially color. Therefore, in this study, we investigated the innate color preference and associative color learning ability of *A. reticulata*. When a green paper and a paper of a different color (yellow, red, blue or black) was offered together to naïve females of the wasp, the females spent less time on a black and blue papers. However, wasps trained to associate black or blue with the presence of a host egg mass showed increased preference for these colors, whereas red- and yellow-trained wasps did not show changes in preference. Our findings indicate that *A. reticulata* uses colors, in addition to chemical cues, in host searching behavior and has the ability to learn colors associated with host presence.

Key words: Associative learning, oviposition, color preference, Hymenoptera, Braconidae, biological control agent

Introduction

Parasitoids and predators are common choices as biological control agents for controlling pest insects. Parasitoids and predators use various cues while searching for hosts and prey. In particular, the use of chemical cues by parasitoids has received much attention (e.g., Godfray 1994; Wajnberg and Colazza 2013). For example, two bethylid wasps, *Cephalonomia stephanoderis* and *Prorops nasuta*, which were released for classical biological control of the coffee berry borer, *Hypothenemus hampei*, in Central and South America, use contact chemicals present in the dust and frass of *H. hampei* larvae (waste products of the host, such as feces, scales, silk and exuviae) to search for their host (Chiu-Alvarado et al. 2009, 2010). Similarly, the braconid wasp *Ascogaster quadridentata*, which is a parasitoid of the codling moth, *Cydia pomonella*, uses volatiles from scales and eggs of the female host moth to locate host eggs (DeLury et al. 1999). Studies have also highlighted the importance of visual cues in host searching behavior (Godfray 1994). For example, the wasp *Trichogramma ostriniae*, which is a parasitoid of the Asian corn borer, *Ostrinia furnacalis*, shows different responses to host egg color as compared to other colors while searching for hosts over short distances (Lobdell et al. 2005).

However, parasitoids not only recognize chemical and visual cues, but also are able to learn which cues lead them to the hosts most efficiently. The braconid wasp *Aphidius ervi* is a biological control agent of the pea aphid, *Acrythosiphon pisum*. When *Vicia faba* is infested by the aphid, the plant emits herbivore-induced plant volatiles (HIPVs) to attract natural enemies of the aphid, including the wasp. The HIPVs attract female wasps that have been exposed to the HIPVs during their developmental stages, but they do not attract naive female wasps, indicating that *A. ervi* learns to use the HIPVs (Gutiérrez-Ibáñez et al. 2007; Takemoto et al. 2009, 2012). The ichneumonid wasp *Pimpla luctuosa*, a parasitoid of the pupae of more than 15 lepidopteran species in different microhabitats, shows a preference for novel odors such as vanilla and strawberry only after several oviposition experiences in the presence of the odors (Iizuka and Takasu 1998). The braconid wasp *Psyttalia concolor,* which is a parasitoid of many tephritid fruit flies, uses the plant structures which conceal the hosts in its host search. Although the wasp does not show innate preference for color nor shape (but size of the hosts), the wasp can learn the color associated with hosts but not the shape (Segura et al. 2007; Benelli and Canale 2012). The pteromalid wasp *Nasonia vitripennis*, which is a parasitoid of pupae of several fly species, does not show innate color preference either, at least, for yellow versus blue. However, after trained, the wasp changes the response to colors associated with hosts and/or honey, although it needs multiple training sessions before exhibiting a preference (Oliai and King 2000).

Such associative learning would improve the host searching efficiency of parasitoids. Therefore, to better understand their host searching behavior, it is important to investigate the learning ability of parasitoids. Furthermore, because parasitoids use both chemical and visual cues, it is also important to understand how they use and learn each type of cue as well as their combination when both types are available. Few such comprehensive studies have been conducted (Wäckers and Lewis 1994; Jang et al. 2000), and our knowledge of natural enemies' use of visual cues is poor compared to that of chemical cues.

