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VERTICAL ORIENTATION AND COLOR CONTRAST AND CHOICES BY BUMBLEBEES (BOMBUS TERRESTRIS L.)

by

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Vertical orientation and color contrast and choices by bumblebees (*Bombus terrestris* L.)

Running headline: Arnon et al.: Perpendicularity & color contrast

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ABSTRACT

The vertical inflorescences of several plant species are terminated by colorful bracts, which attract insect pollinators. The bracts contrast in color with the leaves below them, and are oriented perpendicular to the flowers on the inflorescence. We conducted laboratory experiments to determine the effects of color contrast and perpendicular orientation on the feeding choices of bumblebees. We first trained bees to feeders with color-contrasting perpendicular displays, composed of a horizontal and a vertical display component. We subsequently recorded the bees' choices among feeders that displayed only one of these cues. The bees preferred perpendicular displays that resembled the training model in the color of the horizontal component. None of them chose a color-contrasting display that was not perpendicular. We then evaluated the effects of the horizontal vs. vertical components of perpendicular displays on the bees' choices. After training bees to color-contrasting perpendicular displays, we allowed them to choose between displays that had either the same horizontal or the same vertical component as the training model. Foragers mostly oriented to the horizontal displays to which they had been trained. Our results suggest that (a) bumblebees can learn to associate three-dimensional perpendicular color-contrasting displays with food rewards; (b) these displays are processed hierarchically, with orientation dominating color contrast; (c) The horizontal component of perpendicular displays dominates the vertical component. We discuss possible implications of our findings for the evolution of flower signals based on extra-floral bracts.

Keywords: bee, learning, color contrast, perpendicular orientation, extra-floral display

Extra-floral display structures exist in many insect-pollinated plant species. These displays include secondary structures associated with flowers (e.g. leaves, sepals and sterile flowers), but not composed of flowers themselves. Such structures, collectively called bracts, enhance the visual display of the reproductive organs to potential pollinators (Faegri & Van der pijl 1979; Barth 1985; Gottsberger & Hartmann 1988; Raven 1999). Some extra-floral displays are specialized in that they form colorful flag-like structures at the top of vertical inflorescences. In the Mediterranean flora, for example, flag-like bracts develop in three species of *Leopoldia*, in *Laminum moschatum* Mill., in *Salvia viridis* L. and in *Lavandula stoechas* L. These displays have been shown to attract insect pollinators to varying degrees, depending on species (Herrera 1997; Keasar et al. 2006). They thus may increase the reproductive success of individuals carrying them.

Why are pollinators attracted to flag-like bracts? Previous workers have suggested the bracts' large size as an important attractive feature. Large displays may function as "detective cues", i.e. make inflorescences more conspicuous for pollinators at a distance. They may also provide "selective cues", i.e. signal large floral food rewards to foraging insects (Cohen & Shmida 1993). It was further suggested that one large bract, visible from afar, can advertise many flowers on an inflorescence, and therefore provides a very efficient signal (Faegri & van-der Pijl 1979; Gottsberger & Hartmann, 1988). However, flag-like bracts share other features in addition to their large size. Although composed of leaves, they are colorful, and contrast in color with the foliage and flower petals. They are also located perpendicularly to the plane of the flowers: the bracts form a vertical display, while the flowers are commonly seen as a horizontal surface when viewed from above. This raises the question whether color contrast and vertical orientation may also function as attractive cues to pollinators in flag-like extra-floral displays.

Abundant experimental evidence indicates that pollinators use chemical and visual signals to detect potential food sources, and to select which sources to visit. Color seems to be the most important visual dimension for flower recognition in bees, a major group of pollinators (v.Frisch 1967; Menzel & Lieke 1983 ; Menzel & Backhaus 1991; Menzel & Shmida 1993; Lehrer & Bischof 1995; Giurfa et al 1996; Orth & Waddington 1997). The shape, symmetry, and patterns of the visual display affect the foraging choices of bees as well (Ne'eman & Kevan 2001; Rodríguez et al. 2004; Horridge 2005). Evidence for the attractive role of color contrast arises from the finding that bees prefer displays that contrast strongly with their background over displays with low background contrast (Lunau et al. 1996). In addition, large, high-contrast displays are detected more rapidly than displays that are small and / or low-contrast (Spaethe et al. 2001). The potential of a three-dimensional perpendicular display to attract pollinators has not been investigated before.

