Plant-Pollinator Interactions in an Ecological and Evolutionary Context: The Promising Role of 3D-Printing Technology and Mathematical Modeling

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Abstract

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This dissertation concerns itself with the role of flower shape in affecting the foraging performance of pollinating animals. The pollinator used in this study is a model organism representing crepuscular hawkmoths in research involving the study of flight neuromuscular physiology and plant-pollinator interactions, *Manduca sexta* (hereafter Manduca). The broader goal of the work is to develop a new experimental framework for investigating the ecological and evolutionary consequences of plant-pollinator interactions. To that end, I have combined 3D-printing technology and mathematical modelling to construct artificial flowers, which can be manufactured with great precision and with objective, quantitatively describable shapes.

First, I present a proof-of-concept study to demonstrate the feasibility of collecting foraging data from a real animal pollinator attempting to feed from 3D-printed artificial flowers. I show that Manduca’s foraging performance is extremely sensitive to variation in floral corolla curvature and nectary diameter. These results validate the experimental approach that I describe, justifying

a large investment of time and financial resources into a major iterative improvement of my experimental apparatus.

Next, I describe the improved experimental apparatus and use it to construct a performance landscape of Manduca's innate foraging performance as a function of variation in flower morphology. This landscape suggests that Manduca's foraging performance is contingent on a context-dependent interaction between corolla curvature and nectary diameter.

Finally, I use data from infrared sensors attached to each artificial flower and custom computer vision software (both improvements over the proof-of-concept apparatus) to identify putative proxies of fitness for both the pollinator and the plant (artificial flower). The goal is to examine whether the pollination mutualism between Manduca and the flowers that it visits in nature could represent a scenario of evolutionary conflict or harmony. The results are inconclusive due to opposing conclusions that the various indirect proxies of flower fitness point to. As a result, I make suggestions for improving the experimental apparatus even further by including a physical touch-sensitive sensor into the design of the artificial flowers. Such a sensor would act as a physical analogue for a real flower's reproductive structures, allowing the apparatus to yield a direct rather than indirect measure of flower fitness. Still, the general framework of using 3D-printed flower whose shapes are mathematically specified shows great promise for opening up new areas of experimental inquiry in the field of plant-pollinator interactions.

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EXPRESSION OF GRATITUDE FOR NON-SCIENTIFIC SUPPORT

My journey through graduate school has been punctuated by incredible highs and discouraging lows. Fortunately, they presented themselves in roughly equal measure, and the positive moments came when they were needed most. My doctoral advisors and committee members of course provided critical assistance and guidance in helping me navigate my program, and I am forever indebted to their patience and technical assistance. But I would like to take this opportunity to thank specific individuals and groups who made a difference in my trajectory through graduate school by providing moral support as opposed to the technical guidance that my committee has given me. A small piece of my doctoral degree belongs to each one of you.

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Chapter 1

Introduction to the Dissertation

The sub-field of plant-pollinator interactions has a rich history and has contributed much to our understanding of the processes that have led to current patterns of biodiversity and phenotypic disparity among both flowering plants (angiosperms) and intimately associated species of animals. Pollinating animals interact with flowers, the reproductive structures of angiosperms, in order to acquire some consumable resource - usually nectar and/or pollen. In the process, they unwittingly pick up and transfer pollen from the anthers of one flower to the stigma of another. This creates a mechanism by which flowering plants can achieve efficient cross-pollination and enjoy the benefits of non-selfed sexual reproduction without relying on less directed and potentially shorter-distance pollen vectors such as wind. However, while plant-pollinator interactions may be considered as "mutualisms" due to the mutual benefit of both parties, the interests of the plant and the animal might actually be theoretically misaligned at the level of individual organisms. In fact, mutualisms have even been described as “reciprocal parasitisms,” that is, interactions based on a foundation of mutual exploitation by each party in the interaction (Bronstein 1994). Individual plant fitness is dependent on forcing the pollinator into the appropriate spatial position for animal-anther contact and for animal-stigma contact. In contrast, individual pollinator fitness is dependent on the amount of floral resources it can gain access to. A full understanding of plant-pollinator interactions involves consideration of the fitness interests of both parties. This dissertation primarily focuses on the pollinator's "perspective," with hopes that insights gleaned from this perspective can suggest insights about the plant's perspective as well.

Among animal pollinators, those that employ hovering flight during floral visitation face drastic energetic challenges due to the high metabolic costs associated with this type of locomotion (Norber 1990; Vogel 1994). The task is even more challenging for the subset of hovering nectarivores that lead nocturnal or crepuscular habits, as they must deal with reduced light availability in addition to the physical demands of hovering. Hawkmoths, members of the Lepidopteran family, Sphingidae, contribute substantially to the pollination of many angiosperm species in nature (Groman & Pellmyr 1999; Dar *et al*. 2006; Goyret & Raguso 2006). Simply finding a flower is not the end of the challenge for dark-active hawkmoths, as they are still faced with a difficult sensorimotor challenge involving maintenance of stable hovering flight while managing the physical interaction between the proboscis, a long tubular modification of the insect mouthparts used for fluid uptake, and the surface of the flower itself. Many flowers have phenotypic features, called floral guides, which aid the reward discovery process of potential pollinators. These guides are typically associated with visual features (Medel *et al*. 2003) and olfactory signals (Riffell *et al*. 2009) of a flower. However, there is also evidence that dark-active hawkmoths exploit physical features of a flower's corolla surface to help guide its proboscis to the nectar source (Kevan & Lane 1985; Krenn 1998).

With all of the above in mind, this dissertation seeks to address aspects of nectar discovery of a crepuscular hovering pollinator, the hawkmoth *Manduca sexta*, and how it might be affected by variation in flower shape, potentially explained by the prominence of passive mechanical guidance and/or active mechanosensory feedback between the moth's proboscis and the surface of the flower during proboscis-flower probing. For instance, how does variation in floral corolla curvature and nectary diameter, two salient features of flower morphology, affect the ability of

M. sexta to find the nectar source of a flower? In addition, can the integration of temporally synchronized pollinator position data and proboscis-nectary insertion data during the course of individual floral visits yield additional proxy metrics for both pollinator and plant fitness? If so, then the novel experimental approach introduced in this body of work, which includes mathematical modeling of flower shape, rapid prototyping, and high-throughput data acquisition devices, could open the door to previously unfeasible experimental explorations of plant-pollinator ecology and coevolution.

In Chapter 2 (published in the journal Functional Ecology: Campos *et al*., 2015), I present exploratory data on the effects of variation in flower corolla curvature and nectary aperture size on foraging success in the dark-active hawkmoth, *M. sexta*. I describe the use of mathematical modeling to achieve precise quantitative manipulations of salient features of flower morphology, and the use of rapid-prototyping technology to enable fast production of experimental artificial flowers with high fidelity among "copies." This is, to my knowledge, the first use of such an approach to manufacture experimental artificial flowers, and it has high potential to open up new or previously impractical investigations concerning issues at the interface of plant-pollinator ecology and evolution.

In Chapter 3, I extend the experimental procedure that was validated in Chapter 2 to construct the foraging performance landscape of *M. sexta* as a function of variation in flower corolla curvature and nectary aperture size. This is accomplished through systematic and strategic exploration of these two aspects of flower morphology using an experimental arena in which six different flower morphologies were simultaneously presented to freely flying hawkmoths during unconstrained foraging trials. The experimental arena used in Chapter 2 has been redesigned and expanded in order to accommodate 36 artificial flowers at a time instead of the previous 16. With this change, the artificial flower array now consists of six different flower morphs (six of each morph) instead of the previous two flower morphs (eight of each morph). Key findings include that trumpet-shaped floral curvature significantly improves nectar discovery performance over both flat-disk and bowl-shaped morphotypes. However, this curvature effect is most apparent when flower nectary diameters are small. In fact, as nectary diameter increases, flowers of all curvatures gradually become equally "easy" to feed from.

In Chapter 4, I use automated object-tracking software and infrared sensors attached to the artificial flowers in my experimental arena to attempt to identify aspects of pollinator foraging behavior that might correlate with flower fitness, with two main goals: (1) If such aspects of foraging behavior can be identified, they could be used as proxies for flower fitness in experimental evolution studies of the evolution of flower shape in response to visitation by animal pollinators. This would be significant, as it would lay the foundation for lab-based, experimental tests of the pollination syndrome concept as an explanatory framework within the field of plant-pollinator interactions. (2) The second goal is to use the putative proxies for flower fitness to ascertain whether or not *M. sexta* is in a state of evolutionary conflict or evolutionary harmony with the flowers that it might visit in nature. In this context, “evolutionary harmony” means a situation in which the deployment of flowers of a particular shape simultaneously maximizes the fitness of both the pollinator and the plant. “Evolutionary conflict” means a situation in which plant and pollinator fitness are maximized by the deployment of flowers that are different and distinct from one another. Of five potential proxies for flower fitness that I am able to identify, I find that two suggest evolutionary harmony, two suggest evolutionary conflict, and one is uninformative. As such, no strong conclusions regarding evolutionary harmony or conflict can be made in this case. I make the case for the need to include a physical touch-sensitive sensor into the design of the artificial flowers that I have developed so that the artificial flowers will have an analog to the reproductive structures (stigma and anthers) present in real flowers. Such an "anther sensor" would yield a direct as opposed to indirect proxy of flower fitness. Finally, I discuss other ways in which the apparatus that I have developed could be improved to yield a more robust experimental system for the continued study of plant-pollinator interactions.

