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INSIDE THE TRAP OF A YELLOW LADY'S SLIPPER ORCHID (CYPRIPEDIUM PARVIFLORUM VAR. PUBESCENS): THE EFFECTS OF 'LIGHT WINDOWS' AND FLOWER ORIENTATION ON THE BEHAVIOR OF A NATIVE BEE (ANDRENA MACRA)

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INSIDE THE TRAP OF A YELLOW LADY'S SLIPPER ORCHID (*CYPRIPEDIUM PARVIFLORUM VAR. PUBESCENS*): THE EFFECTS OF 'LIGHT WINDOWS' AND FLOWER ORIENTATION ON THE BEHAVIOR OF A NATIVE BEE (*ANDRENA MACRA*)

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by

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ABSTRACT

Darwin's (1862) famous work on the mechanisms and diversity of pollination in the Orchidaceae highlights the fascinating nature of plant-pollinator interactions in a variety of orchid genera. One particularly interesting aspect of orchid pollination is that about one third of orchid species is deceptive and offers no reward to pollinators. Despite the interest in orchid pollination, relatively few studies have observed pollination in non-rewarding orchids and documented successful pollinator species. This has led to the persistence of various untested hypotheses concerning some pollination mechanisms. For example, the light windows hypothesis posits that the thin tissue beneath the exit holes of the labellum serve as light windows to guide pollinators to the correct escape route out of the labellum. However, many insects are also influenced by geotaxis, and the labellum might also capitalize on innate negative geotactic behavior of visiting insects by providing a vertical escape passageway in the labellum. Despite profuse discussion of the alleged function of light windows in the literature, this is the first study to rigorously test the light windows hypothesis and determine the influence of flower orientation on insect behavior. In order to test these ideas, *Andrena macra* bees were introduced into experimentally manipulated labella of *Cypripedium parviflorum* var. *pubescens* plants in the field as well as choice chambers in the laboratory with the following goals in mind: (1) to determine if light windows are required for pollinators to orient to the correct escape route and exit the plant, (2) to understand the influence of labellum orientation on pollinator behaviors, and (3) to correlate field observations with phototactic and geotactic responses in the laboratory. There were no significant differences observed in bee behaviors for plants with light windows covered compared to control plants, and the bees still oriented to the proper exit even when additional light from the exit holes was blocked. However, there were significant differences in bee behavior when the labellum was abnormally oriented, with bees tending to attempt an incorrect exit much more often when flowers were tilted 90 ° backwards or sideways. For *Andrena macra*, light windows in *C. parviflorum* were not required for their proper orientation to the correct escape route or escape from the plant. Finally, laboratory data were consistent with field data, suggesting that gravity, not light, more strongly influences correct exiting behaviors.

KEYWORDS

Light windows, geotaxis, phototaxis, Cypripedium, Andrena macra, pollination, lady's slipper, orchid

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INTRODUCTION

The unique and complex mechanisms of orchid pollination have enticed naturalists and sparked a wealth of scientific interest for over a century (Darwin, 1862; van der Pijl and Dodson, 1969; van der Cingel, 2001). Almost one third of orchid species are non-rewarding plants that deceive visiting insects into pollinating the flower with advertisement of a reward that is not there (Schiestl, 2005). Despite the interest in the mechanisms of deceitful pollination, relatively few studies have observed the pollination process and documented effective pollinating species (Nilsson, 1979; Case and Bradford, 2009). This is because visiting insects learn to avoid non-rewarding flowers (Dafni, 1984; Nilsson 1992; Bernhardt and Edens-Meier, 2010) which makes pollinations and observations infrequent.

The unique morphology and pollination process of the non-rewarding yellow lady's slipper orchids have been well documented (Darwin, 1862; van der Pijl and Dodson, 1969; Nilsson, 1979; van der Cingel, 2001; Herring, 2007; Case and Bradford, 2009). The common yellow lady's slipper, *Cypripedium parviflorum* var. *pubescens* (Willd.) Knight, is considered a generalist attractor because the bright yellow color and sweet aromas produced by the flower attract a variety of insects (Case and Bradford, 2009). However, despite the high diversity of visiting insects, few are successful pollinators because of the unique trapping mechanism found in this group of orchids. In all members of the subfamily Cypripedioideae, one petal is modified into a slipper-like structure called the labellum (Fig. 1). Visiting insects enter a large entrance hole in the labellum in search of food (or brood place in some species). However, they cannot get

out the way they got in because of the depth of the labellum, waxy curved interior, and/or

incurved labellum margin. To escape, they must orient to the rear of the flower and

climb up a vertical passageway in the back towards the exit holes, one on each side of the

labellum. In this process, pollinators fit tightly and push against the stigmatic surface,

thus widening the exit holes for a more efficient escape and depositing any pollen that is

on their dorsal thorax. Partially blocking each exit hole is a pollinium with a glutinous

mass of pollen that becomes stuck to the insect if it has to squeeze through the exit hole.

When pollinators enter another labellum, they must repeat this escape process and in

doing so deposit the pollen on the stigmatic surface of the new plant. Therefore, although

this species is a generalist attractor, the size and dimensions of visiting insects are critical

factors in determining the ability to pick up and deposit pollen (Nilsson, 1979; Bänziger

et al., 2004; Case and Bradford, 2009). Most often, pollination of *C. parviflorum* occurs

with small, solitary bees in the genus *Andrena.* This is typical of the subfamily

Cypripedioideae, which most commonly use members of the Andrenidae as pollinators

(Cribb, 1997).

A morphological feature of the yellow lady's slipper orchids that has generated a

great amount of debate is the thin layer of tissue just below the exit holes (Fig. 2; van der

Cingel, 1995). This area allows more light to penetrate the labellum than the surrounding

tissue. For over 100 years, these thin areas have been assumed to function as light

windows to guide pollinators towards the correct escape route (Webster, 1886). This idea

has taken hold and is widely disseminated on the web, in books, and in the scientific

literature (e.g., Webster, 1886; Barth, 1991; Argue, 2001; Van der Cingel, 2001;

Bänziger, 2004; http://culturesheet.org/orchidaceae:cypripedium). Based on multi-year

observations, Daumann (1968) noted that the ecological importance of light windows was at least "overvalued." Moreover, he anecdotally stated that plants with covered light windows did not appear to have an effect on bee behavior but also stated that a more rigorous test was needed.

A major goal of this thesis was to determine if light penetration through the windows was required to guide insects towards the correct escape route. This was accomplished by experimentally covering the light windows in a large population of lady's slipper orchids and recording its effects on a bee's ability to exit the proper escape route. In the past, such experiments have been extremely difficult to accomplish due to the paucity of large populations of orchids as well as a large sample size of bees belonging to a single species. Moreover, these bees must be within the size range that would make suitable pollinators of the orchids and preferably be recorded pollinators or at least phylogenetically related to the actual pollinators. In addition, adequate sample sizes of bees and flowers in such experiments would seem to be of paramount importance due to the likelihood of environmental variables that could affect outcomes. In 2009, a bee emergence site was located on the campus of William & Mary that made this experiment possible. The primary bee species used in this study was *Andrena macra* Mitchell, a relatively rare bee species that ranges from West Virginia and Maryland down to Florida and westward to Texas and Oklahoma (Riddick, 1990; Sam Droege, pers. comm.). Like other solitary bees, *A. macra* is a ground-dwelling species known to inhabit sun-exposed roadsides and sandy embankments (Riddick 1992). Even though each *A. macra* bee has its own nest, Riddick (1992) found that nest distribution is clumped, thus resulting in large populations sizes in small plots of land. This is

consistent with the nest site used in this study which contained several thousand bees along a sunny embankment adjacent to a walkway.

During the course of initial experiments, it became evident that the interior orientation of the flower could also play a significant role in assuring that bees exit correctly, particularly by capitalizing on negative geotactic behaviors. Therefore, a second goal of this thesis was to determine how the orientation of the labellum influenced a bee's successful exit. This was accomplished by introducing bees into flowers that were rotated 90 °backwards or 90 °sideways. Finally, in concordance with the field trials, a series of lab experiments was devised in order to test the phototactic and geotactic responses of bees in a more controlled setting. The main purpose of these experiments was to see if bee behavior in the lab correlated with behaviors in the field.

MATERIALS AND METHODS

Bee manipulations:

The bees used in the study were tentatively identified as *Andrena* spp. at the time of the study and were collected from a single nesting site, which was located along a sandy embankment flanking a campus walkway approximately 400 meters from the orchid population. The bees were very active on warm sunny days when many hundreds could be found darting back and forth above their emergence holes as well as climbing in and out of their holes, and flying out of the general area. The vast majority of bees looked morphologically similar during field observations, but occasionally a bee with a larger and slightly more pubescent morphology was evident. Some of these larger bees were observed mating with the smaller bees, and it was therefore assumed that at least

some of the larger bees were females. Bees were captured in sweep nets and transferred to pre-numbered 20 ml glass vials which were placed in a cardboard box with a lid. Fifty to 100 bees were collected in one session and transported to the orchid field site where the bee box was kept in a cool shady place on the forest floor. The vast majority of bees were used in the field trials within one to two hours of capture. For each field trial, one bee was randomly chosen from the bee box and introduced to a flower using a modified syringe that had the needle removed and the narrow end of the barrel sawed off. To get the bees into the syringe, the barrel was placed on top of the open vial containing the bee, and the bee would attempt to escape by crawling out of the vial and into the barrel. The plunger of the syringe was then gently pushed, forcing the bee out of the barrel and into the labellum. The bee's original vial was held near the exit hole of the flower to recapture the bee as it escaped. In trials with the exit hole blocked or in other treatments with the labellum in a different orientation, recapture was considerably more difficult and resulted in a higher frequency of escapes into the field site.

For all field treatments, verbal accounts of each bee's behavior were recorded with a digital recorder from the moment of the bee's introduction to the flower until its exit or after five minutes had elapsed. Behaviors were later transcribed into a notebook and specific response variables were chosen for analysis. These included (1) direction of the first escape attempt, (2) time to orient to the correct escape position in the back of the labellum, (3) time to escape from the flower, and (4) location of the bee's exit (e.g., entrance or exit hole). The correct escape position was defined by a bee assuming a vertical orientation behind the stigma with its abdomen barely visible and its head in the

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center of the two adjacent exit holes. When apparent, it was also noted in the field if bees

removed pollen upon exit.

A total of 298 bees were collected and used for field, lab, and/or identification

purposes. For field trials, 244 of these bees were used. Bees used for the laboratory trials

comprised a subset of those used in the field trials (n=19; approximately 7% of the field

trials) as well as bees from the nest site that were not associated with field trials (n=13).

Most bees were tested on the same day as collection, and the remaining no later than the

following day. Bees were kept in their field vials and stored in the lab at 20 °C until

used. They were provided approximately 0.5ml 10% sucrose solution (w/w) placed on a

small cotton ball in the bottom of their vials which they were observed drinking,

especially after field trials.

To produce a voucher collection, 162 bees were euthanized with ethyl acetate and

pinned. These bees represented a subset (49%) of bees from lab and/or field experiments

with the remainder collected from the nest site but not used in experiments. Almost all of

the large morphotypes that were assumed to be females were recaptured and euthanized.

Bees that exited the flower correctly were examined for the presence of *Cypripedium*

pollen. However, due to the need to use individual flowers several times, pollen was not

always available so quantitative assessments of pick-up rate are not available. Bees were

identified and sexed by Sam Droege, US Geological Survey, Patuxent Wildlife Research

Center, Beltsville, Maryland.