In the present study we investigate the role of color contrast, and a combination of horizontal and vertical display components, on the choices of bumblebees that foraged on flower dummies. Our study extends existing work on the role of color contrast in floral displays, and is the first (to our knowledge) to investigate the possible importance of perpendicular displays. We addressed the following specific questions:

1. Can bees learn to associate perpendicular color-contrasting displays with the presence of a food reward?

2. Which of the display cues affects the bees' foraging choices more strongly?

3. If bees are attracted to perpendicular displays, do they react equally strongly to the horizontal and the vertical components of the display?

METHODS

General

The experimental system and laboratory are described in detail in Keasar (2000). Experiments were carried out in a 3×4 m flight room. Temperatures ranged from 25-30° C, and relative humidity was 20-54%. The room was illuminated during 08:00-20:00 by six pairs of D-65 fluorescent lights of 100-Hz frequency. Experiments were conducted during November 1999-October 2000.

Colonies of naïve *Bombus terrestris* (L.) were obtained from Kibbutz Yad Mordechai, Israel. The queens of the colonies were treated by the suppliers to forego hibernation, so that the colonies were active year-round. Pollen was supplied *ad lib*, directly to the colony. The bees were allowed to fly freely inside the room, and to feed *ad lib* on 30% w/v sucrose solution, between experiments. During the experiments, only one bee, marked by a number tag, was allowed to forage at a time. Computer-controlled feeders, placed on a 1.40×2.40 m green table, were used for the experiments. All feeders had a removable colored plastic landing surface that could be replaced during the experiment. A 30% sucrose solution was used in the feeders as nectar substitute. The feeders dispensed either 1 microliter sucrose solution per visit, or no sucrose solution at all, according to experimental design. Feeders that were programmed to dispense sucrose solution were refilled immediately after the bee left them. Non-dispensing feeders contained sucrose solution that was not accessible to the bees, so that they could not be discriminated by their odor. Head insertions of bees into the feeders were automatically recorded. Horizontal and / or vertical rigid plastic visual displays were attached to the feeders. These displays were blue, yellow or purple in color. Bees from four colonies were used, and each bee participated in one experiment only. The feeders and experimental table were wiped with a water-moistened paper towel between experiments to eliminate odor marks. Feeders were randomly arranged on the table.

Pre-Training

Each bee was pre-trained prior to participating in an experiment. During the first stage of the pre-training, the bees were conditioned to fly to the experimental table to feed. Sucrose solution was first placed in a petri-dish feeder just outside the bee colony. After a bee learned to obtain sucrose solution from this feeder, it was gradually removed away from the colony until it was placed on the experimental table. The bees required 2-4 days for this part of the pre-training. During the next stage, the bee was trained to the morphology of the experimental feeder. The petri-dish feeder was replaced with a computer-controlled feeder, marked by a black circular horizontal landing surface of 3.7 cm diameter. The feeder dispensed 1 microliter 30% sucrose solution whenever probed by the bee. Pre-training on the computer-controlled feeder lasted until more than 5000 visits to the feeder were recorded per bee within 12 hours. This stage typically required 1-3 days.

Experiment 1: Learning of Perpendicular Orientation and Color Contrast as Foraging Cues.

The aim of this experiment was to test whether bumblebees can learn to associate perpendicular color-contrasting displays with the presence of a food reward. If yes, we wished to assess the relative importance of these two cues in the learning process. We used a two-step experimental design. In the *training phase* of the experiment, we exposed each of 17 inexperienced bees, originating from two colonies, to feeders of two types: twenty feeders carried a perpendicular display with color contrast, and dispensed 1 µl sucrose solution whenever probed. Twenty other feeders carried a blue horizontal round display with no vertical component and no color contrast, and contained no food reward (Fig. 1). The round displays were of 5.2 cm diameter. The vertical displays were ellipses with diameters of 4 cm and 2 cm. We used one of four random spatial arrangements of the feeders for each bee. Each bee was allowed to visit the feeders 200 times, and was then caged individually for 45 minutes while the *test phase* of the experiment was prepared.