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Chapter 2

Shape Matters: Corolla Curvature Improves Nectary Discovery in the Hawkmoth *Manduca sexta*[1]

E.O. Campos, H.D. Bradshaw, and T.L. Daniel

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2.1 Abstract

I measured the effects of variation in corolla curvature and nectary aperture radius on pollinator foraging ability using the hawkmoth *Manduca sexta* and 3D-printed artificial flowers whose shapes were mathematically specified. In dimorphic arrays containing trumpet-shaped flowers and flat-disk flowers, hawkmoths were able to empty the nectaries of significantly more trumpet-shaped flowers regardless of nectary aperture size. Interestingly, trumpet-shaped flowers needed to deviate only slightly from the flat-disk morphotype in order to significantly increase hawkmoth foraging ability. Whole-flower three-dimensional shape, particularly corolla curvature, has the potential to act as a mechanical guide for *Manduca sexta*, further implicating direct flower-proboscis contact as an important contributor to foraging success during flower handling in hawkmoths.

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[1] This chapter contains material that constitutes a thesis submitted in partial fulfilment of the requirements for the degree of Master of Science, by Eric Octavio Campos: Shape Matters: Corolla Curvature Improves Nectary Discovery in the Hawkmoth *Manduca sexta*, Department of Biology, University of Washington, 2016.

2.2 Introduction

Many animals consume floral nectar as a primary means of meeting their energetic requirements (Kingsolver & Daniel 1995). Nectar feeding presents an interesting sensorimotor challenge for hovering animals, as they must modulate their aerodynamic forces to precisely position their feeding apparatus in the nectary of a flower that could potentially be swaying in the breeze generated by their flapping wings or by external winds. This mode of flight is metabolically demanding (Vogel 1994; Norberg 1990), making nectar discovery ability all the more important for hovering nectarivores.

Floral guides may aid the process of nectarivory by obligate hovering species. A guide is any phenotypic feature of a flower that acts as a cue to assist animals in finding the floral reward, nectar or otherwise (Sprengel 1793; Sprengel 1996). Floral guides are commonly thought of as visual stimuli (Medel et al. 2003), but the concept can be expanded to include stimuli that could be perceived by other sensory modalities such as chemical (Riffel et al. 2009a) and mechanical sensory pathways (Kevan & Lane 1985; Krenn 1998). Indeed, it has become increasingly evident that pollinators make use of multiple sensory modalities during the foraging process (Goyret & Raguso 2006; Goyret & Kelber 2011). Non-visual sensory information may be particularly critical for nocturnal and crepuscular animals, as they deal with reduced light levels compared to diurnal species. Despite such visual challenges, nocturnal and crepuscular animals effectively forage on night-blooming flowers and are important contributors to the pollination of various angiosperm species (Dar, Arizmendi, & Valiente-Banuet 2006; Groman & Pellmyr 1999; Goyret & Raguso 2006).

The hawkmoth *Manduca sexta* (Fig. 2.1) is a crepuscular pollinator that has an extensive history as a model organism in laboratory and field studies of pollination. It is also noted for its ability to track flower motion while hovering in mid air (Sprayberry & Daniel 2007). Although *M. sexta* sometimes lands on a flower during foraging, it also has the ability to feed by hovering a short distance from a flower, making contact only with its uncurled proboscis (Brantjes & Bos 1980; Raguso & Willis 2002). Previous research has shown that *M. sexta* is able to use both visual and mechanical floral guides during flower handling to aid in nectar discovery: experiments with flat-disk artificial flowers have shown that *M. sexta* preferentially probes with its proboscis at light-coloured areas of a flower, regardless of whether the nectary is present at those locations (Goyret 2010). In addition, groove-like features on a flower's surface may effectively guide the movement of the proboscis along the direction of the grooves (Goyret & Raguso 2006; Goyret 2010).

Recent results by others suggest that foraging by *M. sexta* is under multimodal sensory control, with different sensory modalities, or combinations of them, being important at different stages of the foraging process, depending on a moth's distance from a food source. For example, at large distances, a moth uses olfaction to orient toward a group of flowers (Brantjes 1978). At intermediate distances, the approach to a group of flowers is guided by either olfaction or vision, while extension of the proboscis requires that appropriate olfactory and visual stimuli be present simultaneously (Goyret & Raguso 2006). Once a moth has selected an individual flower, initial placement of the proboscis on the flower is visually guided, with light-colored regions being preferred over dark regions (Goyret 2010). Finally, when the proboscis is in contact with the

Figure 2.1
Manduca sexta, the pollinator used in this study, probing a flower with its proboscis. Photo by Armin Hinterwirth.

flower, mechanoreception through the proboscis ultimately is used to locate the nectar source (Goyret & Raguso 2006, Goyret 2010).

It is possible that corolla grooves are not the only mechanical stimuli used by moths during flower handling. Goyret and Raguso (2006) discussed the possibility that the trumpet-like shape of *Datura* flowers, from which *M. sexta* feeds in nature, may allow for more effective handling by moths in comparison with flat disks of the same diameter, but did not manipulate corolla shape in their study. Indeed, the stereotypical hawkmoth-pollinated flower is deemed to have a funnelform, tubular corolla (Fægri & van der Pijl 1966, Proctor et al 1996). *Datura*, *Petunia*, and *Nicotiana* (tobacco) flowers are a few examples of such funnelform hawkmoth-pollinated flowers.

Here, we test the hypothesis that the trumpet-shaped curvature associated with hawkmoth-pollinated flowers can act as a mechanical floral guide. This was motivated by our initial intuition that, if crepuscular hawkmoths "feel" their way into a flower's nectary with the proboscis, a flat-disk flower would provide no reliable tactile cues as to the location of the nectary, making it more likely that a moth would abandon a visit before the nectary is located. In contrast, the surface of a trumpet-shaped flower slopes toward the nectary and could passively guide the proboscis to the nectary regardless of where the proboscis initially contacts the floral surface.

If physical contact between a pollinator and a flower helps the pollinator find the floral resource of interest, then floral shape might play a significant role in mediating this final phase of pollinator foraging. If that is the case, a deeper understanding of how flower morphology affects foraging performance can help elucidate the present ecology of both plant and pollinator.

The ability to explore the interaction of floral phenotype with pollinator behaviour is difficult in natural systems where genetic correlations among various floral traits may constrain the space of floral shape that can be explored. While it may be possible to create flowers of the desired phenotype through introgressive hybridization of natural populations, even under the most meticulous breeding program, some combinations of floral traits may be difficult or impossible to cultivate due to underlying developmental constraints. As a result, there has been a long tradition of fabricating artificial flowers of exactly the desired phenotype for testing in laboratory or controlled field experiments (Smith et al. 1996, Goyret & Raguso 2006, Sanderson et al 2006, Muchhala 2007, Yoshioka et al. 2007, Temeles et al. 2009, Whitney et al. 2009, Goyret 2010, Kaczoroski et al. 2012). But, with the advent of 3D printing technologies we have access to a powerful tool that can combine computer-aided design (CAD) with mathematical formulation to floral shape. That combination, in turn, can allow us to probe a much wider parameter space than could be made available by earlier fabrication methods or by natural breeding technologies. Thus, the combination of a natural pollinator with CAD-generated flowers presents a unique opportunity to explore the interactions between floral form and pollinator performance, essentially using high throughput technology for exploring the effects of floral morphological parameter space on pollinator foraging performance.

While real hawkmoth-pollinated flowers tend to have some degree of petal dissection and radial grooves leading toward the nectary, we modelled our artificial flowers as having no petal dissection and no grooves. This was done to eliminate any possible contribution of these aspects of flower shape to nectar discovery ability since those variables were beyond the scope of our study and the positive effects of petal dissection and radial corolla grooves has already been demonstrated (Goyret & Raguso 2006).

2.3 Materials and Methods

2.3.1 Animals

Individuals of *M. sexta* were obtained from a colony maintained by the Department of Biology at the University of Washington, Seattle, WA, USA. Moths were allowed to eclose in indoor cages with a 12:12 light:dark cycle. Food-deprived, flower-naïve moths were used in experimental trials 2-5 days post-eclosion. We attempted to collect behavioural data from 70 moths for this study. Of those, 20 moths were excluded from the study because they failed to forage on the artificial flower array and 5 moths were excluded because they emptied all 16 flowers in the array in the allotted time, removing the ability to assess differences in foraging ability based on the metric of number of emptied flowers alone. This left a total of 45 moths from which data were analyzed for this study. Both male and female moths were used in this study (23 males and 22 females). No significant differences were found between the male and female data, so males and females were grouped for the final analysis.

2.3.2 Construction of Artificial Flowers

We designed artificial flowers with fused petals since this is typical of hawkmoth-pollinated flowers in nature. In designing our artificial flowers, we sought to do two things: quantify flower shape, which is often described in qualitative terms (tubular, trumpet-shaped, bell-shaped, funnelform, etc.); and distil the complexity of natural flower shape into a few key parameters that could be systematically and individually varied depending on the hypothesis under investigation. We devised a generalized shape equation that would allow us to control four aspects of floral shape: corolla curvature, nectary radius, flower length, and whole-flower radius (Fig. 2.2). Of these, only corolla curvature and nectary radius were manipulated in this study, but other floral shape parameters may be manipulated in future experiments. The shape equation is:

$$z(r) = L\left(\frac{r - r_0}{R}\right)^{e^c}$$

where z represents the longitudinal axis of our flower model and r represents the radial distance of the corolla from the central z-axis. In addition, c is a curvature parameter determining the lateral profile of the corolla, r_0 is the nectary radius, L is the flower length, and R is the lateral extent of the corolla from edge of the nectary to the outer lip of the flower (note that whole-flower radius is equal to $r_0 + R$). This surface is then given an arbitrary thickness of 1 mm to create a volume. Finally, an adapter was designed at the bottom of the flower to allow a 200 μL PCR tube to be press-fitted onto the flower to act as a reservoir for artificial nectar (20% sucrose solution). The resulting three-dimensional volume was then fabricated in ABS plastic using a uPrint SE 3D printer. The ABS plastic used was white in color and effectively odorless. These flower models are rigid and do not flex or warp in response to subtle contact by *M. sexta* during feeding. Five distinct flower morphologies were used in this study. The values of their shape parameters are shown in Table 2.1.