Orchid field trials:

Field experiments were conducted on a population of *C. parviflorum* var.

pubescens located in a mixed hardwood forest. The population is located on the upper

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portion of a slightly sloped ravine with *Fagus grandifolia* Ehrh., *Liriodendron tulipifera* L., and *Quercus alba* L. as the dominant canopy trees. The most common understory trees are *Ilex opaca* Aiton. and *Cornus florida* L. Common herbaceous species that were evident at this time include *Arisaema triphyllum* (L.), Schott, *Obolaria virginica* L. and *Podophyllum peltatum* L.

The population was approximately 300m² and estimated to contain 45 visible genets in 2010, with each genet producing one or a few ramets. Ramets were assumed to be members of the same genet if their shoots appeared to come from a common point. Most genets were located at least 1 meter apart. There were 30 flowering stems with no more than 2 flowering ramets per genet, with the exceptions of one genet that contained 3 flowering stems and one stem with 2 flowers. There were also several vegetative *C. parviflorum* var. *pubescens* plants in the plot.

As stems emerged, they were checked for the presence of a flowering bud by gently feeling the top for a swelling. Stems that were predicted to flower were covered with mesh bags made of mosquito netting to exclude pollinators before the start of the experiment. Each genet with a bud was assigned a number and each flowering ramet within each genet was assigned a letter. In order to reduce experimental error associated with potential microsite differences in the plot, the closest adjacent flowers were paired and each flower was randomly assigned to a control or treatment group, resulting in 15 control flowers and 15 treatment flowers. In all cases, ramets within a genet were assigned to different experimental groups. Each bee was used only once for the field trials, but the orchids were re-used due to a small sample of flowers relative to the number of trials required. Each flower was used between 1 and 6 times. This variance is

explained by the number of flowers available for experimentation on any given day which was affected by differences in flowering times and bloom longevity. All trials systematically rotated through treatments and flowers in the population to randomize effects of flower, day, time, temperature, and light.

To test the effectiveness of light windows in orienting pollinators to the exit hole, yellow plastic tape (manufacturer 3M, product # 190 YL) was cut according to the shape of the light window and applied to the exterior of the labellum over the light window using Tanglefoot Insect Trap Coating gel. The effectiveness of the cover was examined by bringing a flower (not used in the experiment) into the laboratory and illuminating the bottom of the labellum (Fig. 2). The plastic vinyl cover actually produced a more effective barrier to light than did the surrounding tissue. Insects were introduced into the control and treatment plants alternately. This experiment ran from April 12 through April 22, resulting in a simple size of 61 controls and 60 treatments (called "window treatments" hereafter). Due to a small number of putative female bees collected during the experiment (n = 25, included in the above sample sizes), they were run only through control and window treatments as described above.

When it was apparent from the field trials that the light windows had no noticeable effect, additional experiments were added on April 16. The first of these used the pre-defined window treatment plants by wrapping an opaque black ribbon around the exit holes (the "bandito" treatment) thus blocking light from both exit holes and the light windows. The ribbon was gently clipped together in the back of the labellum using a small metal binder clip (Fig. 3A). This experiment ran from April 16 through April 22 and included 37 bandito treatments and 39 control treatments. The control plants used in

this analysis were a subset of the control plants used in the control vs. window treatment

experiment, comprising only those controls that were concurrent with the bandito trials.

Additional experiments added on the same data as the bandito treatment include the

orientation experiments described below.

The orientation experiments were designed to understand how the physical

orientation of the labellum affects the ability of bees to find the correct exit route. Two

additional treatments were added, the "back bend" and the "side bend" treatments. In

these trials, the treatments as well as the controls were conducted on the same set of

plants (i.e., the "control" set described above) and systematically rotated through

treatments and flowers as described above. The back bend treatment involved bending

the stem back 90 ° such that the distal tip of the labellum pointed upward, placing the

otherwise vertical exit passage in a horizontal configuration (Fig. 3B). The side

treatment involved bending the stem towards either the left or right side 90 ° such that

one exit hole pointed upward and the other downward. This treatment also rendered the

exit passageway horizontal (Fig. 3C). The first flower was randomly assigned left or

right and the following trials alternated sides. Sample sizes for these orientation

experiments were 36 backbend trials and 45 side trials.

Lab trials:

The lab experiments were designed to examine the geotactic and phototactic

responses of bees in choice experiments, and to correlate this behavior with field

observations. A 46 cm long PVC tube of 2.54 cm internal diameter was used as the test

arena. Vial caps were placed over each open end and a ball of cotton was placed on the

inside of each cap for a foothold. The inside of the tube was roughened with clean sand

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and a 2.3 cm wide hole was drilled in the center of the tube, which was used to insert and

recapture the bees. A small cork was placed in the center hole during experiments to

ensure that the bees did not escape.

Five experiments were set up in order to test phototaxis and geotaxis of the bees

(Fig. 4): (1) a horizontal control with no blackout cloth and light all around (to test for

any possible asymmetrical room stimuli that would bias an insect's choice), (2) a

horizontal treatment with blackout cloth on one side (to minimize the stimulus to climb

upward and test the choice of light or dark), (3) a vertical treatment with no blackout

cloth and light all around (to test the geotactic response in the presence of light), (4) a

vertical treatment with blackout cloth on the bottom (to test the preference between up

and light vs. down and dark and, (5) a vertical treatment with blackout cloth on top (to

test the preference between dark and up vs. light and down). The blackout cloth was

constructed from multiple layers of black heavy cotton in which no light visible to the

human eye could penetrate when placed tightly against a bright fiber optic light. For all

treatments, the tube was placed in the center of a growth stand that had full spectrum

fluorescent lights (manufacturer GE, model Ecolux Sunshine 40) above and below the

tube approximately 40 cm away from the center of the tube. For all treatments, each

fluorescent light remained on and all other lights in the room were shut off. To

randomize any possible asymmetries associated with the tube, one end was marked and

randomly assigned a direction in each trial. Likewise, blackout cloth was randomly

assigned a side in each trial using the cloth.

Bees were randomly selected for the trials in groups of five and nearly every bee

was presented with all five treatments. Sample sizes for each experiment ranged from

26-30 bees per treatment. To randomize any potential effects of treatment order or time between an individual bee's trials, each particular combination of bee number and trial number was randomly selected from a bee x treatment matrix (5 x 5) without replacement. In each trial, a bee was placed in the tube and behavior was recorded for approximately two minutes on the digital recorder and later transposed into a notebook. For treatments 1 and 2, trial times were set to the minimum time for each treatment and only behaviors observed in the minimum time were analyzed (60 s for treatment 1, 52 s for treatment 2). The response variable chosen for this analysis was the total time a bee spent in the east, center, or west part of the tube. In vertical treatments, the time each bee spent in the top, center, or bottom of the tube was confounded by the time that bees spent falling to the bottom during a climb and staying there for a period of time before they would resume movement. Therefore, the data were analyzed in a binary fashion with a positive score when bees climbed and reached the top of the tube, and a negative score if they never reached the top (without falling) nor ever climbed.   At the end of trials with the blackout cloth, the cloth was removed to see how far bees had traveled into the dark.

Statistics:

Field contrasts were designed *a priori* and include only orthogonal pair-wise contrasts. These included window treatment vs. control, bandito vs. control, back bend vs. control, and side bend vs. control. Behavioral time data were analyzed with non-parametric Mann-Whitney *U* and Median tests. Categorical data, such as direction and location decisions made by the bees, were analyzed with Fisher's exact tests.

In the laboratory experiments, the total time that bees spent in the east, center, and west parts of the tube was tested for significant differences in distribution using non-

parametric Kruskal-Wallis tests. The three vertical treatments were compared to each

other using Cochran's Q test for comparisons involving the same set of individuals. For

all analyses, Fisher's exact tests were performed using Lowery (1998-2000; 2001-2011)

and all remaining tests were conducted using SPSS (Student version 18.0, 2009).

RESULTS

Bee Data:

One hundred and fifty two (93.8%) of the 162 bees identified were *Andrena*

macra Mitchell. Of the bees identified as *A. macra*, 122 were males and 30 were

females. Several other species of bees were used in the field experiments. These

included one *Andrena banksi* Malloch male, one male and one female of *Andrena*

perplexa Smith, two *Lasioglossum admirandum* Sandhouse females, and one

Lasioglossum coeruleum Robertson female. Other species of bees were collected and

identified from the bee nesting site, but were not tested in the field or the lab trials. These

included two *Colletes thoracicus* Smith males, one *Lasioglossum admirandum* female,

and one *Nomada cressonii* Robertson female. All bees used in the lab experiments were

A. macra.

Sex determination of the bees was attempted during the field trials. One hundred

and seven of the 114 (93.9%) field sex assignments were correct. Six of the seven

incorrect assignments involved other species mistaken to be *A. macra* and the remaining

mistake was made on *A. macra*. Both males and females had similar ranges in time for

orientation to correct escape route and time to escape. They also exhibited similar

behaviors for direction of first escape attempt and escape location. Thus there were no

observable differences in males and females in terms of behavior in the field and lab trials. During the field trials and based on field identifications, it was noted that 16 females and 15 males of *A. macra* successfully picked up pollen. A subset of the bees that were recorded with pollen were identified, sexed, and confirmed to have pollen by Sam Droege.

Orchid Field Trials:

Window Treatment vs. Control

Light windows did not have a significant influence on any of the behavior variables measured for the control and window treatments (medians are reported below for all treatments). There was no significant difference ($p = 0.64$) in direction of first escape attempt between the control and treatments (Fig. 5). In both trials, the majority of bees oriented to the back first (59% for controls and 57% for treatments). Likewise, bees in the control and window treatments oriented to the correct escape route in the same amount of time (4 s for the control and treatment; $p = 0.53$), and there were no significant differences in the distributions ($p = 0.93$; Fig. 6). Bees in the control and the window treatments also escaped in approximately the same amount of time (20 s for control and treatment, $p = 0.77$) and did not differ in distributions ($p = 0.62$; Fig. 7). Lastly, almost all of the bees escaped correctly out of the exit holes regardless of treatment ($p > 0.99$; Fig. 8).

Bandito Treatment vs. Control

As expected, covering the exit holes with a ribbon had a strong effect on the ability to escape correctly with the bandito treatment. In this treatment, only 24% of the bees escaped from the flowers, compared to 84% of bees in the control trials ($p < 0.001$;

Fig. 9). For bees that did escape from the Bandito trials (n = 9), they escaped out of the

entrance hole much more often (22%) than bees in the control treatment (3%; $p < 0.01$;

Fig. 10). However, the ribbon had no effect on other behaviors. A large portion of bees

still went to the back for direction of first escape attempt in both the bandito (60%) and

the control trials (49%; Fig. 11), resulting in no significant difference ($p = 0.24$) between

treatments. Likewise, there were no significant differences between treatments in the

time it took the bees to orient to the correct escape route (Bandito = 6 s, Control = 4 s; p

$= 0.26$ for medians; $p = 0.55$ for distributions; Fig. 12).

   Back Bend Treatment vs. Control

   Orientation of the labellum had a significant and pronounced impact on bee

behavior in the flower. Bees in the back bend treatments went up the front of the

labellum for direction of first escape attempt significantly more often (74%) than bees in

the control trials (23%), which primarily went up the back first ($p < 0.001$; Fig. 13).

Additionally, the bees in the back bend repeatedly tried to exit the front of the labellum

which resulted in a longer period of time to find and orient to the correct escape route (12

s for treatment compared to 4 s for the control; $p = 0.05$; $p = 0.02$ for distributions; Fig.

14). Consequently, some bees in the back bend trials never oriented to the correct escape

route (53% oriented correctly for treatments and 100% for controls; $p < 0.001$; Fig. 15).