In the *test phase*, the bee was exposed to non-rewarding feeders with the following five displays: 1. Horizontal yellow; 2. Horizontal purple; 3. Horizontal purple + vertical purple; 4. Horizontal yellow + vertical yellow; 5. Horizontal, half purple and half yellow (Fig. 1). We did not use blue displays in the *test phase,* because they were associated with both rewarding and non-rewarding feeders in the training phase, hence we did not have a clear expectation regarding their effect on forager choices. Each display was attached to eight feeders. Feeder locations for each bee followed one of seven random arrangements. We recorded the bees' choices on their first visit in this phase.

Experiment 2: The importance of horizontal vs. vertical display components.

The aim of this experiment was to test whether bees orient to the horizontal or to the vertical component of a perpendicular display. Here, too, we conducted a two-step experiment. In the first phase of the experiment, we exposed each of twelve experimentally naïve foragers to feeders of two types: twenty feeders carried a perpendicular display with color contrast (blue horizontal and yellow vertical displays), and dispensed 1 µl sucrose solution whenever probed. Twenty other feeders carried a yellow horizontal display with no vertical component and no color contrast, and contained no food reward (Fig. 2). We used one of four random spatial arrangements of the feeders for each individual. Each bee was allowed to visit the feeders 200 times, and was then caged individually for 15 minutes while the second phase of the experiment was prepared. In the second phase, each bee was allowed 200 additional visits to 20 rewarding feeders with a perpendicular display but no color contrast (yellow horizontal and yellow vertical displays), and to 20 non-rewarding feeders with a blue horizontal display with no vertical component or color contrast (Fig. 2). We recorded the forager's sequence of visits in both phases. We randomly rearranged the feeders on the experimental table between experimental phases, to control for location learning. To control for possible color biases, we replicated the experiment with twelve additional foragers that were exposed to the reciprocal color combinations (Fig. 4). The shapes and sizes of the horizontal and vertical display components were as in experiment 1. Bees originated from two colonies in this experiment.

RESULTS

Experiment 1

Thirteen of sixteen bees directed their first visit in the *training phase* to a feeder that bore a perpendicular and color-contrasting display (the recording of the first visit of the 17^{th} bee was ambiguous). Thus, this display was significantly preferred (one-tailed sign test, $p=0.010$). All bees directed most of their visits to the rewarding feeders (that carried perpendicular, color-contrasting displays) by the end of the *training phase*.

Thirteen individuals probed a feeder with horizontal purple + vertical purple display on their first visit in the *test phase*. We defined this choice as a "success" in a Bernouli experiment. The probability of obtaining 13 out of 16 successful outcomes, if the success probability is 0.2 (i.e., the bees choose randomly among the five displays), is <0.0001 (Binomial test), indicating a strong preference for this display. The four bees that did not visit the purple perpendicular display probed a feeder with a horizontal purple display (Fig. 3).

The foragers' choices in the *test phase* may have been biased by the location of the rewarding feeders in the *training phase*, i.e. bees may memorize locations that had been rewarding, and return to them regardless of the feeders' display. This possibility is however unlikely: at the start of the *test phase*, the majority of bees ($n=9$) visited a location that had not rewarded them during the *training phase*. Only 6 individuals visited a location that had been previously rewarding. Two individuals were excluded from this analysis because of ambiguous recording of the location of the first visit.

Experiment 2

One half of the subjects in this experiment were trained to non-rewarding blue displays, while the other half experienced the reciprocal color combination. The results were very similar for both experimental sets, and were therefore pooled for analysis. In the first phase, 91.6% of the bees (n=24) directed their first visit to a feeder with a perpendicular color-contrasting display. Almost all subsequent visits were also directed to these feeders, which contained sucrose reward (Fig. 4). Three bees stopped foraging before the end of the first phase. Of the remaining 21 individuals, seventeen continued to visit the feeders in the second phase. At the onset of the second phase, all bees (n=17) visited a feeder that had the same horizontal display as the rewarding feeders in the first phase (these feeders did not contain sucrose reward in the second phase). Three individuals persisted in visiting non-rewarding displays only. The remaining bees gradually shifted to visiting mostly rewarding feeders, but chose them less frequently than during the first phase (Fig. 4).