The egg-larval endoparasitoid wasp *Ascogaster reticulata* (Hymenoptera: Braconidae) is a promising biological control agent of pest tortricids (Lepidoptera: Tortricidae) in tea plantations, such as *Adoxophyes honmai*, *Adoxophyes orana fasciata*, and *Choristoneura diversana* (Kawakami and Kainoh 1986). The wasp uses contact chemical cues released by tea plants to search for host eggs and even has the ability to learn the chemical cues to find the eggs efficiently (Kainoh 1997; Seino and Kainoh 2008). Recent research revealed the mechanisms of induction of the contact chemicals associated with *A. honmai* egg deposition (Deshpande and Kainoh 2012; Piyasaengthong et al. 2016). Because *A. reticulata* is diurnal, however, the wasp may use visual cues in addition to chemical cues in its host searching behavior. Color may be especially useful information to detect host eggs because the colors of host egg masses are white to yellow, which differ from the green leaves on which they are laid. However, the use of visual cues and the associative color learning ability of *A. reticulata* are still unknown.

In this study, we investigated the innate color preference and associative learning ability for color of *A. reticulata.* To investigate the innate color preference, we carried out two-choice tests between a green paper and a paper of different color (black, blue, red, or yellow) with naive female wasps. To investigate the associative learning ability for color, we trained the female wasps using the color papers attached to the host egg mass, then carried out two-choice tests as in the experiment for innate color preference.

Materials and Methods

Host insects

We used a colony of *A. honmai* maintained for more than 10 years at the University of Tsukuba. Pupae were collected and placed with a ball of moist cotton wool into a plastic box (25 × 18 × 7 cm) in which the upper inner side was covered by wax paper for oviposition. Some of the egg masses were used for experiments. Other egg masses were collected together with the wax paper and placed into another plastic box (25 × 18 × 7 cm) with pieces of crumpled wax paper (9 × 4.3 cm). Larvae were reared under constant climate conditions (25 ± 1°C, 40–70% relative humidity, and 16:8 h light:dark photoperiod) on an artificial diet containing roasted soybean flour and tea leaf powder.

Parasitoid wasps

We used *A. reticulata* maintained for more than 10 years at the University of Tsukuba. A pair of adult female and male parasitoid wasps was placed into a plastic container (6.5-cm diameter, 4-cm height) for mating. After observing their mating, an egg mass of *A. honmai* (1–3 days old) was placed into the plastic container for 1 h to allow for oviposition. Each pair of wasps was allowed to oviposit once (for 1 h) per day for several days after mating, and 10 to 15 pairs were set up per generation. The parasitized egg masses were placed into a plastic box (25 × 18 × 7 cm) together with the artificial soybean and tea leaf diet and pieces of crumpled wax paper for the host insects. The larvae of these parasitoid wasps spin cocoons when the host insects develop into fourth-instar larvae. The parasitoid cocoons were collected, and the emerged females and males were kept in separate plastic containers (15-cm diameter, 9-cm height) with a ball of moist cotton wool and honey on the inner surface of the container as food. The virgin adult females were reared under constant climate conditions (25 ± 1°C, 40–70% relative humidity, and 16:8 h light:dark photoperiod) and used for the experiments.

Colored papers and other color conditions

Sheets of paper (4 cm × 2 cm) of different colors (black, blue, green, red, and yellow; Muse Inc., Tokyo, Japan) were used in the experiments. All experiments were carried out on a table covered by an ivory white tablecloth under 318-lux fluorescent lights (day white fluorescent lamps, spectrum range of 380–780 nm; FL20SS-ENC/18 LLN, Toshiba, Tokyo, Japan) to provide a consistent background color condition. The flicker frequency of this starter-type lamp was approximately 100 Hz. (The flicker fusion frequency for these wasps is unknown.) The reflectance spectra of the colored papers and the tablecloth were measured at wavelengths between 300 and 700 nm (Fig. 1) using BRC112E CCD array spectrometers and a fiber optic reflection probe (B&W Tek Inc., Newark, NJ, USA). The probe was held at 45° to the surface of each paper and the tablecloth, and the bulb illuminated the measuring area (5-mm diameter). The bulb was connected with a BDS-100 deuterium/tungsten light source (B&W Tek Inc.), and each paper was mounted on a black sheet to eliminate stray reflectance. Spectral reflectance functions were recorded at 10-nm steps and expressed relative to a white standard, which served as a reference for 100% reflectance.