However, bees in the back bend trials escaped from the flowers more quickly (12 s) than

bees in the control trials (28 s; $p = 0.01$; Fig.16). This occurred because bees in the back

bend trials more often used the entrance hole for escape (56%) than in the control (3%; p

< 0.001; Fig. 17); bees could easily step up on the staminode or fall out of the entrance

hole when the labellum was tipped upwards.

Side Bend Treatment vs. Control

The side trials alternated left and right bends to minimize wear on the flowers and also randomize any potential direction effects. No significant differences for any measures between the left and right side bend trials were found and thus the two directions of bending were combined. However, the side bend had significant behavioral effects compared to the controls. As in the backbend trials, the vast majority of the bees in the side bend trials (87%) chose the side of the labellum as the direction for first escape attempt. Bees in control plants primarily went up the back first (49%; $p < 0.001$; Fig. 18). Likewise, bees in the side bend trials took significantly longer to orient to the correct escape route than in the control trials (9.5 s for treatment, 4 s for control; $p < 0.001$), resulting in significant differences in distributions ($p < 0.001$; Fig. 19). Furthermore, the bees in the side bend trials were significantly ($p < 0.001$) less likely to find the correct escape route (61%) than the bees in the control trials, which all oriented to the correct route (Fig. 20). For time to escape, the distributions of the trials differed significantly ($p < 0.001$), but the medians did not (side bend= 10 s, control = 28 s; $p = 0.12$; Fig. 21). Entrance and exit holes were both common out locations for bees in the side bend treatments, which was significantly different ($p < 0.001$) from the control trials. Every bee in the control trial escaped out of the exit holes, with the exception of one bee that escaped out of the entrance hole (Fig. 22). Surprisingly, the bees in the side bend treatments showed no significant preference for the exit holes located on the "top" or "bottom" of the labellum ($p = 0.572$).

Other Bee Species

Although sample sizes were too small to do statistical analyses comparing the other bee species to *A. macra*, the behaviors exhibited by the other species were generally typical of the common behaviors observed in *A. macra*. Four individuals of other bee species were tested in the field experiments and included in the above analyses. These included one *Andrena banksi*, two *Lasiglossum admirandum,* and one *Andrena perplexa*. In a window treatment trial, one *A. banksi* took 1 second to orient to the correct escape route and climbed up the back as the direction for first escape attempt. This individual took 9 seconds to escape from the flower and exited out of the exit hole. In a bandito treatment, one *L. admirandum* took 58 seconds to orient to the correct escape route, climbed up the side as the direction for first escape attempt, and never escaped from the flower. In a back bend treatment, one *L. admirandum* took 18 seconds to orient to the correct escape route, climbed up the front as the direction of first escape attempt, and took 75 seconds to escape from the entrance hole of the flower. Lastly, in a side bend trial, *A. perplexa* took 30 seconds to orient to the correct escape route, climbed up the side as the direction of first escape attempt, but never successfully escaped from the flower.

Lab trials:

Bee behavior in the lab was consistent with the observations made in the field. For treatment 1 (the horizontal tube with light on all sides), when all trials were shortened to the minimum time of 60 s, there were significant differences in distributions ($p = 0.006$) and medians (east = 36 s, center = 16.5 s, west = 0 s; $p = 0.007$; Fig. 23). For treatment 2 (the horizontal tube with one side covered with the blackout cloth), when all

trials were shortened to the minimum time of 52 s, bees spent significantly more time in the light portion of tube (median = 52 s) than the dark side (median = 0 s; $p < 0.001$), which also resulted in significant differences in distributions ($p < 0.001$; Fig. 24). Eighteen of the 30 bees tested never entered the dark side of the tube. In both treatments 1 and 2, almost all of the bees continually tried to climb to the top of the tube, which was the inside circumference in this orientation. When the bees would fall, they would take a few seconds to recover, and then continue climbing up in whichever direction they were facing when they fell. In all of the vertical lab trials, almost all of the bees climbed up [96% for treatment 3 (all light), 96% for treatment 4 (bottom dark), and 85% for treatment 5(top dark)] regardless of the location of the blackout cloth. The slight decrease seen in treatment five did not result in an overall treatment effect ($p = 0.165$; Fig. 25)

DISCUSSION

The experimental reduction of light through the light windows had no effects on bee behaviors measured in this experiment, which included direction of first orientation, time to orient to the correct escape route, and the eventual escape time and location. Additionally, when virtually all light was blocked from permeating the windows and exit holes, there still was no effect on direction of first orientation or time to orient to the correct route. The vast majority of the tests were made with *A. macra*, but a few other species examined in the dataset were all consistent with the most common behaviors displayed in *A. macra*. Most interesting was the case of *A. perplexa* which is the one species in this study that is a known pollinator of *C. parviflorum* var. *pubescens* (Case

and Bradford, 2009). This species was used for a side bend trial and displayed behaviors in this treatment that were consistent with the most common mistake. Specifically, it ran up the interior side of the flower and never found the correct escape route. Thus, the light windows could not overcome the instinct to crawl upward, which suggests that orientation of the flower is more important than light emitted from the windows or exits for this species too. Together, these results indicate that light windows do not help to guide *A. macra* to the correct escape route, and suggest that these results may extend to other species as well.

Despite a lack of empirical tests of the light windows hypothesis, there has been considerable debate concerning the subject (van der Cingel, 1995). Preliminary observations and manipulations made by Daumann (1968) suggested that covering the light windows did not change bee behavior in the plant, but he argued for the need of a more rigorous experimental test. Other biologists suggested that light windows represent an ancestral condition. For example, van der Cingel (1995) notes that the tropical genera *Phragmipedium* and *Paphiopedilum* are partially myophilous containing light windows along with brown sepals, hairs, and a trapping labellum. He suggests that this set of features may be ancestral in the Cypripedioideae, however he also acknowledges that anatomical data places *Cypripedium* as ancestral to *Phragmipedium* and *Paphiopedilum*. Molecular data also places *Cypripedium* basal in the Cypripedioideae (Cox et al., 1997), which would then require the evolution of the light windows in a principally bee pollinated group.

All the discussions to date mention the light windows as a mechanism to guide pollinators (Webster, 1886; Barth, 1991; Argue, 2001; van der Cingel, 2001; Bänziger et

al., 2004). However, another possibility exists. Although solitary bees are the main pollinators of *C. parviflorum*, the bright yellow color and sweet aroma of the labellum attract a diversity of insects (Nilsson, 1979; van der Cingel, 1995; Schiestl, 2005; Case and Bradford, 2009). A labellum that already contains a large insect may prevent pollinators from entering the plant or prevent their escape out of the exit holes by blocking the correct route. Therefore, light windows could function to allow other phototactic visitors such as flies to escape effectively. This hypothesis could be tested by using insects that are known to have a strong phototactic response using the methods outlined in this experiment. An interesting candidate for such an experiment could be syrphid flies, although their phototactic responses are largely unknown. These flies are known pollinators of *Phragmipedium*, *Paphiopedilum* and *Cypripedium reginae* (Dressler, 1993). Previous observations of strong phototatic responses in flies (Chiang, 1963) suggests that flies may have stronger responses to the amount of light penetrating through the light windows than *A. macra*.

It is possible that light windows have hypothesized effects in other bees that act as pollinators of the orchids. Perhaps bees that are highly phototactic may exhibit differences in orientation or escape times in orchids with various amounts of light penetration through the light windows. Even so, it is difficult to imagine how a slight difference in the emission of light could have substantial qualitative effect on the fitness of *C. parviflorum*. *Cypripedium parviflorum* is a non-rewarding trap flower, and non-rewarding flowers are often visited less frequently as compared to rewarding flowers (Nilsson, 1992; O'Connell and Johnston, 1998; Case and Bradford, 2009). The fairly low visitation rate for non-rewarding flowers, such as *C. parviflorum*, suggests that frequency

of pollinator visitation is not as important as the effectiveness of pollinators that do enter the flowers. In order for an insect to be an effective pollinator, it must be able to contact the anther to pick up pollen and the stigmatic surface to deposit pollen. Thus, the manner in which visitors navigate through the plant seems to be more important than the frequency of visitation or quickness of escape while insects are in the plant. As observed in our study as well as by Bänziger et al. (2004) and Nilsson (1979), despite the ability of bees to quickly orient to the correct escape route, the process of escaping out of the exit holes is progressively difficult for larger bees. This results in some bees backing back down into the plant despite being in the correct position for escape. Based on the life history traits of *C. parviflorum* as well as pollinator behavior observations in the field, it seems that even if light windows did have an effect on bee behavior, the difference would not have a strong impact on overall pollination success.

Finally, it is possible that light windows have no effect at all. The adaptationist program encompasses the mindset of attributing particular functions to all traits in an organism. Moreover, there is an underlying notion that organisms are simply a conglomeration of independent traits that have all been under selection to fulfill a certain purpose. In a well-known paper by Gould and Lewontin (1979), the authors argue against the adaptationist program and suggest several other critical factors that may explain why a particular trait arose. For example, a trait may be the result of random events, a by-product of another adaptation, or become fixed in a population even if there is selection against it. Therefore it is not safe to assume that every trait has a particular function. The light windows hypothesis was first discussed over a century ago (Webster, 1886) and has appeared in numerous unpublished and published literature (Webster,

1886; Barth, 1991; Argue, 2001; van der Cingel, 2001; Bänziger et al., 2004; http://culturesheet.org/orchidaceae:cypripedium) yet this controlled experiment is the first one known to test the idea. Although it may seem logical that light windows should have the particular function of guiding pollinators towards the exit holes, the data from this experiment showed that light windows do not have a significant effect on behavior for *A. macra* and potentially other bee species.

However, while the loss of light in or near the exit holes had no demonstrable effect on bee behavior, orientation of the flower had a strong effect. *Andrena macra* is strongly influenced by gravity, as supported by the data from both the field and the vertical lab experiments. When the labellum was oriented either upwards (in the back bend trials) or to the side (in the side bend trials) the bees had a strong tendency to climb in whichever direction was up, regardless of the location of the exit holes. In a normally-oriented labellum, the urge to climb up results in bees climbing up the only completely vertical surface inside the labellum, which is behind the stigma and in route to the correct escape passage. Changing the labellum's orientation would likely result in a drastic change in pollination success. For example, the bees in the back bend and side bend treatments escaped more quickly and often escaped out of the entrance hole instead of the exit holes. Others never found the correct escape exit. When bees exit out of the entrance hole, they do not come in contact with the anthers or stigma, resulting in unsuccessful pollination of *C. parviflorum*. Moreover, the most dramatic change was seen in the back bend treatment where the vast majority of the bees attempted to climb up the front of the labellum, which was the longest vertical climb inside the labellum in that orientation. These results nicely mirrored the results of the lab where experiments

showed that bees climb up in the tube regardless of light availability. Over half (18 of

30) of the bees in the horizontal trial (trial 2; testing light and dark preference) never

entered the dark side of the tube, but when given a choice of gravity or light (trial 5), all

of the bees (with one exception) chose to climb to the top of the tube even though it was

completely covered in blackout cloth. Although bees may have a preference for light in

some situations, gravity apparently has a much stronger influence on their behavior under

the field and lab conditions tested. If gravity has the strongest influence on bee behavior,

then bees would be expected to climb in whichever direction faced upwards. This

hypothesis is consistent with the observations made in the field; bees climbed up the back

in normal orientation trials, up the front in the back bend trials, and up the side in the side

bend trials. Even though bees could escape faster and more easily in the back bend and

side treatments, they chose to climb upwards, supporting the conclusion that gravity is

highly influential on bee behavior.