DISCUSSION

Our results indicate that bees can learn to associate a three-dimensional perpendicular, color-contrasting display with the presence of a food reward. Experiment 1 was designed to test whether both of these cues contribute equally to the bees' choices, or whether they are processed hierarchically. In this experiment, bees were first trained to perpendicular, color-contrasting displays. In the *test phase*, they were presented with feeders that had only one of these display cues (either color contrast or perpendicular orientation), or none of them. We expected the bees to choose the color-contrasting display in the test phase if they learned to associate color contrast with reward during training. Similarly, we expected them to visit the two uniform-colored perpendicular displays in the test phase, if they formed an association between perpendicular orientation and sucrose reward during training. None of the bees chose a color-contrasting feeder, suggesting that their choices were not primarily guided by the color-contrast signal. However, the possibility that the bees learned to orient to perpendicular displays, was not clearly supported either: most foragers chose the purple perpendicular displays, but none of them chose the yellow perpendicular display.

Two interpretations could explain this choice pattern. First, the foragers may have been attracted to any display with a large purple area, regardless of their experiences in the training phase. This interpretation is compatible with the fact that a few individuals chose purple horizontal displays in the test phase, and with evidence for innate preference for blue color components in bumblebees (Keasar et al. 1997; Gumbert 2000; Chittka et al. 2004). Alternatively, bees that had been trained to blue horizontal displays may have oriented to displays of a similar color (horizontal purple) in the test phase. This interpretation is consistent with previous evidence that color dominates shape in the hierarchy of cues that affects foraging decisions of bees (Gould 1993). It is also compatible with the tendency of bees to generalize learned color-reward associations, i.e., to visit displays that are similar in color to those that had rewarded them in the past (Gumbert 2000; Dyer & Chittka. 2004a). The ability of bees to discriminate between pairs of similar colors diminishes if they are not exposed to both colors concurrently (Dyer & Chittka 2004b; Giurfa 2004; Dyer & Neumeier, 2005). This was the case in experiment 1, since foragers were first trained to blue displays, and only later exposed to the purple feeders. This design may have increased the bees' tendency visit purple feeders on the basis of their color similarity to the training feeders. This interpretation implies that the horizontal component of the perpendicular display in the *training phase* (blue) may have affected the bees' later foraging choices more than the vertical component (yellow). Thus, bees in experiment 1 may have learned to orient to a blue/purple horizontal surface with any kind of vertical attachment. This possibility is explored in experiment 2, and supported by its results.

Experiment 2 was constructed as a binary choice design to reduce the variability generated by multiple choices in experiment 1. An additional improvement of experiment 2 over experiment 1 lies in its reciprocal design: in the *training phase* of experiment 2, one half of the bees foraged on color-contrasting, perpendicular display feeders with a blue horizontal component, while the remaining bees experienced similar feeders but with a yellow horizontal component. This design allowed us to assess whether the bees associated the reward with a particular color (e.g. blue) or a particular orientation (e.g. horizontal) during training.

In the first phase of this experiment, as at the onset of experiment 1, almost all experimentally naïve bees preferred a color-contrasting perpendicular display to a uniformly colored horizontal display. This finding hints at an innate preference for these display features, which requires further investigation. In the second phase, on the other hand, all bees chose a uniformly colored horizontal display that had the same color as the horizontal display of the first phase. This finding implies that (a) the bees learned the color of the horizontal component of the rewarding feeders during the first phase, even though the vertical component was also available as a cue. (b) This learning led them to discriminate against a color-contrasting perpendicular display that did not match the learned horizontal display at the beginning of the second phase. (c) Yellow horizontal displays were learned as effectively as blue horizontal displays.

A competing interpretation of the bees' choices at the onset of the second phase is that they had learned to avoid horizontal displays with no color contrast, since these displays did not reward them in the first phase. This interpretation suggests that negative reinforcement in the first phase strongly affected the bees’ choices in the second phase. We consider this interpretation less probable, because eight of the bees did not probe the non-rewarding feeders at all throughout the first phase, hence did not have an opportunity to associate their display with lack of reward. These bees nevertheless visited feeders with perpendicular display at the start of the second phase.

The results of experiments 1 and 2 are consistent in that they suggest a strong effect of the horizontal display component on later choice. The bees landed on the horizontal display components, and stood on them while imbibing sucrose solution. This prolonged exposure to the horizontal component may have strengthened its association with food reward for the bees. Moreover, bees may have learned primarily the horizontal-component color, because color discrimination is significantly better in the lower half of their visual field than in the upper half (Lehrer 1998). The effect of the horizontal display was strongest in experiment 2, where all subjects chose the color of the horizontal component that had previously rewarded them. In experiment 2, only 13 out of 16 bees showed this choice pattern. This difference may be due to the binary design of experiment 2, as opposed to the five-choice design of experiment 1. Reducing the number of choices may facilitate decision-making for bees, enabling them to choose their preferred displays more accurately.