Training for associative color learning

A sheet of colored paper (4 cm × 2 cm) was placed in a colorless, transparent glass Petri dish (9-cm diameter), and a host egg mass was placed on the sheet. A female wasp was released into the Petri dish and allowed to oviposit for 2 min after she had detected the egg mass and started oviposition. The training was carried out three times per female at 10-min intervals, in accordance with the previous paper about enhancement of learned response to plant chemicals in this wasp (Honda et al. 1996). Between training sessions, the female was kept in a Petri dish with honey lined on the inner surface. At 10 min after the last training session, each female was used in two-color choice tests.

Two-color choice tests

To investigate the innate color preference and the effect of training for associative color learning, we carried out two-color choice tests using naive female wasps and color-trained female wasps. A sheet of green paper and a sheet of black, blue, red, or yellow paper (4 cm × 2 cm) were placed in a colorless, transparent glass Petri dish (9-cm diameter) in parallel with a 2 cm gap between them, and then a female wasp was released into the Petri dish with an aspirator farthest to the color papers and close to edge of the Petri dish. The behavior of each female wasp was observed for 3 min after introduction, and the number of visits and the residence time on each sheet during the test period were recorded using The Observer ver. 5.0 (Noldus Information Technology, Wageningen, The Netherlands). In nature, the host egg masses are on a green background (i.e., tea leaves), hence we assumed green was the standard color and allowed female wasps to choose between green and the four other colors (hereafter, “alternative colors”).

Data analysis

Although it would be straightforward to analyze the number of visits and residence time on the alternative colored papers (black, blue, yellow, or red) rather than those on green paper, several female wasps did not visit the alternative colored papers at all. Therefore, we analyzed the number of visits and residence time on the green paper to avoid a complete or a quasi-complete separation.

We first checked the correlation between relative residence time on a green paper (residence time on a green paper / sum of residence times on green and alternative colored papers in a trial) and relative number of visits on a green paper (number of visits on a green paper / sum of the numbers of visits on green and alternative colored papers in a trial) using Spearman's rank correlation. Because we found a significant correlation between them, we analyzed the color preference based only on the residence time.

A generalized linear model (GLM) was used to analyze the residence time on green paper, in which the experimental variables were female experience (naive, green-trained, and alternative-color-trained), alternative color (black, blue, red, or yellow), and their interaction; a Gamma distribution was used as the error structure. Because Gamma GLMs do not accept values that are not positive, we replaced 0 with 0.001 in the model. We incorporated an offset with log-transformed total residence time (sum of residence times on green and alternative color in a trial) in the model, because we were interested in the relative residence time on green rather than the residence time itself. The effect of each explanatory variable was tested by a F-test in analysis of deviance (ANOVA) for GLM fit. To assess the wasp's innate color preference, we constructed a Gamma GLM using data only from naive wasps and tested the effect of alternative color in the same way. To assess the effect of training for associative color learning, we constructed a Gamma GLM for each alternative color and compared the relative residence times of green-trained wasps and of alternative-color-trained wasps with those of naive wasps using

Wald’s test. All statistical analyses were performed with the statistic software R version 3.2.0 (R Core Team 2015).