A MATLAB script for exploring artificial flower shape parameter space using the shape equation described above is available at the following GitHub repository: https://github.com/eocampos/artificial_flower_shape_exploration

2.3.3 Behavioral Assays

Artificial flowers were placed in a 4 by 4 square array of 16 flowers with flower centres spaced 10 cm apart. A polystyrene foam box was used as the structural base for the flower array. The flower array was positioned so that the long axis of the flowers was orthogonal to the ground. The array was always dimorphic, populated by two distinct flower morphologies, present in equal numbers (8 of each). Flower position was randomized before each moth foraging trial using a custom Python script (https://github.com/eocampos/random_flower_array_shuffler_16flowers). Each nectar reservoir was filled with 20 μL of 20% sucrose solution, which is typical of many hawkmoth-pollinated flowers (Raguso *et al*. 2003), and of lepidopteran-pollinated flowers in general (Watt et al 1974; Heinrich 1975; Kingsolver & Daniel 1979). The flower array was placed inside of a 90 x 90 x 65 cm (height x width x depth) glass-walled flight chamber. A white light LED array illuminated the flight chamber from above at an illuminance

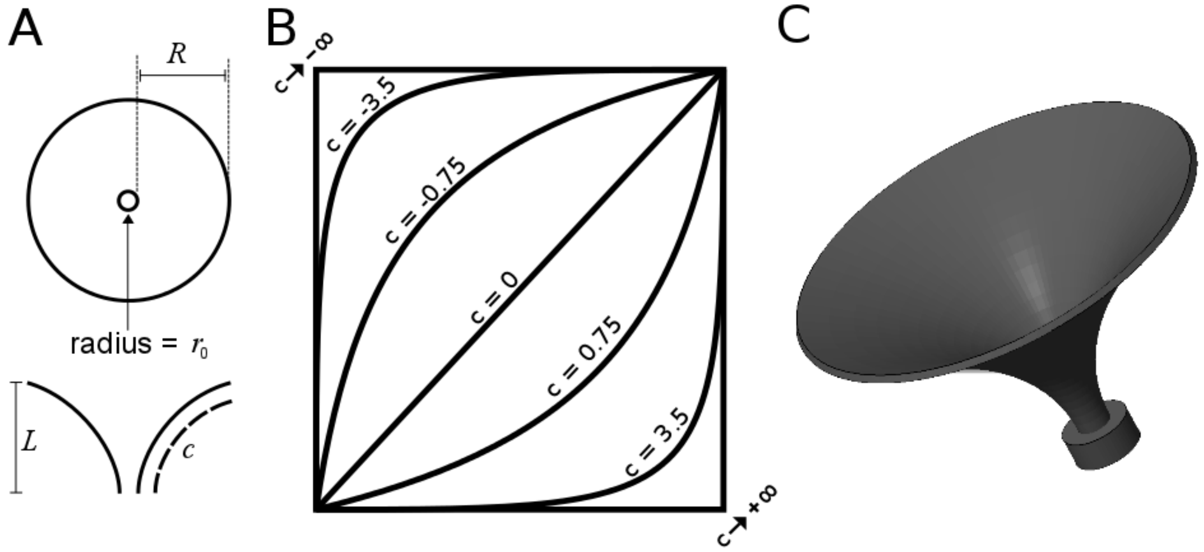

Figure 2.2
(A) Bird's eye view (top) and lateral section (bottom) of generic artificial flower depicting what each of our four shape parameters represent. R is the linear distance between the nectary aperture edge and the edge of the "corolla" and r_0 is the radius of the central aperture. Note that this means that whole-flower radius is equal to $r_0 + R$. (B) Illustration showing how altering the value of the corolla curvature parameter, c, in our flower shape equation affects the lateral profile of our artificial flowers. When c is equal to 0, the corolla forms a straight line between the base of the flower and its outer lip, forming a truncated cone if revolved around the vertical axis. Negative values produce curved corolla profiles that lead to trumpet-shaped flowers if revolved around the vertical axis. As c approaches negative infinity, the corolla approaches a vertical path that turns suddenly at a 90-degree angle, producing a flat-disk flower with cylindrical nectar tube if revolved. Positive values of c produce bowl-shaped flowers if the resulting path is revolved. The given values of c are only for illustrating the trend. (C) A 3D computer rendering of what the flower in (A) would look like.

Table 2.1 Shape parameter values of the five artificial flower morphologies used in this study.

Flower Morph	Length L (mm)	Nectary radius r_0 (mm)	Corolla Lateral Extension R (mm)	Curvature Parameter c
M1	30	2.5	25	-0.75
M2	30	2.5	25	$\to -\infty$
M3	30	1.25	26.25	-0.75
M4	30	1.25	26.25	$\to -\infty$
M5	30	1.25	26.25	-3.5

of 0.1 lux to simulate moonlight conditions. Illuminance was measured with a Gossen Mavolux 5032C lux meter at flower level on the centre of the flight chamber. The flight chamber was also illuminated with two bright infrared lights (Magnalight **LEDLB-16E-IR;** 790-880 nm flat emission peak) for recording foraging behaviours with an infrared video camera (Sony DCR-TRV310).

Moths were placed one at a time into the flight chamber and allowed to forage from the flower array for 5 minutes following the first visit to a flower. Each individual moth only took part in one foraging trial. Moths that did not take flight within 5 minutes of being placed in the flight chamber were replaced and not used again.

Manduca sexta requires olfactory cues to initiate its appetitive behaviour (Brantjes 1978; Goyret & Raguso 2006), so we placed a piece of filter paper impregnated with 8 μL of a 7-component mixture of floral scent inside of the flight chamber 5 minutes before the first foraging trial of a data collection session. The filter paper remained in the flight chamber between trials to keep the air in the chamber as saturated with scent as possible. This artificial flower scent is designed to be representative of a host of hawkmoth-pollinated flowers. Within a mineral oil vehicle, the floral scent mixture contained benzaldehyde (0.6%), benzyl alcohol (17.6%), and linalool (1.8%). These three compounds are sufficient to elicit behavioural responses similar to those elicited by natural *Datura wrightii* scent. *Manduca sexta* has an innate bias toward *D. wrightii* scent (Riffell *et al*. 2009b). The mixture also contained methyl salicylate (2.4%), nerol (3%), geraniol (9%), and methyl benzoate (0.6%), the latter being present in significant quantities in the headspace of many hawkmoth-pollinated flowers (Riffell *et al*. 2013).

After a foraging trial was completed, the moth was recaptured and the number of emptied flowers of each morph was recorded. Flowers were always either completely full or completely empty following a foraging trial, so the resulting data were composed of integer values ranging from 0 to 8 for each flower morph. Video of the foraging trials was also used to count the number of visits that the moths made to each flower morph.

For each of the three experiments described below, the sample size was equal at N = 15 foraging trials.

2.3.4 Experiment 1

In the first experiment, the two morphs present in the flower array were a trumpet-shaped morph (M1) and a flat-disk morph (M2) with 2.5 mm nectary radii. These two morphs will be referred to as M1 and M2 throughout the rest of this paper (Fig. 2.3; Table 2.1). Corolla curvature was the only difference between the two morphs. The design goal for these two morphs was to create a pair of flowers that differed markedly in their corolla curvature, with the flat-disk morph being putatively more difficult to exploit than the trumpet-shaped morph. The nectary radius of 2.5 mm was chosen so that we could be sure that nectary constriction was not a limiting factor to nectary discovery, as the proboscis of *M. sexta* is on the order of 1 mm wide. A flower length of 30 mm was chosen so as to ensure that moths could theoretically reach the nectary, their proboscis length ranging from 50-70 mm.

2.3.5 Experiment 2

The second experiment contained flower morphs that were identical to the flowers used in Experiment 1, except that nectary radius was reduced by half to 1.25 mm for both morphs (Fig. 2.3, M3 and M4). This is slightly larger than the nectary aperture sizes of *Datura wrightii* and *Petunia axillaris* (measured by us using digital callipers on one *D. wrightii* flower and six *P. axillaris* flowers taken opportunistically from the University of Washington greenhouse in February of 2014), two flowers that are visited by *M. sexta* in the wild (*D. wrightii* nectary radius: 1 mm; *P. axillaris* nectary radius mean: 0.7 mm ± 0.06 SEM).

2.3.6 Experiment 3

The third experiment again contained a flat-disk morph and a trumpet-shaped morph. The flat-disk morph was identical to the one used in Experiment 2 (Fig. 2.3, M4). The trumpet-shaped morph in this experiment had a corolla curvature such that it deviated from the flat-disk morph only slightly, having a sharp curve at the transition between the upper disk portion of the flower and the "nectar tube" portion of the flower instead of the exact 90-degree angle of the flat-disk morph (Fig. 2.3, M5).