Preference for the rewarding feeder formed more slowly in the second phase of experiment 2 than in the first phase. Three bees visited only the rewarding feeders throughout the first phase. Interestingly, in the second phase, these individuals visited (rather languidly) only feeders with the horizontal display color that had rewarded them initially (i.e., they were never rewarded in the *test phase*). This finding agrees with previous reports on strong effects of naïve experience on later foraging choices in bees (Gumbert 2000). Other bees did modify their display preferences, but the rate of change varied greatly between individuals, as reflected in the large error bars of Fig. 4. This suggests that individuals vary in their tendency to abandon their innate preferences and/or early learning experience when making foraging choices. Individual variability in choice behavior among bumblebees has been documented before, and was ascribed to a tradeoff between foraging speed and choice accuracy (Chittka et al. 2003; Dyer and Chittka 2004c), and to differences among colonies (Raine et al. 2005).

Our experiments were inspired by field studies that suggest a role for extra-floral displays, such as flag-like bracts, in pollinator attraction (Herrera 1997; Keasar et al. 2006). We recognize the difficulty of extrapolating results obtained in a sterile laboratory setting to the behavior of animals in complex "real-life" situations. Nevertheless, a few features of bee behavior that emerged in the laboratory experiments may aid in the interpretation of field observations. First, several features of floral displays may have coevolved with the foraging preferences of their pollinators. A case in point is the high prevalence of blue-violet flowers in the European flora, which coincides with an innate preference for these colors in bees (Chittka et al. 2004). Similarly, the color-contrasting perpendicular display in flag-like bracts may have coevolved with an innate pollinator preferences for such displays suggested by our results. Second, our experiments show that bees respond to the horizontal component of the display (inflorescence) more strongly than to the vertical display (flag-like bract). This behavior could be adaptive, because inflorescence development is not completely synchronized with bract development (Herrera 1997; Keasar et al. 2006). In situations of imperfect synchrony, pollinators may enhance their foraging success by assigning greater weight to inflorescence visual cues than to the visual signal of the vertical bracts.

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FIGURE LEGENDS

Figure 1: Design of experiment 1. In the *training phase* (top), we allowed each of 17 naïve bees 200 foraging visits to feeders of two displays that were either rewarding (+) or non-rewarding (-). In the *test phase*, we exposed each bee to feeders of five displays and recorded their first choice. Feeders are drawn schematically from a side view.

Figure 2: Design of experiment 2. In the *training phase* (top), we allowed each of 24 naïve bees 200 foraging visits to feeders of two displays that were either rewarding (+) or non-rewarding (-). Rewarding feeders carried color-contrasting, perpendicular displays, while non-rewarding feeders had horizontal one-color displays. One half of the bees were trained to blue horizontal displays (left figure), and the remaining bees were trained to horizontal yellow displays (right figure). In the *test phase*, the bees made 200 additional visits to feeders that carried either the same horizontal or vertical display components as rewarding feeders in the *training phase*. Feeders are drawn schematically from a side view.

Figure 3: The frequency of choices of the five displays offered in the test phase of experiment 1. First choices of 17 bees, which had completed the training phase, were recorded.

Figure 4: Mean visit frequency to rewarding feeders in the first (dashed line) and second (solid line) phase of experiment 2. Data are based on visit records of 24 bees in the first phase, and 17 individuals in the second phase. Error bars are 1 SEM.