Results

Number of visits and residence time

The relative residence time on green paper (residence time on a green paper / sum of residence time on green and alternative colored papers in a trial) was significantly correlated with the relative number of visits (number of visits on a green paper / sum of the numbers of visits on green and alternative colored papers in a trial) (Fig. 2; Spearman’s rank correlation: rho = 0.926, $S = 34576$, $P < 0.001$). Therefore, we analyzed the color preference based only on the relative residence time. The relative residence time was not always consistent with the relative number of visits; there are one to two plots largely deviated from the linear relationship between the residence time and the number of visits in Figure 2. For example, one naive wasp visited a green paper once and a blue paper once in a trial, but the wasp stayed on the green paper for 44.33 sec and on the blue paper for only 0.97 sec. However, most of plots were on the line of relationship between the residence time and the number of visits.

Innate color preference

In naive female wasps, the preference for green differed significantly depending on the alternative colors (ANOVA for Gamma GLM: $F = 4.645$, df = 3, $P = 0.006$). The preference for green was strongest when the alternative color was black, and the preference was significantly greater than when the alternative color was yellow and red (Table 1; Fig. 3). Naive females also showed a preference for green over blue (Fig. 3b), but the preference for blue was not significantly different than that of black (Table 1).

Effect of associative color learning training

There was a significant effect of the interaction between female experience and alternative color on the relative residence time on green (ANOVA for Gamma GLM: $F = 2.3699$, df = 6, $P = 0.033$), indicating that the effect of associative learning training differed depending on the alternative color. Thus, we tested the effect of associative color learning training on the relative residence time for each alternative color.

When the alternative color was black, black-trained wasps showed significantly lower preference for green compared to naive wasps ($P = 0.028$), whereas the preference of green-trained wasps was not significantly different from that of naive wasps ($P = 0.826$; Table 2a and Fig. 3a). When the alternative color was blue, blue-trained wasps showed significantly lower preference for green compared to that of naive wasps ($P < 0.001$; Table 2b and Fig. 3b). Surprisingly, in this trial green-trained wasps seemed to show lower preference for green compared to that of naive wasps, although the difference was not significant ($P = 0.085$; Table 2b and Fig. 3b). When the alternative color was red, neither red-trained nor green-trained wasps showed a different preference for green compared to that of naive wasps (red-trained: $P = 0.771$, green-trained: $P = 0.239$; Table 2c and Fig. 3c). Likewise, when the alternative color was yellow, the relative residence time on green did not differ significantly depending on female experience (yellow-trained: $P = 0.180$, green-trained: $P = 0.103$; Table 2d and Fig. 3d).

Discussion

When a black or blue paper was offered together with a green paper, naive *A. reticulata* females showed greater preference for green over black and blue. When a yellow or red paper was offered together with a green paper, they did not show specific color preference. After training for associative color learning, *A. reticulata* changed its color preference; black-trained and blue-trained females showed lower preference for green compared to the naive wasps. However, such changes were not observed in red-trained and yellow-trained females. These results suggest that *A. reticulata* is able to distinguish black and blue stimuli from a green stimulus, prefers green over black and blue, and is able to modify the color preference after trained. The results also suggest that *A. reticulata* might not have color preference in the choice of green and yellow / red, and it might not be easy for the wasp to learn the association between the colors and the presence of hosts. Otherwise, the wasp might not be able to distinguish yellow and red stimuli from green stimulus.

Why *A. reticulata* showed greater preference for green over black might be explained by the egg color of the host. Innate color preference for yellow has been reported in several parasitoid wasps. For example, the egg parasitoid wasp *T. ostriniae* shows preference for yellow over white, green, and black, and the preference is explained by the yellow egg color of its lepidopteran host, *Ostrinia nubilalis* (Lobdell et al. 2005). Two other egg parasitoid wasps, *Telenomus podisi* and *Trissolcus basalis*, show preference for yellow over green, brown, black, and white, and the preference is also explained by the yellow egg color of their preferred hosts, *Euschistus heros* and *Nezara viridula*, although both parasitoid wasps are oligophagous and can parasitize various species of stink bugs (de Aquino et al. 2012). The egg-mass color of *A. honmai*, the host of *A. reticulata*, is also yellowish white or yellow, and the host egg-mass becomes blackish just before hatching. Considering the color change of the host egg-mass, the female wasps should avoid black, because blackish egg-masses are too old for their oviposition.