The importance of labellum orientation has been well documented in the

Orchidaceae. This structure orients insects to enter a particular place (e.g., all

Cypripediodeae), grip the labellum in a certain way (e.g., *Orchis*, *Drakaea*), or even fall

off of the labellum in a precise manner (e.g., *Coryanthes*; Dressler, 1993, van der Cingel,

1995). As seen in this study, orientation of the labellum has a strong effect on bee

behavior, specifically in the direction that bees choose to climb. *Cypripeidum*

parviflorum flowers that may be tilted could have much reduced pollination success

because insects would more often escape out of the entrance hole, thus never

encountering the stigma or anthers. The importance of orientation for pollination success

suggests that over time there may have been selection for various mechanisms to ensure

correct orientation during development. One such mechanism is resupination, or twisting

of the flower during development so that the labellum is always the lowermost petal.

This trait is found in all orchid subfamilies and is suggested to be a basic feature of the

family (Dressler, 1993). The process of resupination is a twisting of the pedicel during

development that occurs in many orchids and is driven by gravity (van der Cingel, 1995).

The flexibility of the flower may also help to correct a bloom that has been knocked over

or pushed sideways. Case (pers. comm.) has noted that even though blooms may

experience perturbations in the field, the labellum quickly orients itself to the proper

orientation. Dressler (1993) discusses how many nonresupinate orchids also orient their

flowers in relation to gravity in order to maintain a normal posture.

Andrena macra has not been documented as a natural pollinator of *C. parviflorum*

var. pubescens, thus raising the question of whether *A. macra* is a suitable test subject for

this experiment. *Cypripedium parviflorum* and related species are considered to be

generalist attractors (Nilsson, 1979) by virtue of their bright yellow color and aromatic

fragrances that suggest food (Nilsson, 1979). The high diversity of insect visitors spans

many taxa including Thysanoptera, Coleoptera, Diptera, Hymenoptera, and Lepidoptera

as observed by Nilsson (1979) in a European population of *Cypripedium calceolus* L. In

the population used for this study, Case and Bradford (2009) found several bee families

(Andrenidae, Apidae, Halictidae, and Megachilidae) trapped in the labellum, but also

Diptera and Coleoptera. Thus, it is a reasonable assumption that *A. macra* could visit

yellow lady's slippers in search of food. In fact, one test subject voluntarily entered the

flower again immediately after it exited. Moreover, the genus *Andrena* is one of the most

important genera for pollination in *Cypripedium* and is well documented (van der Pijl and

Dodson, 1969; Nilsson, 1979; van der Cingel, 1995; Herring, 2007; Case and Bradford, 2009).

Although there is high diversity of insect visitors, only a few are successful pollinators because the dimensions of the insect are thought to be critical in determining the effectiveness of pollinators (Nilsson 1992; Bänziger et al., 2004). In this study, both males and females successfully picked up pollen from *C. parviflorum*, but females appeared to be more effective at removing it; nearly every female that was tested successfully picked up pollen if it was available, probably due to their larger size and subsequent tighter fit through the exit hole. The majority of males, however, often slipped past the pollinia without picking up anything. This observation is consistent with van der Cingel (1995) and Nilsson (1979), both of whom emphasized the importance of medium-sized female *Andrena* bees as effective pollinators of *Cypripedium*. In Case and Bradford (2009), the insects recorded with *Cypripedium* pollen were in the size range of 11-14 mm long, 2.5-3.0 mm wide, and with a thoracic height of 2.5-3.0 mm. *Andrena macra* females are 11-12 mm long and 3-4 mm wide and *A. macra* males are 8-11 mm long and 2.5-3.0 mm wide. Therefore, both females and males fit the size range of previously reported effective pollinators.

Lastly, very few studies have documented pollinators of *C. parviflorum* var. *pubescens* because observations of pollination are rare (Case and Bradford, 2009). However, all studies suggest while there may be some overlap in individual species, the documentation of novel pollinators is generally the rule. This is probably because the orchids exploit a variety of suitable insects that are present in the local pollinator community (Case and Bradford, 2009). In summary, in the absence of a known

emergence site of a known pollinator, the use of *A. macra* is not an unreasonable test

subject given its morphological dimensions, phylgogenetic position and ability to pick up

pollen. In concordance with the similarities in pollinator sizes and dimensions noted

above, there are a variety of other behaviors observed for *A. macra* that are similar to

previously reported behaviors of other bees in the labella of *Cypripedium* flowers. For

example, we noticed that upon insertion into the labellum, a large portion of the bees

would rapidly run around the labellum, climbing up the sides and front, until they

oriented to the back and escaped from the exit hole. Similar observations of "frantic"

behavior once a bee entered the labellum were made by Bänziger et al. (2004) in a study

of pollination in the Chinese slipper orchid, *C. guttatum*. Moreover, when the flower was

correctly oriented, it was difficult for bees to escape out of the entrance hole even if

flying was attempted. This observation was also noted in *C. guttatum* (Bänziger et al.,

2004). Another observation made in the field was the involved process of escape out of

the exit holes. In this study, many bees quickly oriented to the correct escape route, but

then slowed down to either sit with their heads popping out of the exit holes or backed

down into the labellum before exiting the flower. When the bees did escape, a portion of

the bees (that were not recaptured) rested on the dorsal sepal, the forest floor, or other

parts of the plant before flying away. Despite the lengthier (1-4 minutes) escape process

in *C. guttatum,* Bänziger et al. (2004) described similar observations of heads emerging

from the exit hole, backing down into the plant, pollen acquisition, and resting on the

sepal after escape. One to four minutes inside the labellum of *C. guttatum* is much longer

than the average time observed in this experiment, which was 38 seconds in the control

plants. Nilsson's (1979) study of pollination in *C. calceolus* documents the amount of

time bees spent in the labellum according to various bee species. He found that smaller bee species that escaped from the exit holes spent on average 1.28 minutes in the labellum, also longer than this experiment. Quicker exit times are most likely related to the use of smaller bees in this experiment. Other than exit times, the observed behaviors were very similar to reports of bees across many *Cypripedium* species. Moreover, these life history and behavioral observations provide further insight into the process of pollination in these orchids.

CONSERVATION NOTES

Due to the low occurrence of pollinator visitation and success in *C. parviflorum,* it is essential to understand what factors influence plant-pollinator relationships for conservation practices (Bernhardt and Edens-Meier, 2010). A greater knowledge of the mechanisms that enhance pollination success, such as correct orientation of the labellum, the size of effective pollinators, how far pollinators travel and the ecological conditions that support native bee nest sites can help conservation biologists develop the best management strategies to prevent further declines in this species as well as many other orchids. Furthermore, this study provides data on the behavior and distribution of a relatively rare native bee species, *Andrena macra* (Sam Droege, pers. comm.). Overall, native bee species are understudied as compared to honey bees and other species crucial for economically important crops. One of the few known bee nests available on campus provided a great opportunity to document the variety of solitary bee species present at this site, as well as study similarities in pollination behaviors trapped in *C. parviflorum*. Since *A. macra* and several other species tested in the field fits the size range to be an

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effective pollinator of *C. parviflorum*, this study provides insight into plant-pollinator interactions and outlines the mechanisms that can significantly transform this interaction. In the face of climate change and anthropogenic effects on the environment, it is important to understand not only the effects on individual species, but between interacting species such as native bee species and the flowers they pollinate (Soulé et al., 2005).

Currently, the orchid population in the College Woods and the native bees emerge in approximately the same time frame over several weeks. However, it has been noted (Case and Bradford, 2009) that the vast majority if not all pollination occurs in the first half of the bloom period of the orchid when pollinators have few food plants available and are still learning where food plants are. This is important for pollination because the orchids exploit naïve pollinators in an environment where there is less floral competition for the attention of the insects. Future changes in climate may have serious impacts on the phenological synchrony of these organisms and could result in the pollinators either emerging too early or too late to be effective pollinators of this orchid population. Even if changes occur synchronously, a change in temperature alone can influence the turgidity of the labellum as well as the length of time that a plant is in flower (Nilsson, 1979; pers. obs.). Nilsson (1979) discusses the importance of labellum stiffness in the first few days of blooming and asserts that the floral "mechanism was maximally hard to pass during the first days of flowering which resulted in efficient early removal of the pollen smear by bees [correctly exiting the plant]." The same tendency of the labellum was noted in this study; hot days resulted in less efficient pollen pick up and deposit because of reduced turgor pressure in the labellum, creating a loose fit for insect escape. Climatic

changes could result in a quicker loss of turgidity or shortened bloom period and thereby affect pollination rate.

Final factors to comment on are anthropogenic effects on the environment and how these influence plant-pollinator interactions. For instance, habitat loss has seriously reduced the number of orchid populations (Case, pers. comm.) and most likely has also reduced populations of solitary, ground-dwelling bee species. The population of *C. parviflorum* used in this study is located just off a path through the woods and is quite close to development on campus. Moreover, this area has been targeted for future development by the University. Temperate orchids in general and the yellow lady's slipper in particular, are notoriously difficult to transplant. A good example of this is in Great Britain where *Cypripedium calceolus* has been hunted to extinction by enthusiastic hobbyists. Virtually all attempts at reintroduction fail, and most of the genotypes from transplants into gardens are extinct. These difficulties resulted in only one remaining genotype left in the wild in Great Britain (Case, pers. comm.). In addition, yellow lady's slippers grow only in calcareous substrates; here it is where the Yorktown formation reaches the surface layers of soil. This occurs in only a few places throughout the College Woods. The orchids also take a decade or more to go from seed to flower, and even when they mature they can decline from robust to tiny individuals over the course of one year (pers. obs.). Artificial seed germination is accomplished using sterile tissue culture techniques but results in extremely low germination and strong selection; germination rates can be well under 1%. They are disturbance intolerant (e.g., they are especially poor competitors and are intolerant to changes in moisture and light). Even more difficult are the steps to introduce the symbiotic fungus, and then introduce the

seedling to growth outside of sterile culture. Thus, relocation because of climate change

or other assault is not feasible for these plants. It is therefore critical to educate the

academic community about the biological importance of this area and develop necessary

protocols to protect it. Likewise, the bee site borders a paved walkway that runs right

through campus and is also in danger of walkway development (e.g., plantings such as

grass) or intentional pesticide application from the sight of swarming bees. Again,

education is critical, even here at William & Mary.

In conclusion, understanding the mechanisms that influence pollinator success in

C. parviflorum and increased knowledge of the interactions between native bee species

and these flowers are important components for conservation practices. Overall

increased knowledge about these two systems will aid in developing conservation

awareness and practices that can ensure the survival of *C. parviflorum* and many native

bee species.

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Figure 1. Front and lateral views of the *Cypripedium parviflorum* var. *pubescens* labellum showing its large entrance hole (A), the pathway that an insect must traverse inside of the labellum to properly exit the plant (B) and the exit hole adjacent to a pollen mass (C).