2.3.7 Statistical Analysis

For each of our three experiments, we tested two null hypotheses: 1) the mean number of flowers of each morph that were emptied in the array is equivalent; and, 2) the mean number of visits to each flower morph in the array is equivalent. We used a series of permutation tests (Edgington

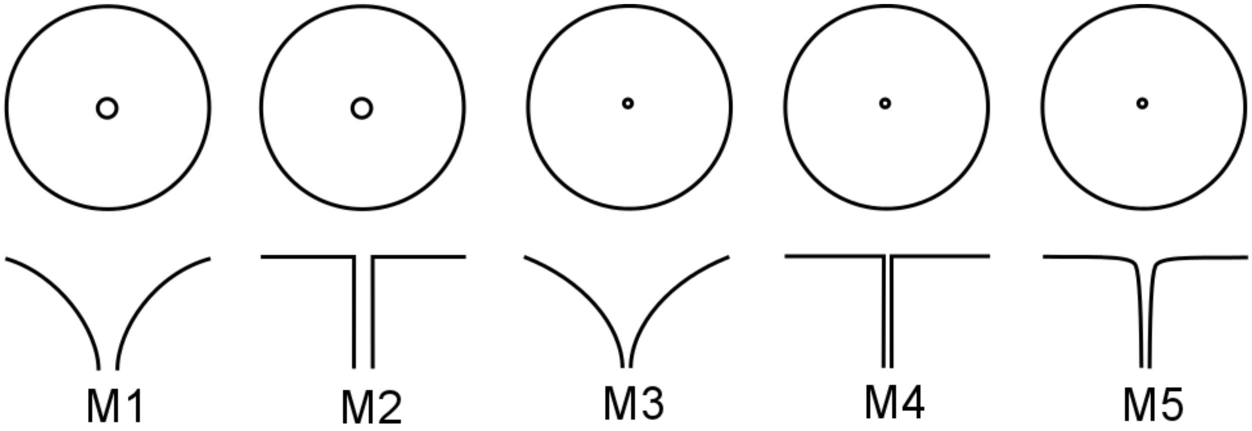

Figure 2.3
Bird's eye view (top) and lateral section (bottom) of the five different artificial flower morphs used in our experiments. They are labeled M1 through M5 and referred to as such in the main text for convenience. All are drawn to the same scale.

1969) to test these hypotheses. The data in each of our experiments took the form of two pairs of columns of numbers: the number of trumpet-shaped flowers with emptied nectaries and number of flat-disk flowers with emptied nectaries in each foraging trial; and the number of visits to each morph in each foraging trial. Under the null hypothesis, the difference between the means of each pair of columns should be zero. We used a custom MATLAB script (https://github.com/ eocampos/permutation_testing) to randomly permute (shuffle) the assignment of each data entry to the two categories (trumpet-shaped *vs*. flat-disk flowers) in the data table and then recalculate the difference between the means of the two permuted columns of data. The data were permuted 10,000 times and the resulting mean differences were used to construct the null sampling distribution against which the observed data were compared.

Once our null sampling distributions were constructed, we performed 1-tailed tests of the null hypothesis by mapping our observed mean differences onto the corresponding sampling distributions and determining the fraction of the sampling distributions that rested between the observed mean difference and the end of the tail. This fraction is the 1-tailed p-value. We chose an α level of 0.05 for each of our individual tests.

2.4 Results

In our first experiment comparing a trumpet-shaped flower against a flat-disk flower with 2.5 mm nectary radius, we found a significant difference in the number of emptied flowers, with trumpet-shaped flowers emptied more frequently than flat-disk flowers (Fig. 4A; M1 trumpet-shaped mean = 5.7 ± 0.57 SEM; M2 flat-disk mean = 3.9 7 ± 0.56 SEM; $p = 0.0443$). Moths did not show a tendency to visit one morph more than the other (M1 trumpet-shaped mean = 21.2 ±

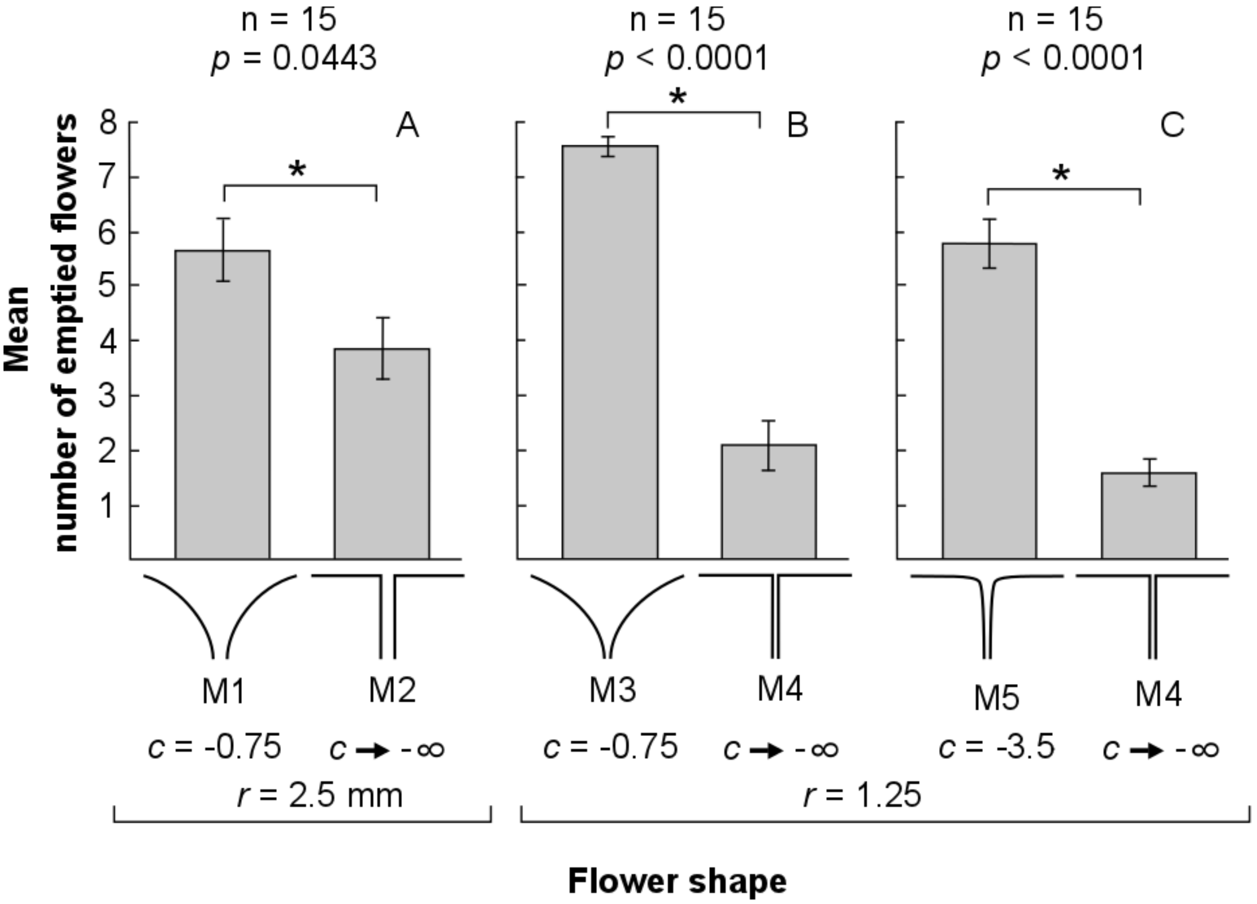

Figure 2.4.
Mean number of emptied flowers of each flower morph per foraging bout (± SEM) in each of our three experiments. Parts A-C correspond to Experiments 1-3 respectively. Significant differences are denoted with an asterisk.

2.9 SEM; M2 flat-disk mean = 19.2 7 ± 2.4 SEM; $p = 0.6134$) and did not exhibit constancy during foraging to a significant degree as measured by Bateman's index (Bateman 1951, and see also Waser 1986) (mean BI = -0.35 ± 0.10 SEM; $p > 0.99$). The subsequent two experiments were done with flower models with nectary radii reduced by half to 1.25, corresponding to a four-fold reduction in nectary size in terms of area. This reduction in nectary radius was the only difference between the flower models used in Experiment 1 and Experiment 2. With this one change, we saw an even greater difference in the number of emptied flowers of each morph (M3 trumpet-shaped mean = 7.6 ± 0.16 SEM; M4 flat-disk mean = 2.1 ± 0.44 SEM; $p < 0.0001$), but still no tendency to visit one morph more than the other (M3 trumpet-shaped mean = 31.9 ± 2.6 SEM; M4 flat-disk mean = 31.1 ± 3.3 SEM; $p = 0.4287$), and still no tendency towards constancy during foraging (mean BI = -0.09 ± 0.05 SEM; $p = 0.97$). In Experiment 3, we kept the disk morph unchanged from the previous experiment but changed the curvature parameter of the trumpet-shaped morph so that it more closely approached the sharp 90-degree transition in lateral profile of the flat-disk morph. However, even with this change, we still saw the same pattern of flower exploitation heavily biased toward the curved trumpet-shaped flower (M5 trumpet mean = 5.8 ± 0.46 SEM; M4 flat-disk mean = 1.6 ± 0.25 SEM; $p < 0.0001$) and with no tendency to visit one morph more than the other (M5 trumpet mean = 26.7 ± 2.4 SEM; M4 flat-disk mean = 26.1 ± 2.5 SEM; $p = 0.4253$). Again, moths did not exhibit constancy during foraging during Experiment 3 (mean BI = -0.06 ± 0.03 SEM; $p = 0.79$).

2.5 Discussion

We set out to test whether the centrally sloping morphology of trumpet-shaped flowers might act as a mechanical floral guide for the long proboscis of hawkmoths. Our results show that hawkmoths were indeed able to exploit trumpet-shaped flowers far better than flat-disk flowers in all three of our experiments (Fig. 2.4).

We excluded the possibility that this result could be due to an initial preference for either morph by showing that both flower morphs were visited with equal frequency and without constancy, supporting our hypothesis that the differences in foraging success were due to interactions between the moth's proboscis and the different tactile cues offered by trumpet-shaped and flat-disk flowers.