Yellow color is also important for *A. reticulata*, because of the host egg-mass color as mentioned before. Besides, some papers reported that starved female wasps show preference for yellow, because yellow is a common flower color, and flowers supply nectar and other substances to the wasps (Wäckers 1994; Uefune et al. 2013). In this study, we did not starve the female wasps during the experiment; however, we did not check if they were full. Since green color is also important for *A. reticulata* because the host lays egg-mass on leaves (= green), the wasp might have showed no preference between green and yellow. If so, innate color preference might explain why *A. reticulata* modified its color preference after black- and blue- trained but not after yellow- and red-trained. The monarch butterfly, *Danaus plexippus*, can learn any colors associated with the presence of honey; however, it takes much longer for it to learn the innately preferred color than non-innately preferred colors (Blackiston et al. 2011). It might not be easy to increase the preference for innately preferred color. For tests of these possibilities, it is necessary to train the wasp more intensively to see if the wasp eventually learns the innate preferred color or not.

Our discussion so far has assumed that *A. reticulata* is able to distinguish the tested four colors from green. However, if *A. reticulata* is not be able to distinguish yellow and red stimuli from green stimulus, all results might be explained simply by its color vision system. Insects have several different receptor types in their eyes, and detect colors based on an inhibitory interaction between these short-wavelength and long-wavelength sensitive receptor types (Kelber 2001). Many insects have three receptor types: receptor sensitive to the ultraviolet part of the spectrum (maximum close to 350 nm), receptor sensitive to blue (maximum at ca. 440 nm), and receptor sensitive to green (maximum at ca. 540 nm) (Kelber 2001). The yellow and red stimuli would also activate only the green receptors, making it difficult to distinguish either yellow or red stimuli from the green stimulus. In contrast, blue and black would induce a lower activation of the long-wavelength receptors, and thus *A. reticulata* would easily distinguish the color from green. Alternatively, the wasp might use achromatic (brightness) cues instead of chromatic one (wavelength, that is, colors) and thus simply chooses the brightest stimuli. Considering that the fluorescent lights we used is in the range of 380-780 nm (that is, without UV) and also the flicker rate might be below this wasp's flicker fusion frequency, we should consider this a possibility.

In conclusion, our experiments revealed that *A. reticulata* uses colors in the search for its host egg-mass and has an ability to learn some associated colors. As described previously, the wasp uses and learns contact chemicals released by tea plants, which are induced by *A. honmai* egg deposition (Deshpande and Kainoh 2012; Piyasaengthong et al. 2016). *Adoxophyes honmai* uses more than 90 species of host plants, including 44 families of broad- and needle-leaved trees and herbaceous plants (Minamikawa and Osakabe 1979). Hence, the wasp needs to respond to chemical cues from various host plants. In that case, the use of visual cues in addition to chemical cues would be helpful for its host search. This leads to the question of how the wasp uses and learns the combined cues when both chemical and visual cues are available. In future research, we will investigate the effects of combined cues on the host searching behavior of *A. reticulata* and its associative learning.