Figure 2. *Cypripedium parviflorum* var. *pubescens* showing light penetration through light windows in control (A) and window treatment plants (B). The flowers were backlit to show the amount of light penetration through the windows and reduced light penetration from the vinyl tape.
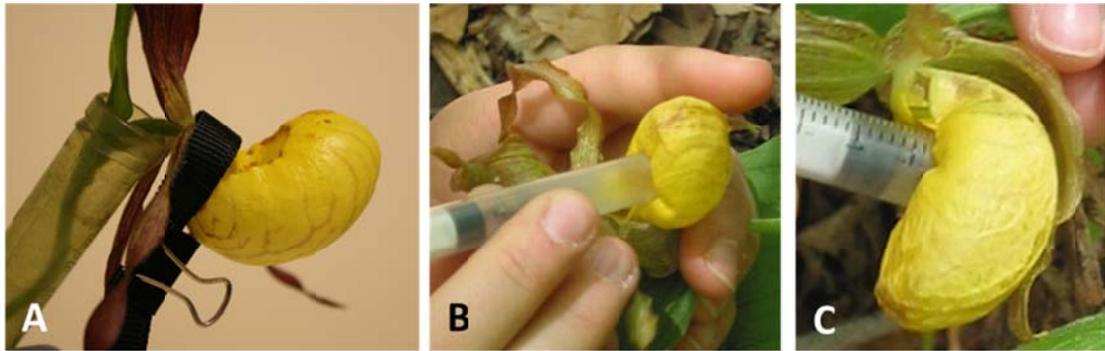
Figure 3. Additional treatments to test light windows and orientation effects on bee behavior. A-Bandito treatment showing the clipped ribbon around the labellum, which was used to cover the light windows and exit holes. B-Back bend treatment showing upwards orientation of the labellum. C-Side bend treatment showing sideways orientation of the labellum.
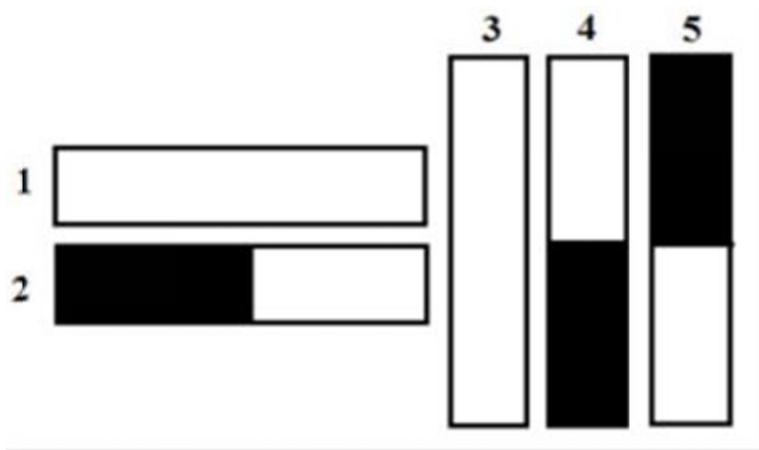
Figure 4. Visual representation of the five treatments in the lab trials. Dark sections are parts of the tube covered with blackout cloth.
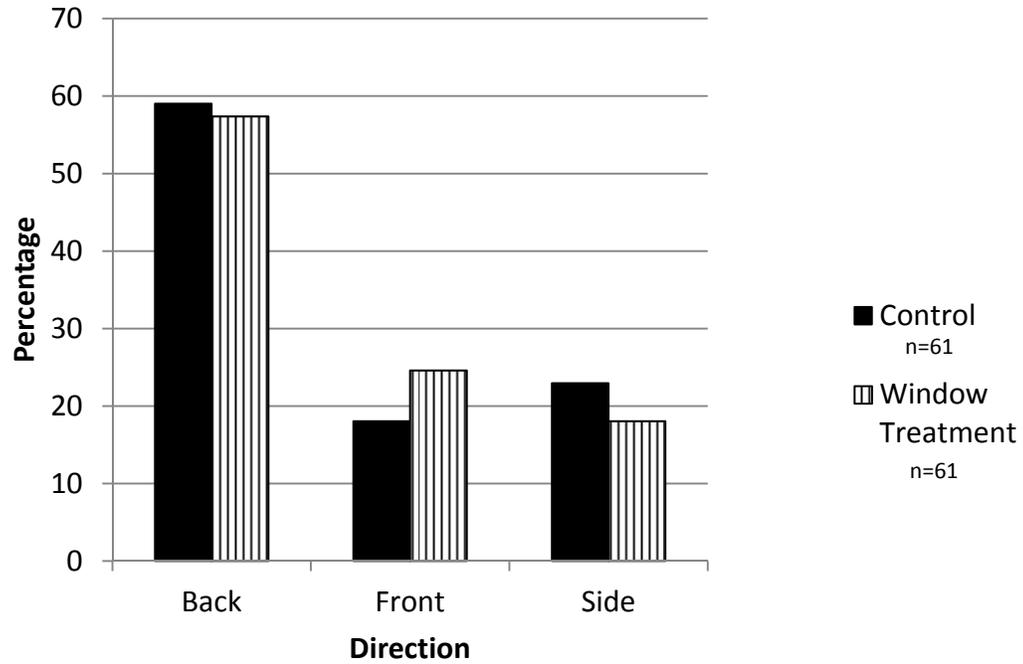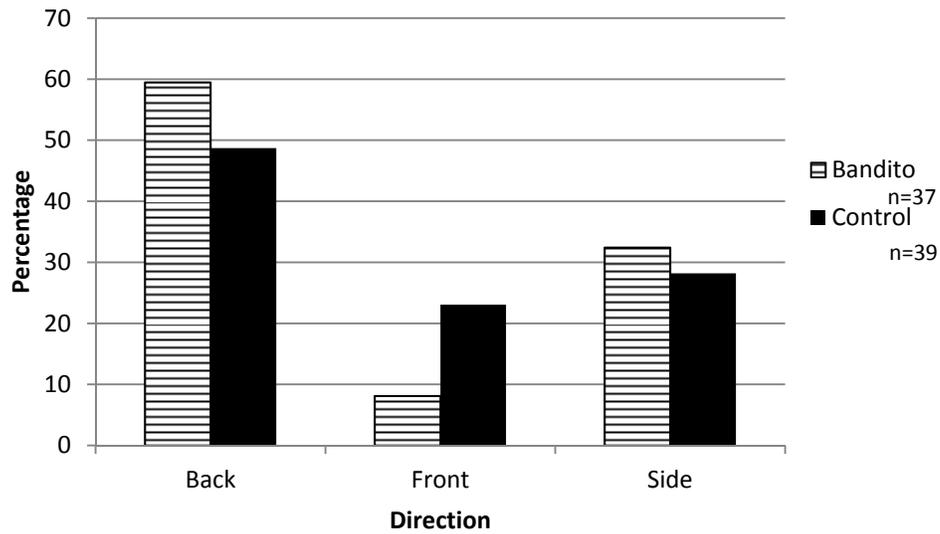
Figure 5. A comparison of the control and window treatment trials for the percentage of bees choosing the back (the correct escape route), front or side of the labellum as a first attempt at escape from the labellum. The choice of direction and treatment are independent ($p = 0.64$; Fisher's Exact test).
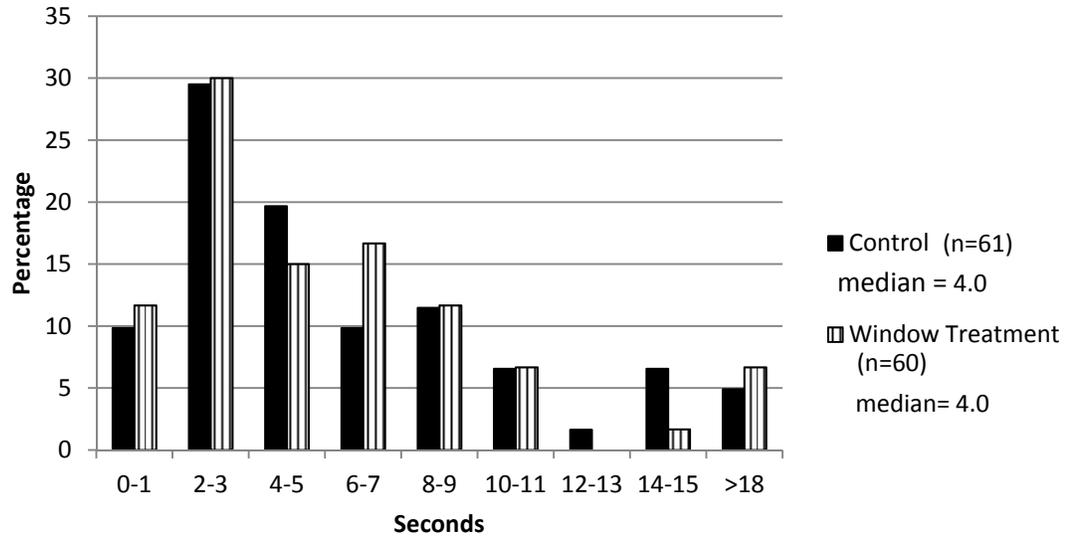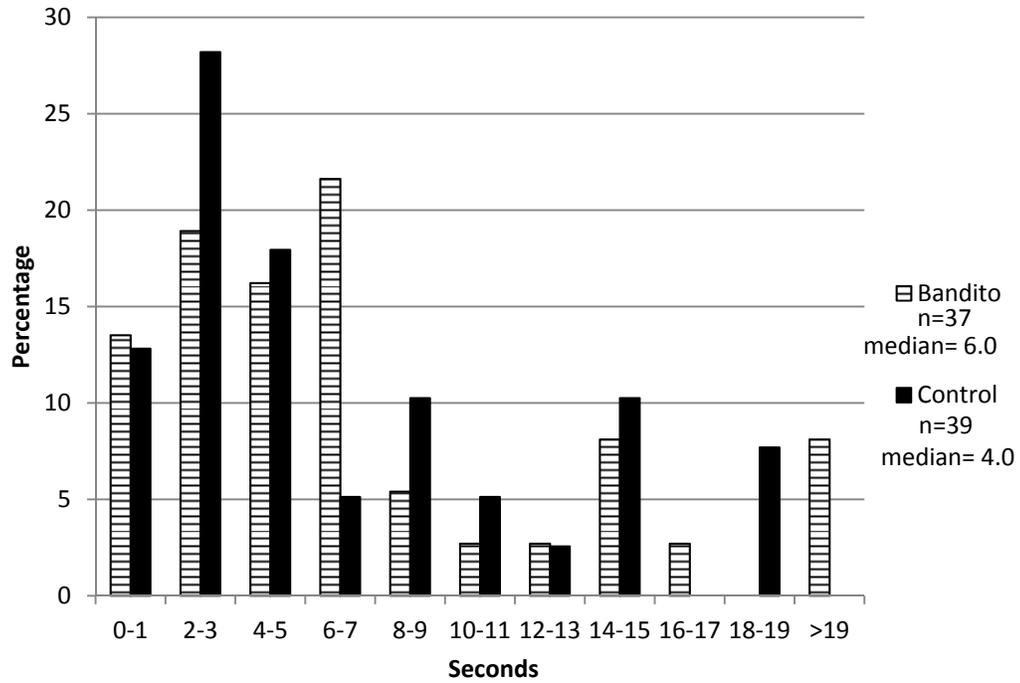