Krenn (1998) showed that the lepidopteran proboscis contains mechanoreceptors, especially at the proboscis tip where contact with a flower would initially be made. However, our data do not allow us to discriminate between the alternatives of: 1) active mechanical sensing of a curved surface by *M. sexta via* the proboscis; and, 2) passive motion of the proboscis directed by the curved landscape of a trumpet-shaped corolla. This issue remains an open topic, particularly in the domain of sensory ecology where multiple sensory modalities mediate interactions between animals and plants.

Interestingly, moths presented with a flat-disk flower and a trumpet-shaped flower whose curvature was so slight that it qualitatively resemble the flat-disk morph to a high degree (Experiment 3) showed that even this small deviation from a flat-disk corolla towards the trumpet shape characteristic of hawkmoth-pollinated flowers is enough to significantly increase nectar discovery ability (Fig. 2.4C). Such sensitivity to slight curvature was unexpected and is indicative of the potential importance of corolla curvature as a mechanical floral guide. Future experiments will better define the strongly nonlinear relationship between corolla curvature and foraging success.

Results from others suggest that foraging by *M. sexta* is under multimodal sensory control, with different sensory modalities or combinations of them being important at different stages of the foraging process, depending on a moth's distance from a food source. At large distances, a moth uses olfaction to orient toward a group of flowers (Brantjes 1978). At intermediate distances, the approach to a group of flowers is guided by either olfaction or vision while extension of the proboscis requires that appropriate olfactory and visual stimuli be present simultaneously (Goyret et al. 2007). Once a moth has selected an individual flower, initial placement of the proboscis on the flower is visually guided, with lightly coloured regions being preferred over dark regions (Goyret 2010). Finally, when the proboscis is in contact with the flower, mechanoreception through the proboscis ultimately is used to locate the nectar source (Goyret & Raguso 2006, Goyret 2010). Our results corroborate recent conclusions that *M. sexta* primarily uses tactile cues to find floral nectar during visits to individual flowers. However, while the experimental manipulations of Goyret & Raguso 2006 and Goyret 2010 focused on the role of floral surface features such as grooves, our results suggest that whole-flower shape might also function as a mechanical nectar guide for nocturnal/crepuscular moths foraging in low light.

We have shown that trumpet-shaped corolla curvature assists moths in exploiting floral resources. But this phenotypic feature might also facilitate pollination for the flower by helping to align the animal with the flower's reproductive parts. If, for example, the anthers and stigma are located along the centre-line of the flower, then the trumpet shape could force a visiting animal toward the reproductive parts while still allowing sufficient access to the reward. In that case, the -3.5 curvature flower (Fig. 2.4C) flower could have been pollinated more, and consequently would have been more fit, than the -0.75 flowers (Fig. 2.4A-B). Flower petals have indeed been shown to act as positioners for hummingbirds, facilitating pollen deposition (Temeles & Rankin 2000). Given this, why do many night-blooming flowers qualitatively resemble the -0.75 corolla curvature? One explanation is that corolla curvature could be under balancing selection due to interactions with other animal species, pollinator or otherwise, as in the finding that phenotypic variation in flower form in the alpine wildflower *Polemonium viscosum* was maintained by conflicting demands for pollinator attraction and enemy avoidance (Galen 1999).

It is important to note that there is increasing evidence that nectarivory is influenced not only by the host of floral morphological parameters mentioned above, but also by the dynamics of floral motion (Sprayberry & Daniel 2007). Thus the ability of a moth to exploit a flower depends as well upon how effectively it can track a moving flower. That tracking could depend upon both the visual tracking capability of a pollinator and upon any mechanosensory signals (at the level of the proboscis) they may process. Both sensory stimuli are potentially influenced by floral shape. Thus while Sprayberry & Daniel (2007) showed that motion and morphology combine to determine pollinator foraging success there are a host of open topics that include the roles of luminance, mechanosensing, and even the frequency characteristics of the natural motions of flowers.

As mentioned previously, we did not need to manipulate all of the floral shape parameters in our generalized shape equation in order to test the hypotheses presented in this paper. Many different experiments investigating the role of floral form in determining pollinator foraging effectiveness are still possible. In particular, nectary width and length are known to be important determinants of pollination success in Malagasy long-spurred orchids (Nilsson et al. 1985, Nilsson 1988). Our ability to manipulate mathematically-specified artificial flower morphology means that we have the ability to investigate the role of minute but potentially important differences in floral form on pollinator foraging performance, independent of evolutionary and developmental constraints on the shape of flowers in nature. Furthermore, additional floral features such as separated petals, bilateral symmetry, and rotation of the corolla along the long axis of a flower are all possible *via* the addition of more parameters to the shape equation. We hope that such precise, well-defined, reproducible flower construction techniques can open the door to otherwise inaccessible or difficult manipulations of floral form.

Using the power of computer-aided design and rapid prototyping technologies, it is increasingly possible to explore how individual aspects of floral morphology influence plant-pollinator interactions. These interactions, mediated by physical contact between the flower and the animal, are a major determinant in the effectiveness with which pollen is transferred to conspecific flowers. Using the methods and 3D-printing technologies in this study, it is possible to construct not only foraging performance landscapes as a function of variation in flower morphology, but also flower fitness landscapes as pollinators attempt to forage from populations of computationally-derived floral forms. Such studies can help elucidate the details of how pollinator visitation influences the evolution of floral shape in nature, and the extent to which floral forms are the result of specializations between one plant and one pollinator species, or generalization due to multiple species of visitors exerting significant but non-similar selective pressures.

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2.7 Data Accessibility

Data are deposited in the University of Washington Research Works Archive. http://hdl.handle.net/1773/27082 (Campos, Bradshaw & Daniel 2015).

2.8 Supplemental Software Accessibility

Script for shuffling positions of artificial flowers in the 16-flower array:

https://github.com/eocampos/random_flower_array_shuffler_16flowers

Script for exploring shape parameter space of the artificial flowers described in this study:

https://github.com/eocampos/artificial_flower_shape_exploration

Script for carrying out permutation tests:

https://github.com/eocampos/permutation_testing

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Chapter 3

The Foraging Performance Landscape of a Crepuscular Hawkmoth in Response to Variation in Flower Shape

3.1 Abstract

I constructed a foraging performance landscape for dark-active hawkmoths (*Manduca sexta*, hereafter Manduca) attempting to feed from artificial flowers that vary in two aspects of their shape: corolla curvature and nectary diameter. The artificial flowers were designed in computer-aided design (CAD) software and their shapes were determined by parameters in a mathematical equation and then 3D-printed to achieve high precision, reproducibility, and throughput of the physical models.

In a series of foraging trial experiments involving hexamorphic, 36-flower arrays, Manduca foraging performance exhibited sensitivity to both nectary diameter and corolla curvature. When nectary diameter was small (2.5 mm or less) foraging performance peaked at corolla curvatures corresponding to trumpet-shaped flowers, with performance dropping steadily toward the two extremes of curvature (flat-disk flowers and bowl-shaped flowers). When nectary diameters were larger (3.25-7 mm), foraging performance plateaued at a high level, independent of corolla curvature. Interestingly, these data suggest that Manduca can maximally exploit trumpet or cone-shaped flowers regardless of how narrow the nectary is, until it becomes narrower than the width of the moth's proboscis. These results support earlier studies characterizing Manduca's

nectar discovery strategy as heavily reliant on the physical interactions between the proboscis and the surface of the flower.

The success of generating this performance landscape using precisely engineered flowers holds promise for future iterative improvements to this kind of experimental design, which could open up new investigations in the field of plant-pollinator interactions, particularly their coevolution.

3.2 Introduction

The flowering plants constitute over 250,000 described species, accounting for over 87% of land plant diversity (Palmer *et al*. 2004). For decades, the concept of the pollination syndrome has been the leading framework for understanding the evolution of floral phenotypic disparity. This concept hypothesizes that pollinating animals can be classified into one of several ecologically equivalent “guilds” whose members visit a common set of flowering plant species (Ollerton *et al*. 2009). Such specificity provides a mechanism by which relatively unrelated flower species can independently evolve similar phenotypes, in response to selection by pollinators of a common guild (Fægri & van der Pijl 1979).

Pollination syndromes are typically named according to the animal taxon that is broadly representative of that syndrome, and are described in terms of the phenotypic traits that the flowers within each syndrome commonly exhibit (Fenster *et al*. 2004). For example, flowers belonging to the hummingbird pollination syndrome commonly exhibit reddish colors, tubular shapes, weak fragrances, and produce large amounts of dilute nectar (Fenster *et al*. 2004). Hummingbirds putatively select for this combination of floral traits. However, recent research has shown that traditionally-defined pollination syndromes are poor predictors of the most frequent visitors to a species of flower, casting doubt on the explanatory power of pollination syndromes (Hingston & McQuillan 2000; Ollerton *et al*. 2009), although consensus remains that floral phenotype has been heavily influenced by flower-visiting animals in general (Feinsinger 1983).

But the evolutionary pressures exerted by pollinators on flowers are not unilateral. Through their close ecological associations, flowers and pollinating animals are hypothesized to often be locked into close coevolutionary relationships in which both the plant and the pollinator mutually influence each other's evolutionary trajectories (Feinsinger 1983; Futuyma & Slatkin 1983). Although morphological adaptations for specialized fluid feeding by insects may predate the radiation of the angiosperms (Ren *et al*. 2009; Ollerton & Coulthard 2009), fossil evidence suggests that the diversifications of major lineages of modern flower-visiting insects co-occurred with that of the angiosperms in the Cretaceous (Grimaldi 1999).