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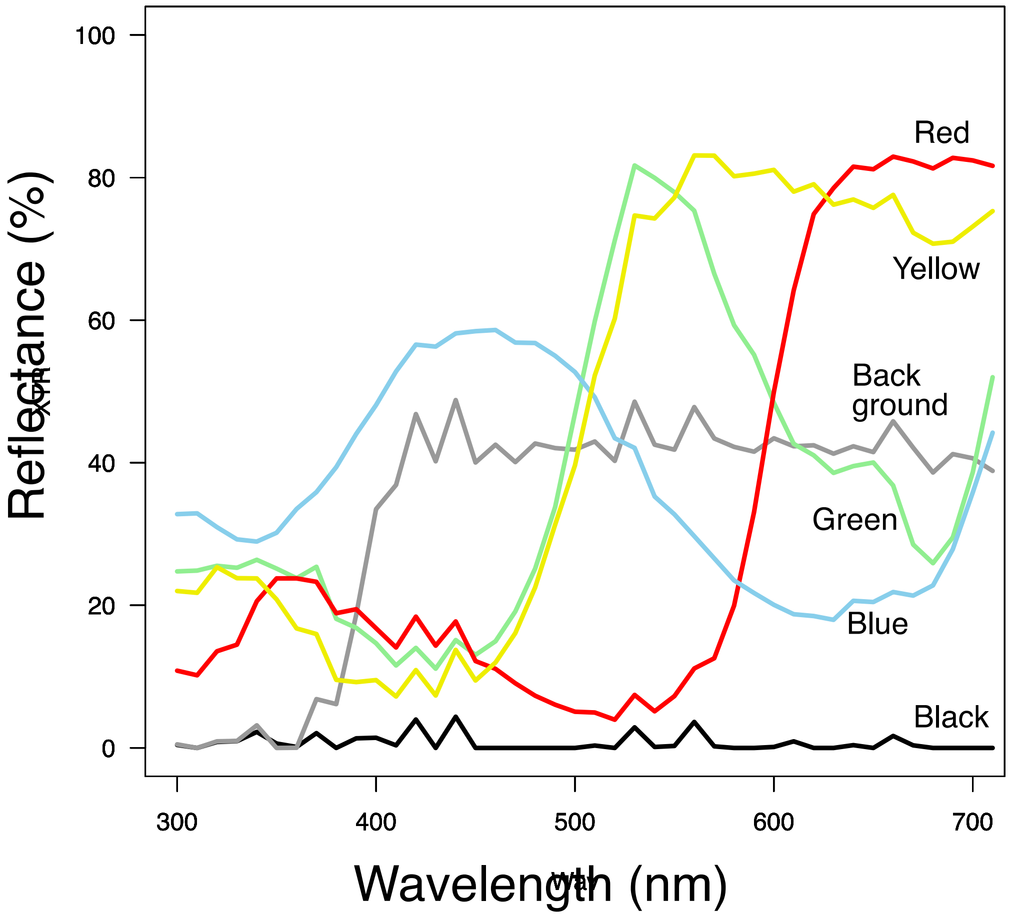
Figure legends

Figure 1. Reflectance spectra of colored papers (green, black, blue, red, and yellow) and of the tablecloth (ivory white) used as the background in the two-color choice tests.

Figure 2. Relationship between the relative number of visits and relative residence time on a green paper in naive and alternative-color-trained female *Ascogaster reticulata*.

Figure 3. Box plots of relative residence time on a green paper (residence time on a green paper / sum of residence times on green and alternative color papers in a trial) in two-color choice tests using naive, green-trained, and alternative-color-trained female *Ascogaster reticulata*, when the alternative color was black (a), blue (b), red (c), and yellow (d). In each box plot, the whiskers mark the minimum and maximum values, the dark line shows the median, and the box edges represent the first and third quartiles. The numbers of replicates are shown in parentheses. Asterisks show significant differences from naïve females (GLM, *significant at $P <$ 0.05;***significant at $P < 0.001$; for details, see Table 2).