Figure 6. Distributions of the control and window treatment trials for the time it takes a bee to orient correctly to the escape route. The distributions ($p = 0.93$) and medians ($p = 0.53$) do not differ (Mann-Whitney U test).
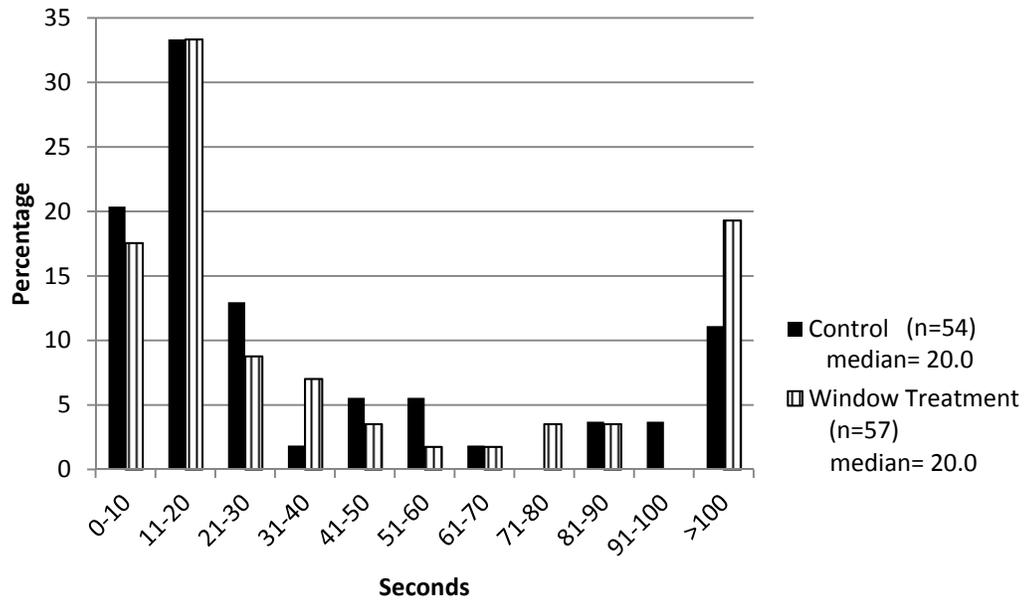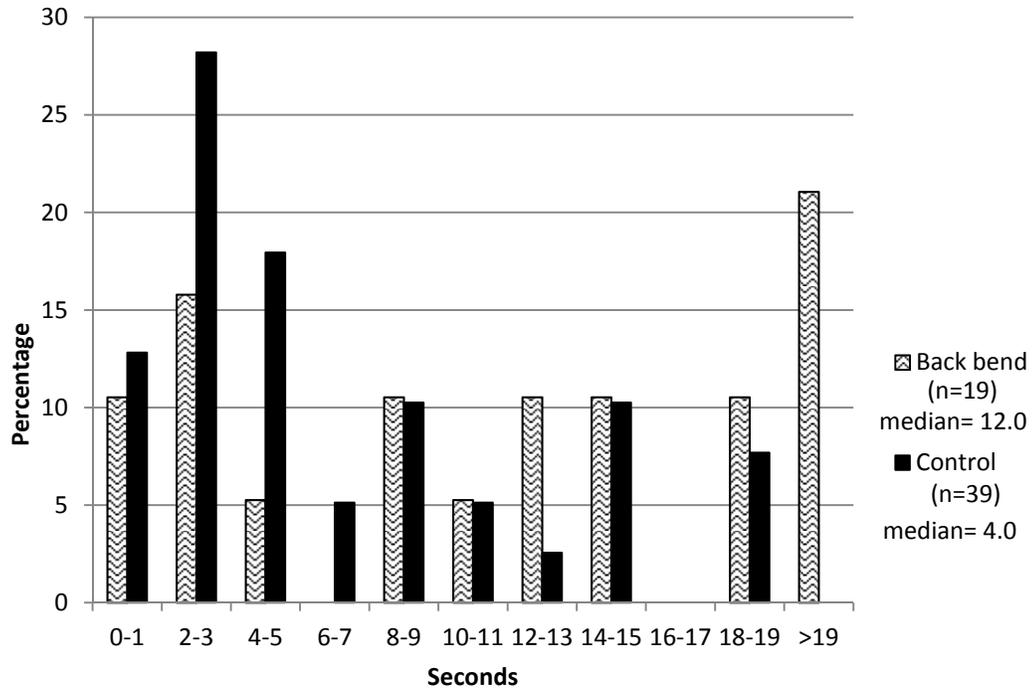
Figure 7. Distributions of the control and window treatment trials for the time it takes a bee to escape from the flower. The distributions ($p = 0.62$) and medians ($p = 0.77$) do not differ (Mann-Whitney U test).
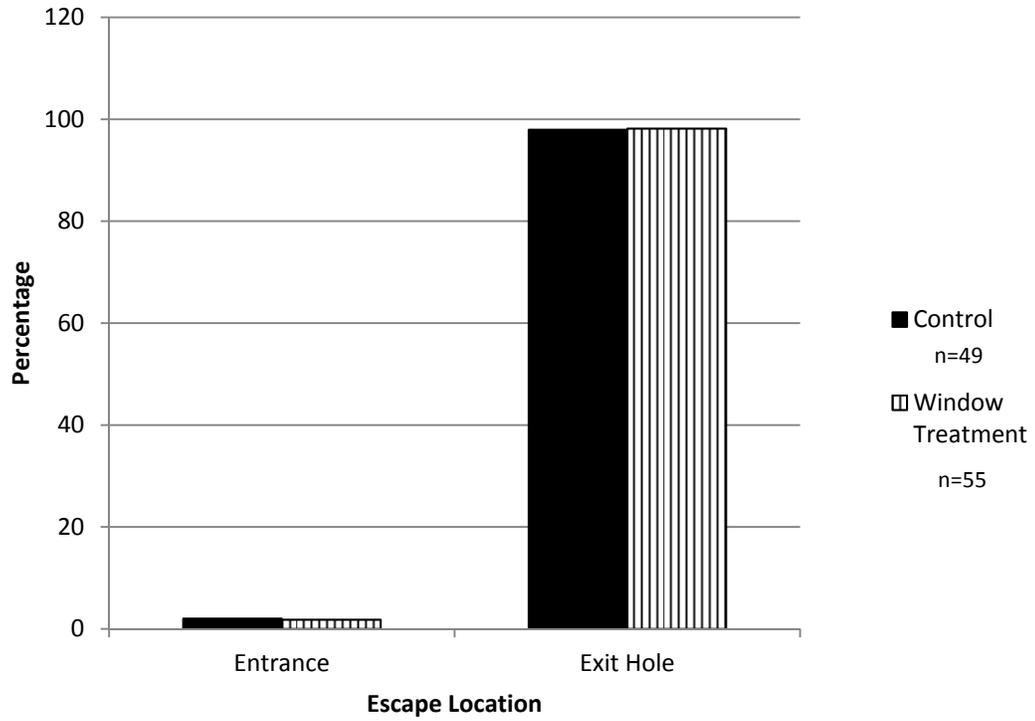
Figure 8. A comparison of the control and window treatment trials for the percentage of bees escaping out of the entrance and exit holes. The escape location and treatment are independent ($p > 0.99$; Fisher's Exact test).
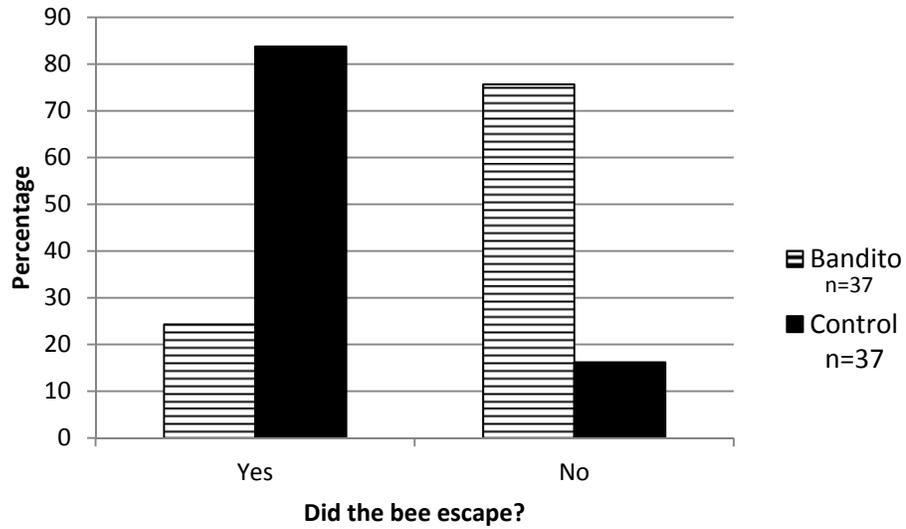
Figure 9. A comparison of the bandito treatment and control trials for the percentage of bees escaping from the flowers. The probability of escape depends on the treatment ($p <$ 0.001; Fisher's Exact test).
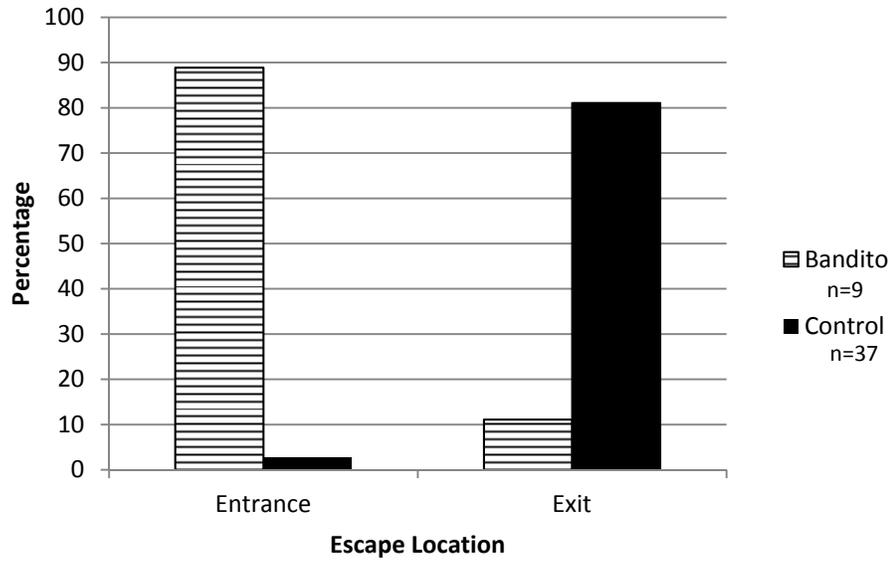
Figure 10. A comparison of the bandito treatment and control trials for the percentage of bees escaping out of the entrance and exit holes. Escape location depends on the treatment ($p < 0.001$; Fisher's Exact test).