Perhaps one of the most extreme putative examples of plant-pollinator coevolution (see a counter-explanation by Wasserthal 1997) is the mutual selection for long appendage length in the Malagasy epiphytic orchid, *Angraecum sesquipedale* (the nectar spur) and the East African hawkmoth, *Xanthopan morgani* (the proboscis). When sent a sample flower of *A. sesquipedale*, Charles Darwin observed the unusually long nectar spur of the flower, which could be anywhere from 27-43 cm in length (Wasserthal 1997), and famously surmised that there must exist a moth in the orchid's range with a proboscis long enough to reach the nectar (Kritsky 2001; Darwin 1862). Alfred Russel Wallace later predicted that the exceptionally long proboscis of *X. morgani* might be a likely candidate (Wallace 1867), and in 1903 a subpopulation of this hawkmoth was indeed found in Madagascar (Rothschild & Jordan 1903). This example is often touted as one of the great predictive success stories of Darwin's (at the time) still fledgling theory of evolution by natural selection (Kritsky 2001). In support of this coevolutionary scenario, recent data have shown that hawkmoths can evolve innate olfactory preferences for flower species whose lengths match the length of the moth's proboscis (Haverkamp *et al*. 2016). This task represents an extreme form of nectarivorous foraging in which a hovering moth probes with a long, compliant tube (the proboscis) to find a flower's nectar reservoir in low light conditions. Foraging on nectar while hovering may present a significant sensorimotor challenge to the animal, but is nonetheless effectively performed in nature (reviewed in Campos *et al*. 2015).

The final phase of crepuscular (twilight active) hawkmoth foraging, nectar discovery, is sensitive to variation in the visited flower's corolla curvature and nectary diameter (Campos *et al*. 2015). But the details of how these aspects of flower shape affect foraging performance individually and in concert have not been empirically demonstrated. With nocturnal hawkmoths representing a major guild of pollinating insects (Dar *et al*. 2006; Goyret & Raguso 2006), an evaluation of their foraging performance relative to variation in flower shape may yield ecological and evolutionary insights for both the animal and the plant.

One fruitful approach to the study of organismal phenotype evolution involves the construction of a morphospace. Morphospaces are representations of organismal phenotype that can be useful in distilling a prohibitively complex multidimensional concept into its most salient parameters, thereby facilitating pattern discovery. Although organism phenotypic diversity is vast in nature, it is always the case that some theoretically possible phenotypes will never be realized. This could be for a variety of reasons including developmental pathway constraints, fitness tradeoffs with other traits, or historical contingency - i.e. because the random mutation(s) necessary for a particular phenotype have simply not yet occurred despite the existence of a potential fitness advantage.

One of the earliest landmark contributions to the morphospace literature was a group of papers addressing the theoretical and realized landscape of mollusk shell shape (Raup & Michelson 1965; Raup 1966; Raup 1967), in which shell shape was described by a logarithmic spiral model with four shape parameters. Even with just four parameters, this model was able to capture the large-scale aspects of gastropod shell shape (minus ornamentation details, for example having to do with surface textural features). Armed with a theoretical morphospace, one can begin to explore the relationships between geometry and function or, in an eco-evolutionary perspective, the relationship between phenotype and fitness.

Following these landmark papers, morphospace analyses have been employed extensively in animal systems, but relatively rarely in plant systems, and more rarely still in studies of flower morphology in particular (reviewed by Chartier *et al*. 2014). In this study, I use a combination of mathematical modeling and 3D printing to construct artificial flowers for use in free-flight pollinator foraging experiments. Because flower morphology is explicitly quantified by the parameters in my mathematical model, pollinator foraging performance can be plotted explicitly within the context of a flower morphospace.

In order to build off of the major result from Chapter 2, namely that 3-dimensional aspects of floral form affect foraging performance in a dark-active hawkmoth, as a next step I wanted to more fully characterize this dependence by measuring foraging performance in a greater volume of flower morphospace. However, the experimental setup used in Chapter 2 was inadequate for this, because it would take a prohibitively large amount of time to sample the desired number of flower morphs using a dimorphic flower array and manual collection of nectar removal data. Therefore, I redesigned my experimental arena in order to facilitate data collection for this study's goals. At this point, it could be helpful to define a few terms within the context that they are used in this study:

Flight arena: the enclosure (4 walls and a roof) inside which foraging trials take place. The flower array exists inside the flight arena. The dimensions of the flight arena were 2.4 m width by 4.0 m length by 2.5 m height.

Flower array: The physical structure within the flight arena, which holds individual artificial flowers in place while hawkmoths attempt to forage during foraging trials. The flower array, as designed for this study, holds 36 flowers in a 6 by 6 square pattern.

Flower morph: an artificial flower with a distinct combination of flower shape parameters.

Flower population: A distinct group of 6 flower morphs and their duplicates, forming the 36 flowers that populate the flower array.

With the above terms explicitly defined, I return to describing the changes that I made in my experimental setup relative to Chapter 2. Specifically, I increased the size of the flower array from a 4x4 16-flower array that could accommodate 8 of one morph and 8 of another morph simultaneously, to a 6x6 36-flower array that could accommodate 6 different flower morphs at equal frequency of 6 of each. This larger artificial flower array allowed me to sample flower morphospace faster, enabling me to construct a performance landscape of hawkmoth foraging as a function of variation in flower corolla curvature and nectary diameter. To detect successful nectary probing events, each artificial flower was held in place by a custom 3D-printed bracket that housed an occlusion-triggered infrared sensor, composed of an infrared-emitting diode and an infrared-detecting diode. A moth's porboscis would need to pass though the IR beam to reach the nectar source of the flower, partially occluding the beam and signaling the probing event to a data acquisition device.

The major questions to be addressed here are:

1.) How sensitive is hawkmoth foraging performance to variation in flower nectary diameter?

2.) Does "trumpet-shaped" represent an optimum corolla curvature in terms of hawkmoth foraging performance?

3.) Do nectary diameter and corolla curvature have independent effects on hawkmoth foraging performance, or do they interact to produce a synergistic or context-dependent pattern of foraging performance?

Many animals meet their daily energetic needs exclusively or in large part by consuming floral nectar. The last stage of the foraging process of Manduca, a representative of the crepuscular/nocturnal moth pollinator guild, seems to rely on physical contact between the proboscis and the surface of the flower. Nectary diameter and corolla curvature are two of the most salient features of a flower's morphology that might influence nectar discovery in a probe-dominated search strategy. In addition, these aspects of flower morphology might also play an important role in positioning a pollinator so that contact with the flower's reproductive parts is most likely, which has implications for the flower's fitness.

Experimental explorations of how flower morphology might affect pollinator performance can be difficult to execute using real flowers. Genetic correlations and developmental constraints conspire to make some flower phenotypes practically impossible to achieve, even under the most meticulous of greenhouse breeding programs (Campos *et al*. 2015). In contrast, the mathematical modeling approach that I employ in this study gives me complete quantitative control over experimental morphology treatments while eliminating subjectivity from the descriptions of flower shape. Furthermore, the choice of 3D printing as the manufacturing method of my artificial flowers allows for rapid and precise construction of my artificial flower populations, allowing for large experimental populations sizes. Such attributes of my experimental design might have been prohibitively difficult to execute using other established artificial flower construction techniques (Smith *et al*. 1996; Goyret & Raguso 2006; Sanderson *et al*. 2006; Muchhala 2007; Yoshioka *et al*. 2007; Temeles *et al*. 2009; Whitney *et al*. 2009; Goyret 2010; Kaczorowski *et al*. 2012; Policha *et al*. 2016). The combination of computer-aided design (CAD), mathematical modeling, and 3D printing is a start toward the implementation of high-throughput technologies to address questions at the interface of plant-pollinator ecology and evolution.

3.3 Materials and Methods

3.3.1 Animals

Individual Manduca were obtained from a colony maintained by the Department of Biology at the University of Washington (see Materials and Methods from Chapter 2 for relevant details). Moths were flower-naïve and unfed for 2-3 days post-eclosion. The moths used in foraging experiments were obtained haphazardly with respect to sex. The lack of any statistically significant differences between the foraging behavior of males and females in Chapter 2 informed my decision to pool the data from males and females. Any moth that failed to fly or feed from any artificial flowers within 5 minutes of being released into the experimental arena was excluded from the study. In total, I attempted to collect data from 190 moths. Of those, 125 moths (68 males, 57 females) successfully foraged from the artificial flowers and were included in the analysis.

3.3.2 Shape Parameters of Artificial Flowers

The artificial flowers used in this study were made using a 3D printer, using the same methods described in Chapter 2 (Campos *et al*. 2015). Briefly, they were designed in a computer-aided design application based on an equation that allows for the manipulation of up to four aspects of flower shape: corolla curvature, nectary radius, flower length, and whole-flower radius. Of these, only corolla curvature and nectary radius were manipulated in this study, but other floral shape parameters may be manipulated in future experiments. The shape equation is:

$$z(r) = L\left(\frac{r - r_0}{R}\right)^{e^c}$$

where z represents the longitudinal (often vertical) axis of our flower model and r represents the radial distance of the corolla from the central z-axis. In addition, c is a curvature parameter determining the lateral profile of the corolla, r_0 is the nectary radius, L is the flower length, and R is the lateral extent of the corolla from edge of the nectary to the outer lip of the flower (note that whole-flower radius is equal to $r_0 + R$).

3.3.3 Experimental Apparatus and Flight Arena

Artificial flowers were arranged in a square array that could hold 36 evenly spaced flowers. Flower centers were spaced approximately 30.5 cm apart. Before a foraging trial began, the artificial flowers were placed in random positions in the flower array. Flower position randomization was implemented by a custom Python script making use of a pseudorandom number generating function (GitHub repository: https://github.com/eocampos/random_flower_ array_shuffler_36flowers). Modular T-slot extruded aluminum (80/20® Inc.) was used as the structural support of the flower array. The 36-flower array was populated with 6 distinct flower morphs, present at equal frequencies of 6 of each.