Reflectance (%)
100
80
60
40
20
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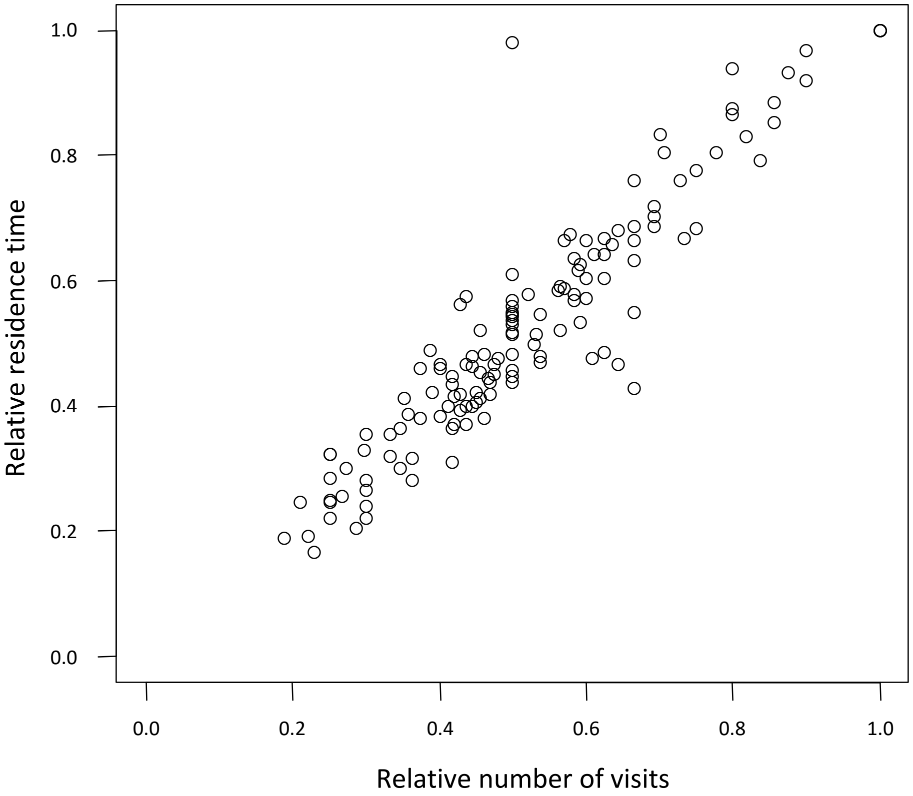
300
400
500
600
700
Wavelength (nm)
Red
Yellow
Back ground
Green
Blue
Black

Fig. 1

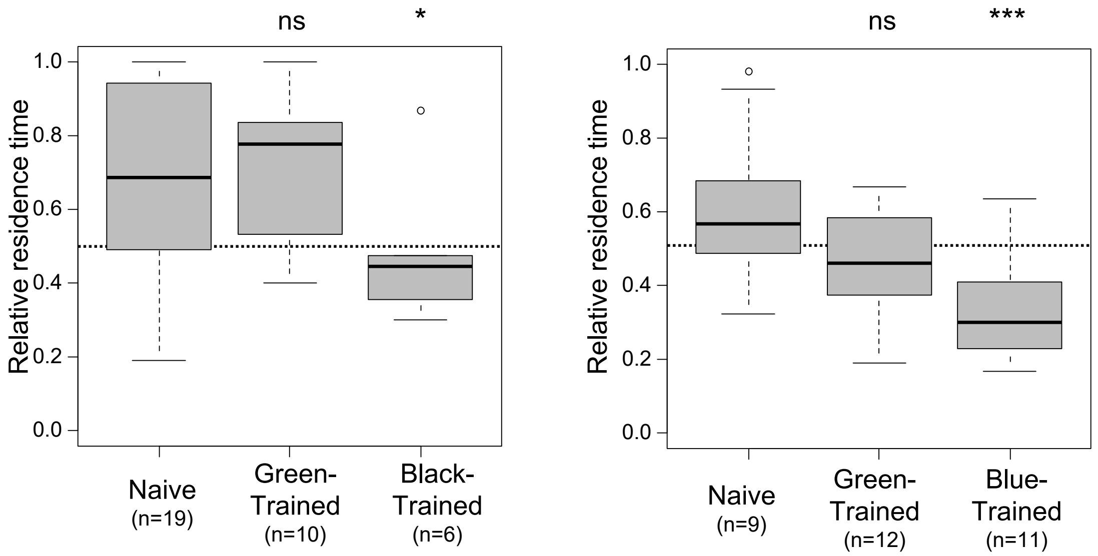


1.0
0.8
0.6
0.4
0.2
0.0
Relative residence time
0.0
0.2
0.4
0.6
0.8
1.0
Relative number of visits