Figure 11. A comparison of the bandito treatment and control trials for the percentage of bees choosing the back (the correct escape route), front or side of the labellum as a first attempt at escape from the labellum. The choice of direction and treatment are independent ($p = 0.24$; Fisher's Exact test).

Figure 12. Distributions of the bandito treatment and control trials for the time it takes a bee to orient correctly to the escape route. The distributions ($p = 0.55$) and medians ($p = 0.26$) do not differ (Mann-Whitney U test).
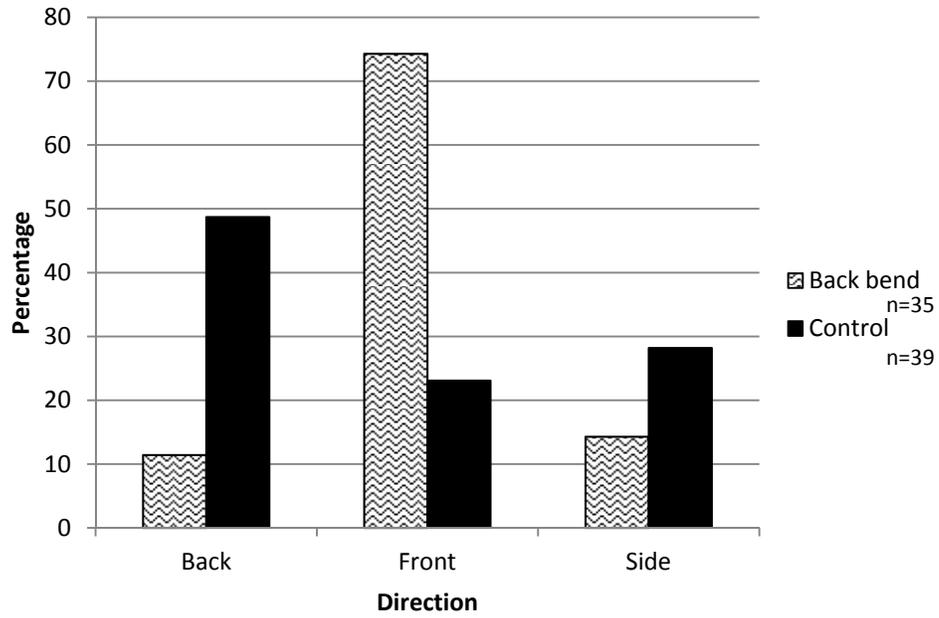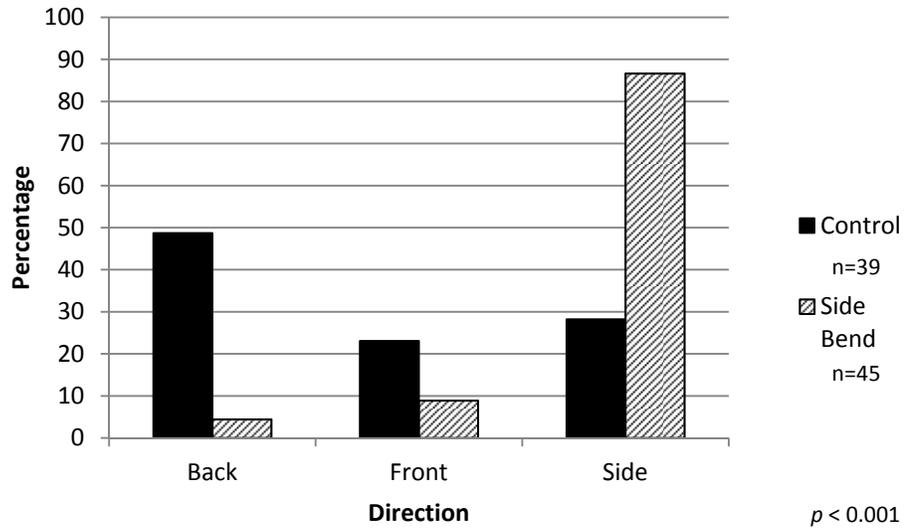
Figure 13. A comparison of the back bend treatment and control trials for the percentage of bees choosing the back (the correct escape route), front or side of the labellum as a first attempt at escape from the labellum. The choice of direction depends on the treatment ($p < 0.001$; Fisher's Exact test).
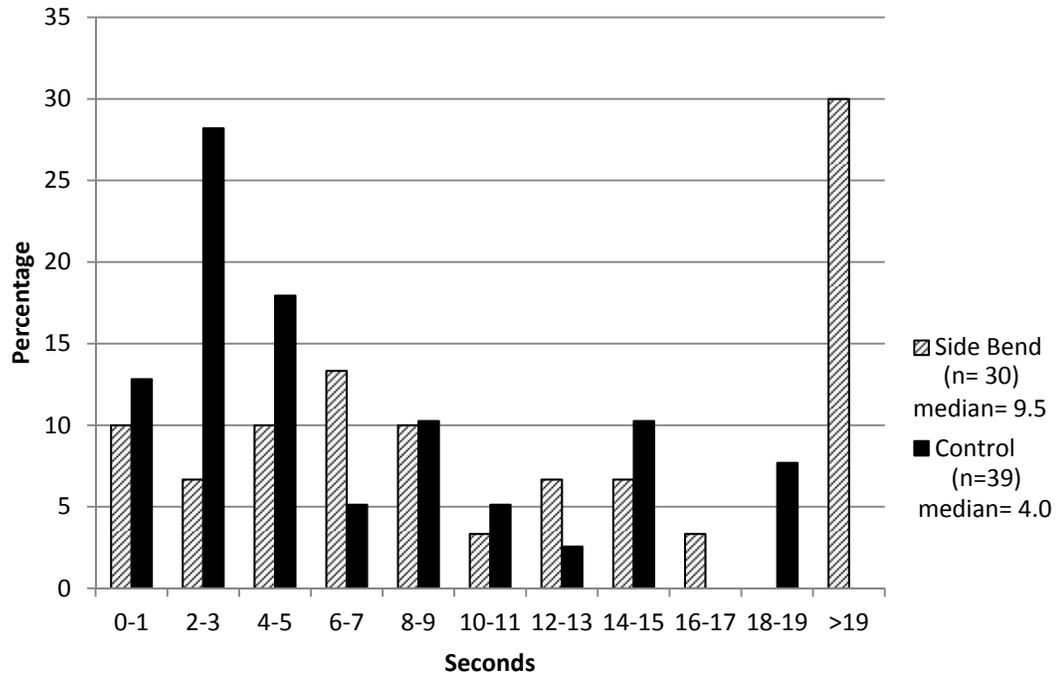
Figure 14. Distributions of the back bend treatment and control trials for the time it takes a bee to orient correctly to the escape route. The distributions ($p = 0.02$) and medians ($p = 0.05$) differ significantly (Mann-Whitney U test).
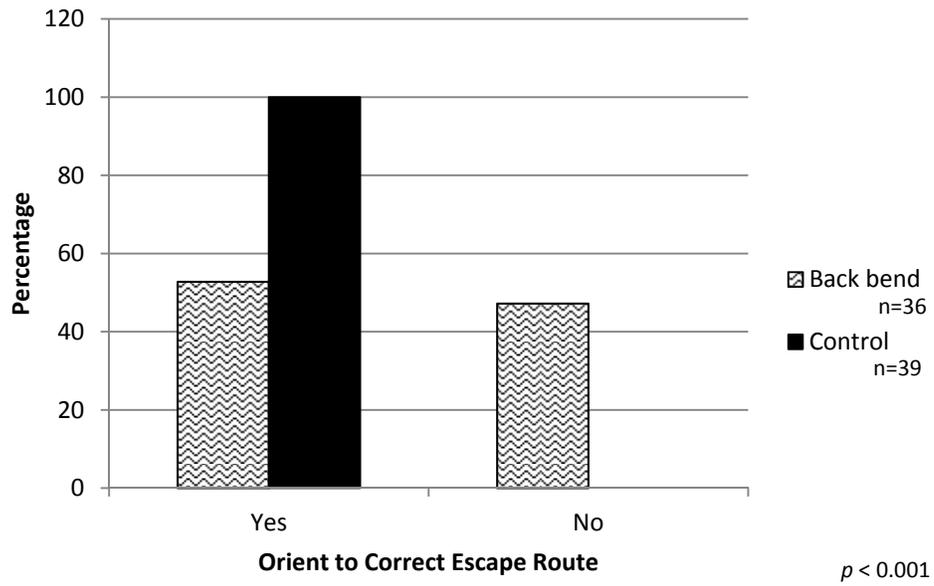
Figure 15. A comparison of the back bend treatment and control trials for the percentage of bees orienting to the correct escape route. The probability of orienting to the correct escape route depends on the treatment ($p < 0.001$; Fisher's Exact test).
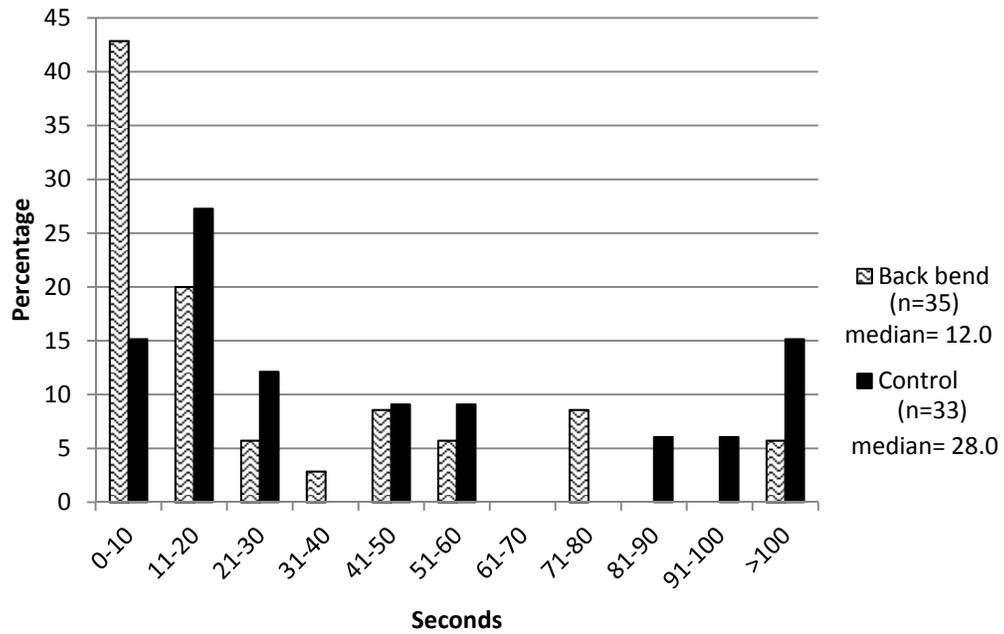
Figure 16. Distributions of the back bend treatment and control trials for the time it takes a bee to escape from the flower. The distributions ($p = 0.01$) and medians ($p = 0.02$) differ significantly (Mann-Whitney U test).
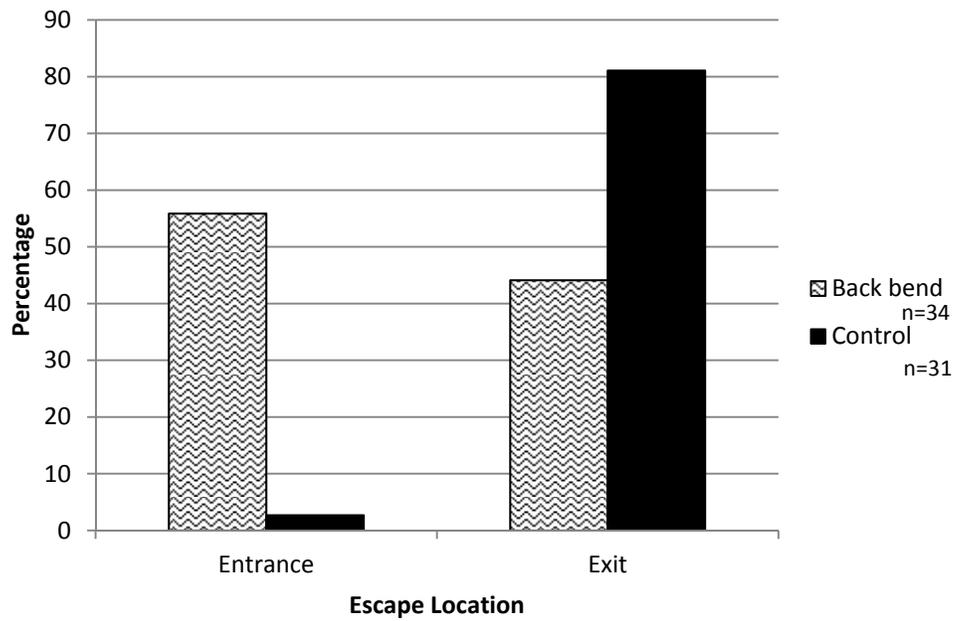
Figure 17. A comparison of the back bend treatment and control trials for the percentage of bees escaping out of the entrance and exit holes. The escape location depends on the treatment ($p < 0.001$; Fisher's Exact test).
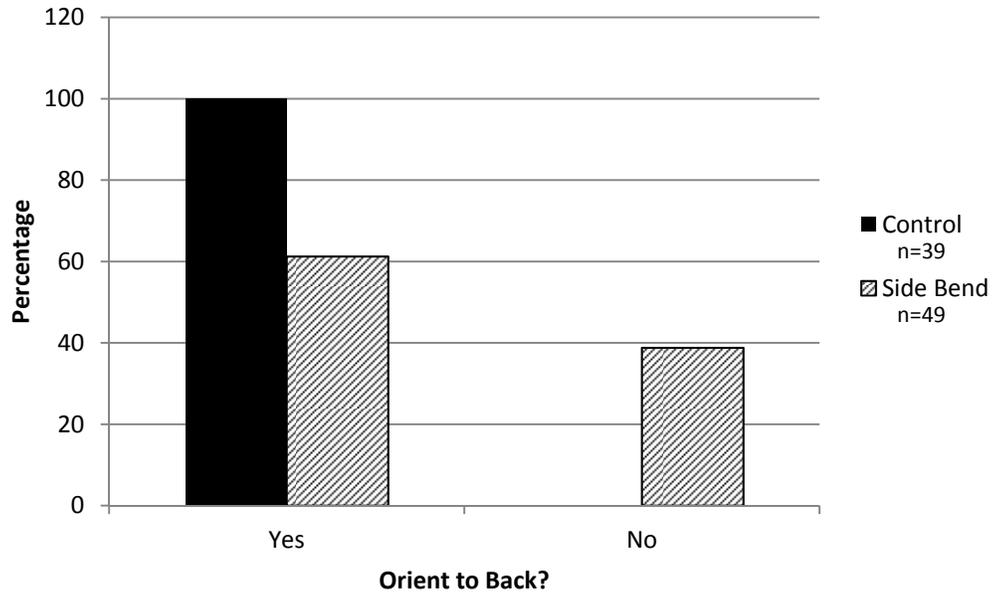
Figure 18. A comparison of the side bend treatment and control trials for the percentage of bees choosing the back (the correct escape route), front or side of the labellum as a first attempt at escape from the labellum. The choice of direction is dependent on treatment ($p < 0.001$; Fisher's Exact test).