Only corolla curvature and nectary diameter were varied in this study. Flower positions were randomized with a custom Python script before each foraging trial. Each artificial flower's nectar reservoir was filled with 20 μL of 20% w/v sucrose solution. Two dim white LED lights illuminated the flight arena from above, calibrated to a combined illuminance of 0.1 lux at flower level to simulate moonlight conditions (Gossen Mavolux 5032C lux meter, Gossen Foto- und Lichtmesstechnik GmbH, Nuremberg, Germany). The flight arena was also illuminated from above with a single infrared light (Magnalight LEDLB-16E-IR; 790-880 nm flat emission peak, modified by removing the light's focusing lenses to create even, diffuse lighting) to allow video recording of the moth's foraging trajectory (Fig. 3.1).

Each artificial flower was held in the flower array by a 3D-printed bracket that contained an infrared (IR) emitter-detector (Fig. 3.2), creating an infrared beam sensor. When a moth's proboscis enters the nectar reservoir of any artificial flower, the infrared beam is broken, and this event is recorded through a data acquisition system consisting of an Arduino® microcontroller and a laptop computer (Fig. 3.1). The IR beam sensor data were used in this study to check after each foraging trial for whether or not all 6 of any flower morph were exploited. If so, then any flower emptied after that time was not included in the analysis, to ensure that time was not a factor in the relative exploitation ability of the flowers. In other words, if given enough time, a moth might eventually manage to empty all flowers of all morphs in the array even though some

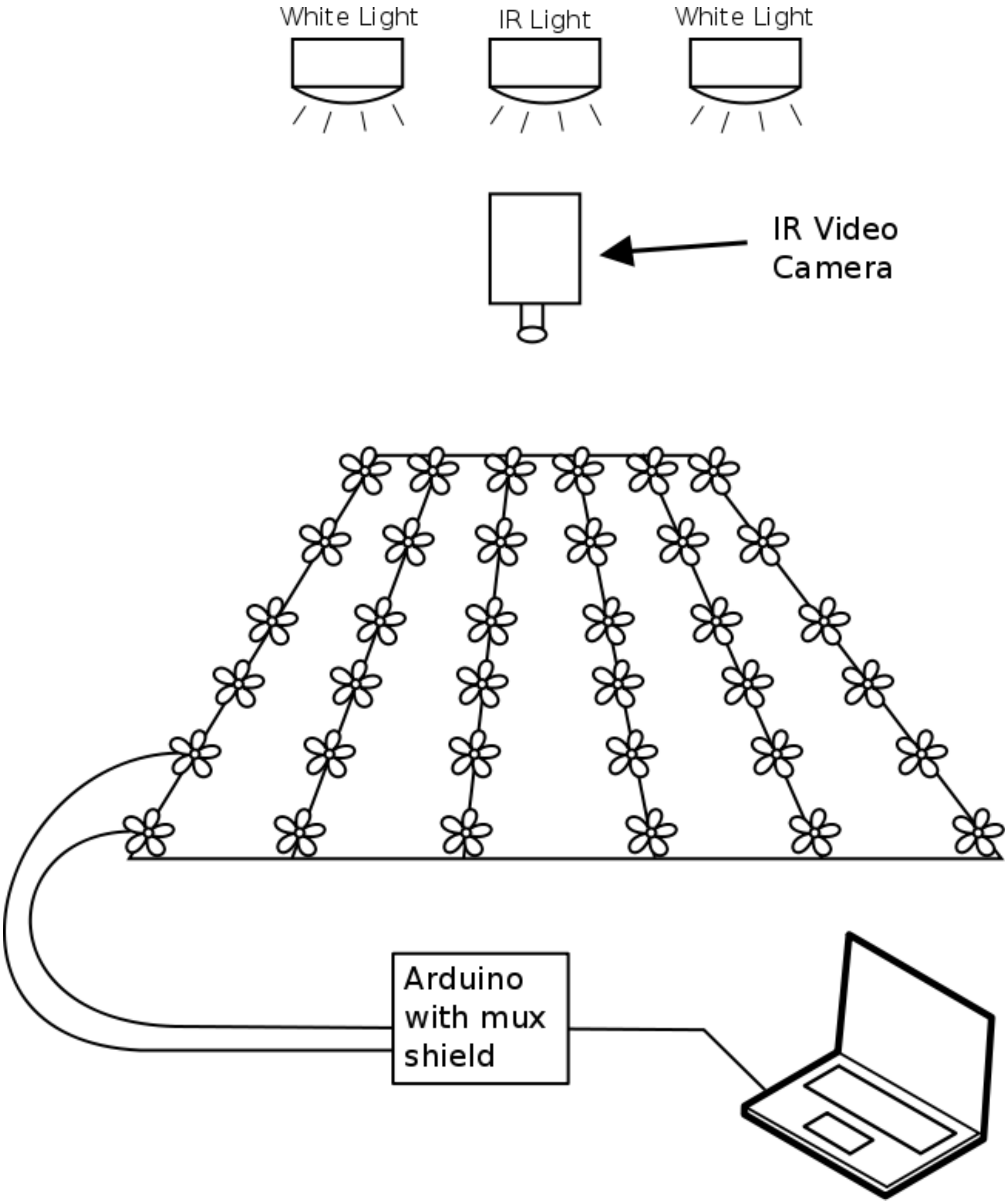

Figure 3.1 Line drawing depicting major elements of my experimental arena. The morphological disparity of the artificial flowers populating the array is not reflected here. Each artificial flower was attached to an infrared sensor to detect when proboscis insertion into the nectary occurred at each flower, and to calculate the duration of each probing event. The signal from each flower was routed to an Arduino® microcontroller and then relayed to a laptop via USB. For simplicity, only two of the flowers are shown "wired up" in this graphic.

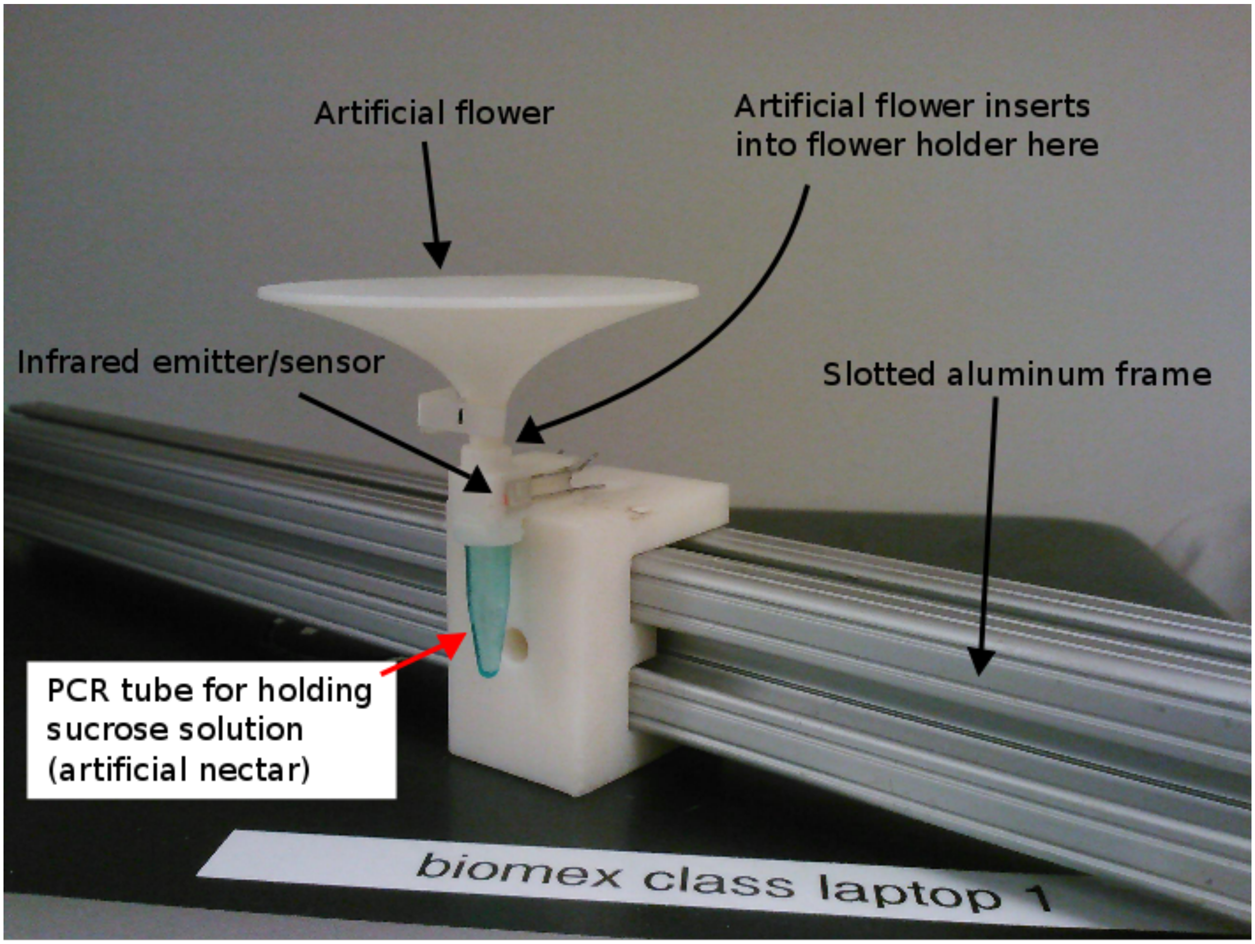

Figure 3.2
Photograph depicting the custom 3D-printed bracket used to hold artificial flowers in the 36-flower array with integrated IR emitter/detector diodes. Note the electrical leads of this demonstration model are not connected to anything.