Fig. 1

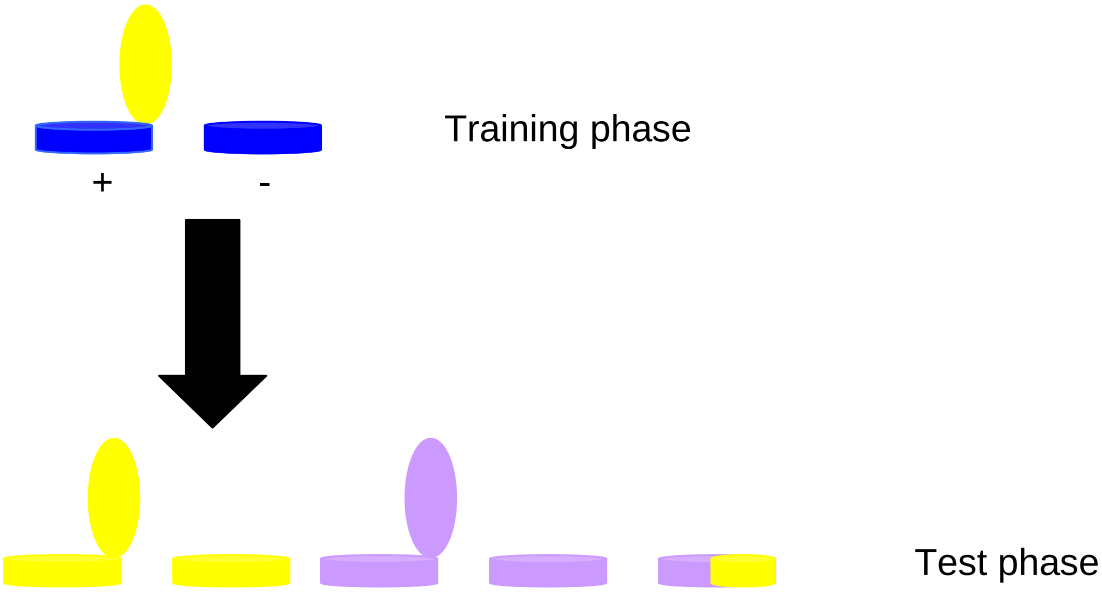


Fig. 2

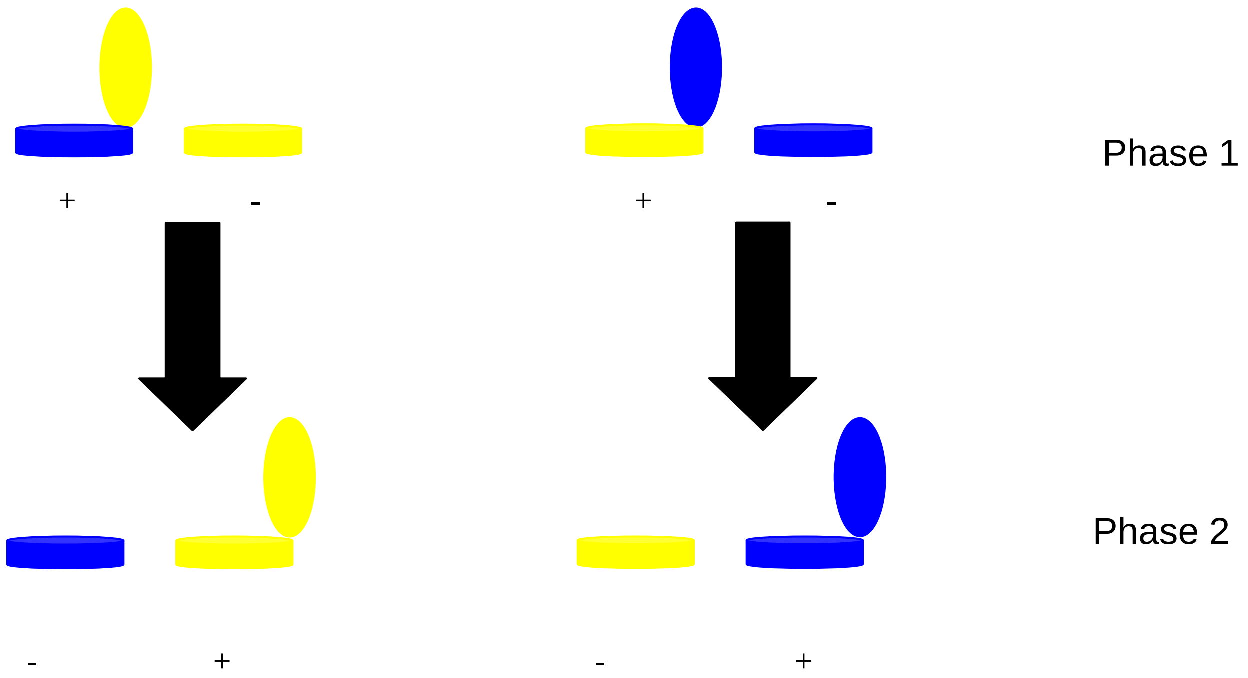


Fig.3

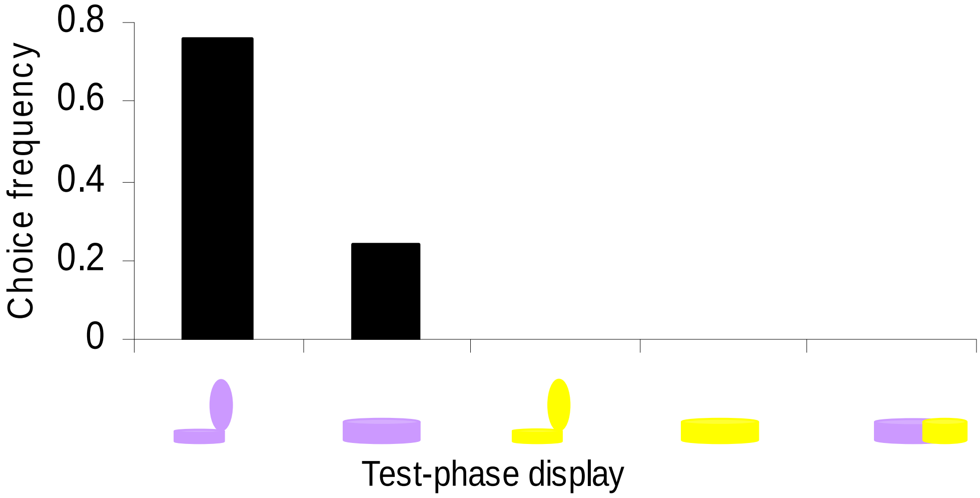
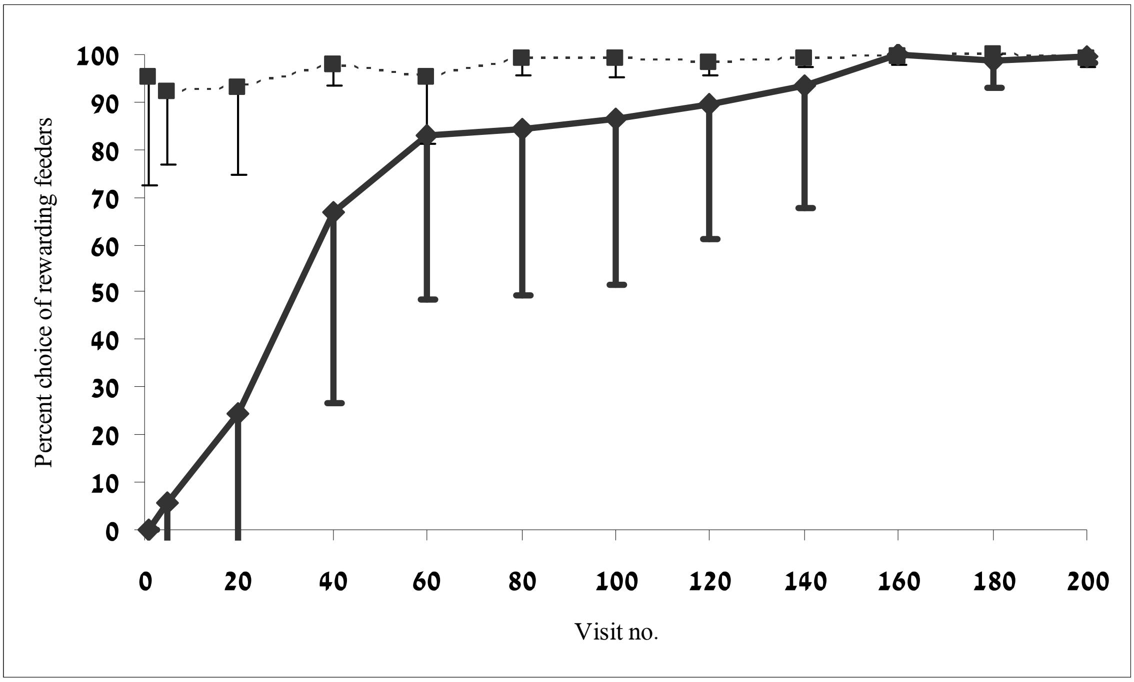


Fig. 4



Percent choice of rewarding feeders
0
10
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90
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140
160
180
200
Visit no.