Figure 19. Distributions of the side bend treatment and control trials for the time it takes a bee to orient correctly to the escape route. The distributions ($p < 0.001$) and medians ($p = 0.047$) differ significantly (Mann-Whitney U test).

Figure 20. A comparison of the side bend treatment and control trials for the percentage of bees orienting to the correct escape route. The probability of orienting to the correct escape route depends on the treatment ($p < 0.001$; Fisher's Exact test).
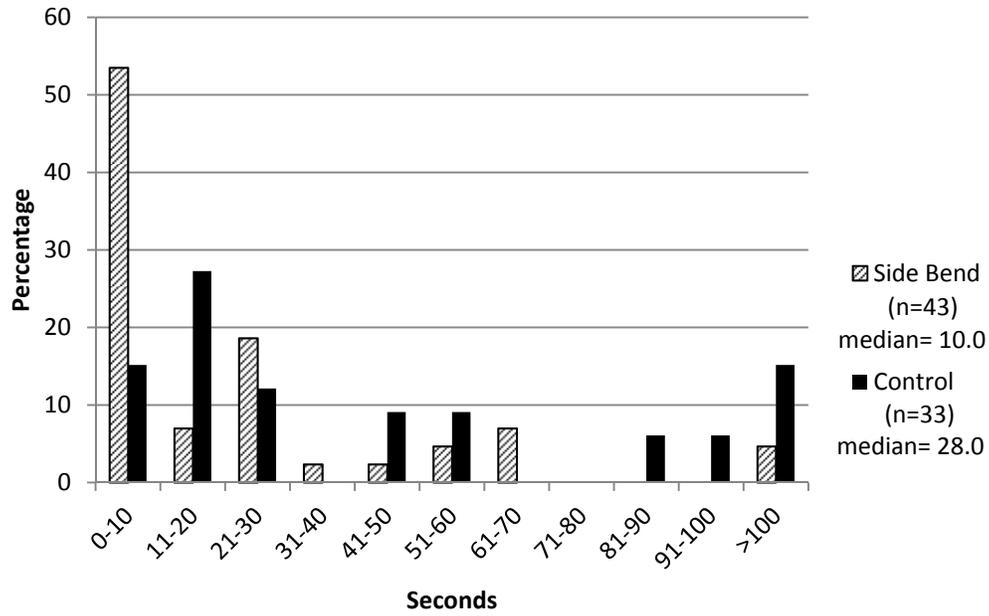
Figure 21. Distributions of the side bend treatment and control trials for the time it takes a bee to escape from the flower. The distributions ($p < 0.001$) differ significantly, but the medians ($p = 0.12$) do not differ (Mann-Whitney U test).
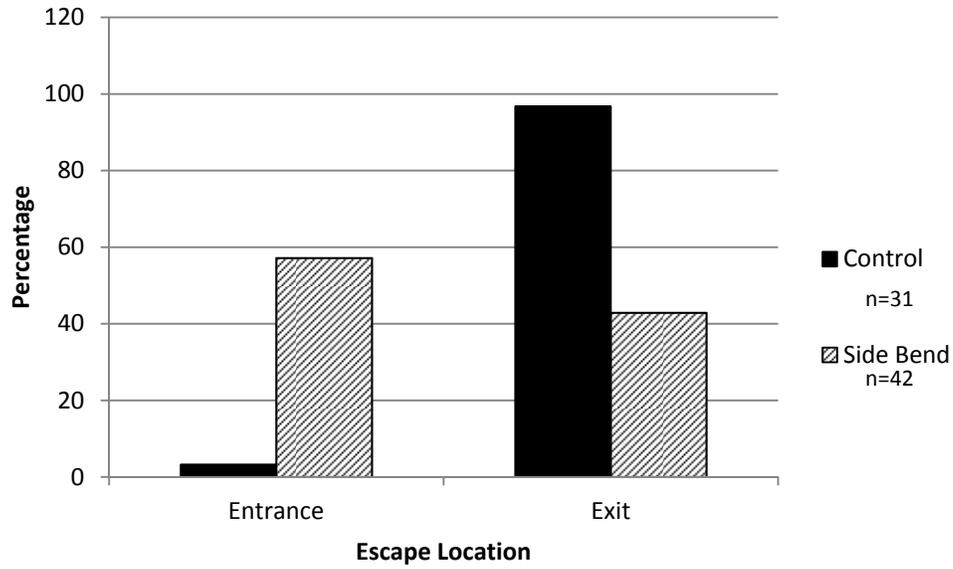
Figure 22. A comparison of the side bend treatment and control trials for the percentage of bees escaping out of the entrance and exit holes. The escape location depends on the treatment ($p < 0.001$; Fisher's Exact test).
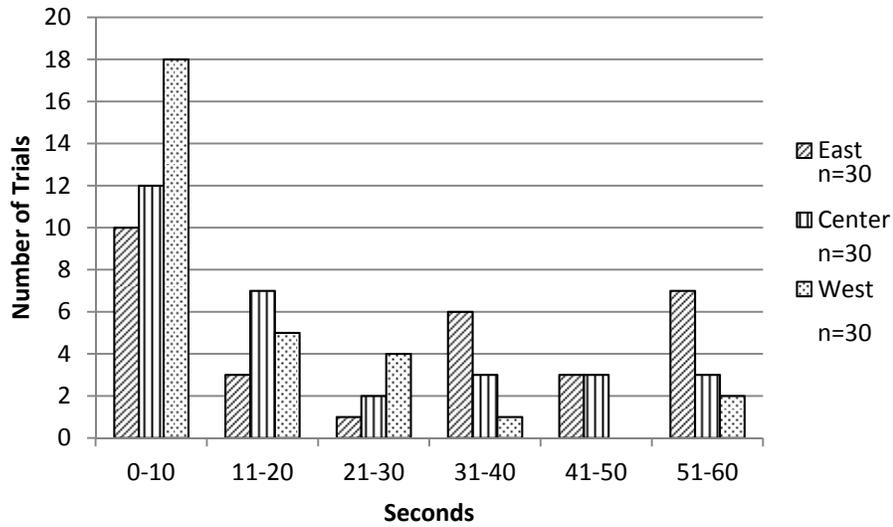
Figure 23. Lab Treatment 1: Time bees spent in the east, center, and west portions of the tube in 60 seconds (minimum time for this trial). The distributions ($p = 0.006$) and the medians ($p = 0.007$) differ significantly (Kruskal-Wallis test).
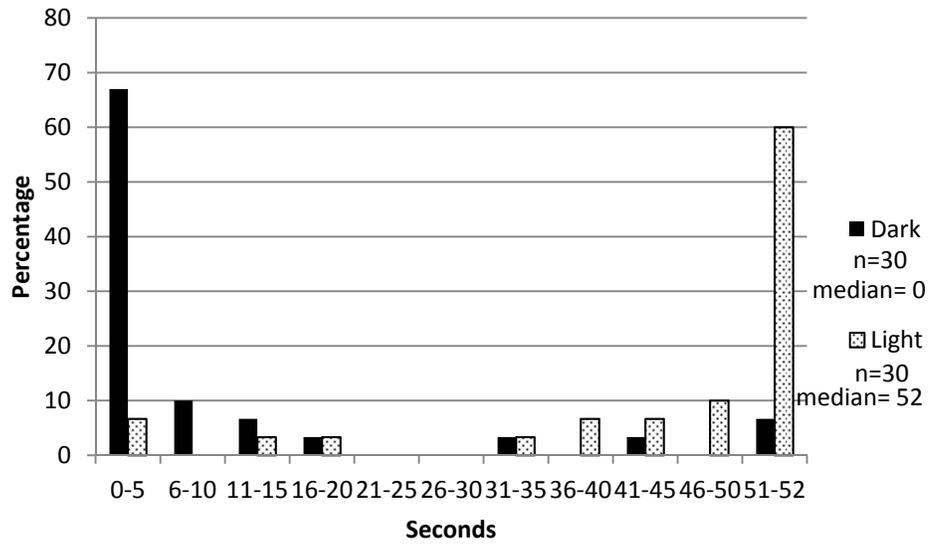
Figure 24. Lab treatment 2: Distribution of time bees spent in the dark and light portions of the tube. Distributions ($p < 0.001$) and medians ($p < 0.001$) differed significantly.
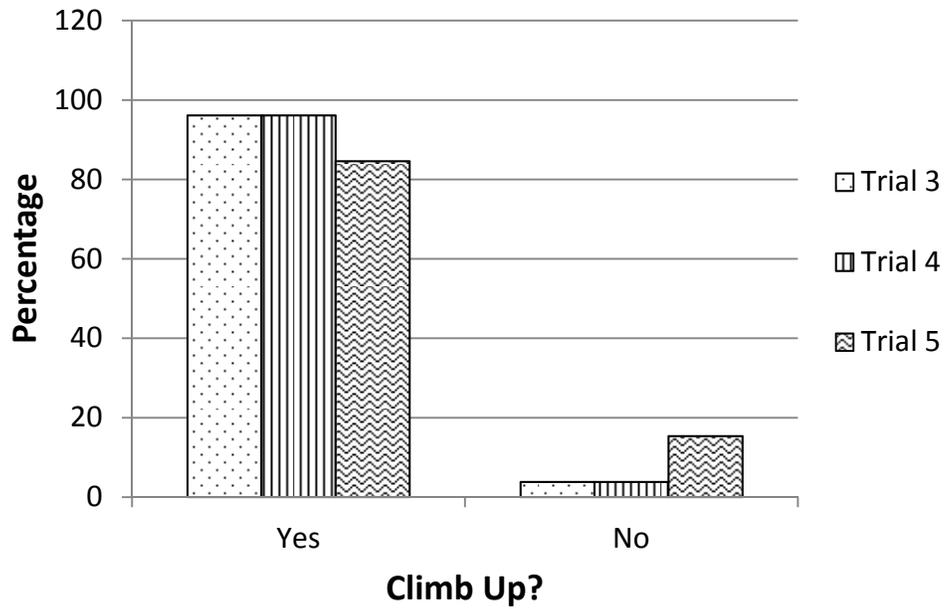
Figure 25. Lab Treatments 3, 4, and 5: The percentage of insects that reached or attempted to reach the top of the tube when light entered the tube from all around (trial 3), just the top (trial 4), or just the bottom (trial 5).   There was no significant association between light treatment and the number of climbing insects ($p = 0.165$; Cochran's Q).