Fig. 2

(a) Green vs. Black

(b) Green vs. Blue



(c) Green vs. Red

(d) Green vs. Yellow

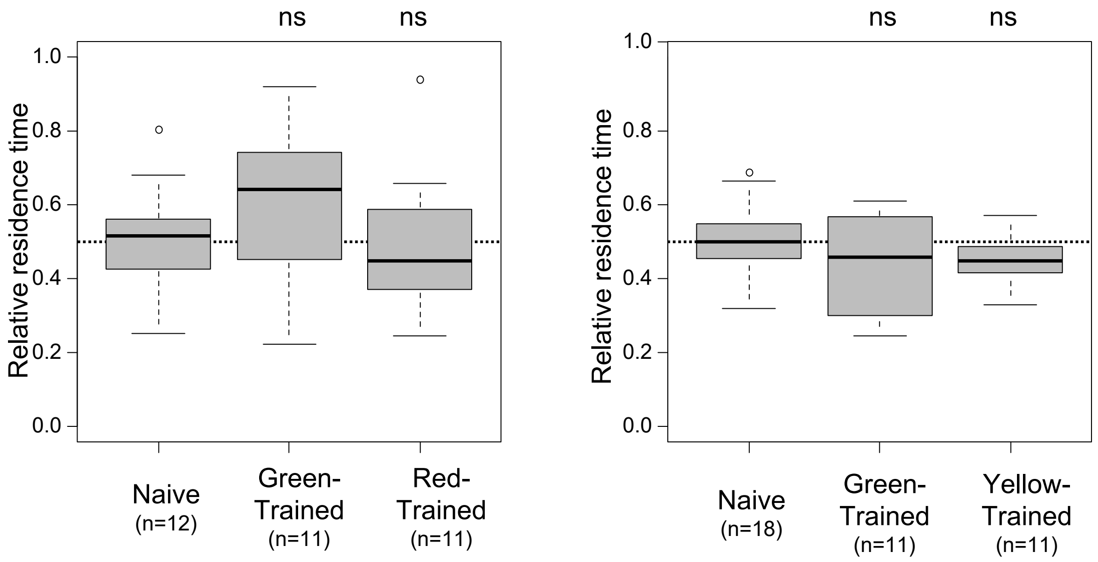


Fig. 3

Table 2. Generalized linear models of residence time on a green paper with varying levels of female experience (naive, green-trained, or alternative-color-trained) when the alternative colored paper was black (a), blue (b), red (c), or yellow (d). An offset with the log-transformed total residence time in a trial was incorporated into the model to assess the relative residence time in the choice test, and a Gamma distribution was applied as the error distribution.

(a)

Fixed effects	Estimate	SE	*t*	*p*
(Intercept)	-0.353	0.081	-4.382	< 0.001
Green-trained (vs. Naive)	0.030	0.137	0.222	0.826
Black-trained (vs. Naive)	-0.380	0.164	-2.309	0.028

(b)

Fixed effects	Estimate	SE	*t*	*p*
(Intercept)	-0.476	0.124	-3.836	< 0.001
Green-trained (vs. Naive)	-0.293	0.164	-1.785	0.085
Blue-trained (vs. Naive)	-0.619	0.167	-3.705	<0.001

(c)

Fixed effects	Estimate	SE	*t*	*p*
(Intercept)	-0.669	0.100	-6.717	<0.001
Green-trained (vs. Naive)	0.173	0.144	1.200	0.239
Red-trained (vs. Naive)	-0.042	0.144	-0.293	0.771

(d)

Fixed effects	Estimate	SE	*t*	*p*
(Intercept)	-0.681	0.055	-12.428	<0.001
Green-trained (vs. Naive)	-0.149	0.089	-1.673	0.103
Yellow-trained (vs. Naive)	-0.122	0.089	-1.368	0.180