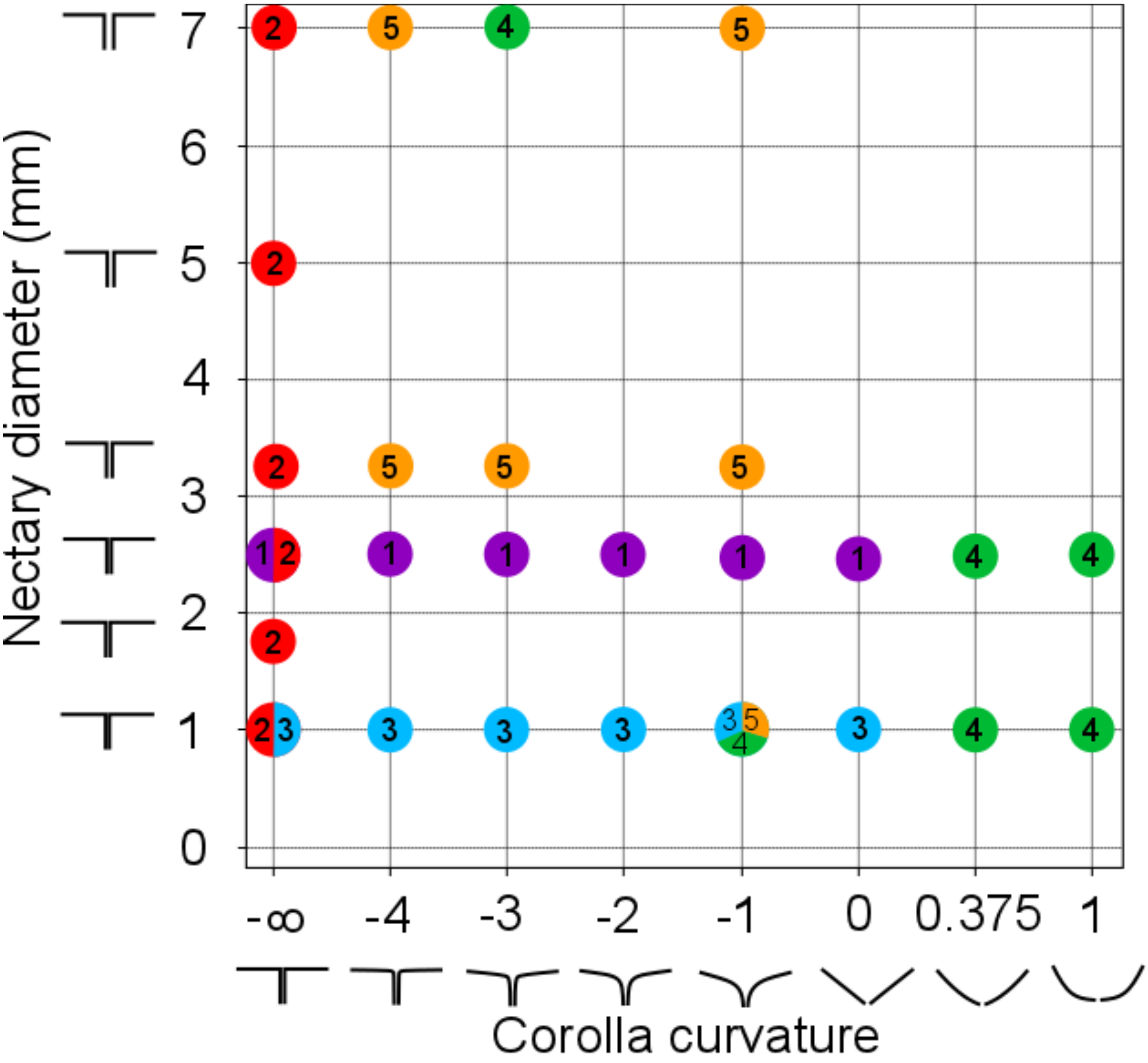

Figure 3.3
Graphic depicting the locations (colored circles) in the theoretical flower morphospace of the flowers used in each of my 5 experimental artificial flower populations. Numbers inside colored circles represent the population (numbered 1 through 5 in the main text) that each flower was used in. Exact values of the shape parameters are given in the Methods, Section 3.3.5 of the main text. Three flowers were used in two or more populations each, represented by the chimeric colored circles and two-digit numerical code. Example: the flower location coded 12 was used in populations 1 and 2. In future figures in this chapter, moth foraging performance (average number of emptied flowers per foraging trial) will be plotted within this same set of axes.

are easier to exploit than others, e.g. if a moth depletes flowers in ranked order of their ease of exploitation until all are eventually consumed.

An infrared-sensitive USB video camera (Point Grey Firefly® MV, model FMVU-03MTM-CS, 640x480 resolution) was mounted above the flower array looking straight down, to capture a bird's eye view of the moth's flight trajectory as it foraged on artificial flowers. In preparation for a foraging trial, the IR sensors/flower holders were covered with a sheet of black felt to provide a low-glare, high-contrast background against which the moth could easily be seen in the video footage of each foraging trial. All IR sensor wires and the video camera cable were routed under the door of the flight arena and relayed to a laptop computer, allowing me to monitor the live video feed and IR sensor states from outside of the arena, so as to not disturb a moth during its exploration of the arena.

The air temperature of the flight arena was kept at a constant 24°C using an electric space heater located under the flower array, out of view of the moth. The olfactory cue necessary for initiation of Manduca’s appetitive behavior was provided by the presence of a 7-component artificial flower mixture, as described in the Methods of Chapter 2 (Campos *et al*. 2015).

3.3.4 Behavioral Assays and Response Variables Measured

To assess differences in moth exploitation ability as a function of variation in flower shape, I exposed moths one at a time to my hexamorphic flower array. After a moth successfully probed the nectary of any flower, I allowed it to continue foraging on the artificial flower array for as long as it was willing to visit flowers. A foraging trial was ended when the moth left the flower array. Foraging trials typically lasted from 4 to 12 minutes. No moth participated in more than one foraging trial. After each trial I counted the number of flowers of each shape that were emptied of their nectar. As in Chapter 2 (Campos *et al*. 2015), flower nectaries were always either completely full or completely empty after a foraging trial, suggesting that a nectary was completely drained the first time it was successfully probed. Each flower population (described in section 3.3.5 below) was foraged upon by 25 hawkmoths. In other words, I ran 25 foraging trials on each flower population.

If a moth ever emptied all 6 of any particular flower morph during a foraging trial, I used the data from the IR sensors of the flowers to find the time at which the 6th flower of that morph was first probed, and considered this the end of the foraging trial. In this way, I could be sure that my exploitation data accurately reflected relative ease of exploitation among the various flower shapes present in the array, rather than a response to the absence of a nectar reward.

After emptied flowers were counted, empty nectar tubes were replaced with fresh nectar tubes and the positions of the flower morphs were re-randomized in preparation for the next foraging trial.

In addition to flower exploitation data in the form of counts of emptied flower morphs, I also recorded the number of visits paid to each flower morph by analyzing the video footage of each foraging trial. This was used to assess whether differences in exploitation frequency between different flower morphs could be explained by the moths' potential innate preference for some flower shapes over others.

3.3.5 Experimental Treatments and Sample Size

I exposed moths to 5 different artificial flower populations (25 moths per flower population). All flowers had a length of 20 mm. The flower holder that each flower plugged into added 10 mm of overall length from the top of the flower to the beginning of the nectar tube, making overall flower length 30 mm, the same as for the artificial flowers used in Chapter 2. All flowers also had overall diameters of 55 mm, the same as in the artificial flowers used in Chapter 2. I varied the nectary diameters and corolla curvatures of the flowers in my 5 experimental populations as follows:

Population 1: Nectary diameter ($2*r_o$) of all flowers held constant at 2.5 mm; corolla curvature parameters, $c = -\infty, -4, -3, -2, -1$, and 0. With this population, I explored the effects of variation in corolla curvature on hawkmoth foraging performance with flowers ranging from perfectly flat disks ($c \rightarrow -\infty$), through various degrees of trumpet-like curvature, and finally ending in a perfect cone ($c = 0$).

Population 2: Corolla curvature of all flowers held constant at $c \rightarrow -\infty$; nectary diameters $2*r_o=$ 1, 1.75, 2.5, 3.25, 5, and 7 mm. With this population, I explored the effects of variation in nectary diameter on a flat disk floral surface.

Population 3: Same as Population 1, except nectary diameter (2r) held constant at 1 mm instead of 2.5 mm. With this population, I explored the effects of corolla curvature on foraging performance in a set of flowers whose nectary opening barely larger than the moth's proboscis (mean proboscis width proximal to the head = 0.84 mm± 0.008mm SEM, N = 30).

Population 4: A mix of curvatures and nectary diameters mostly intended to sample the bowl-shaped part ($c>0$) of flower morphospace. $c = -1$ and $2r = 1$ mm; $c = 0.375$ and $2r = 1$ mm; $c = 1$ and $2r = 1$ mm; $c = 0.375$ and $2r = 2.5$ mm; $c = 1$ and $2r = 2.5$; $c = -3$ and $2r = 7$ mm.

Population 5: Another mix of curvatures and nectary diameters, this time intended to sample regions of the "large nectary diameter" (3.25-7 mm) parts of the flower morphospace. $c = -4$ and $2r = 7$ mm; $c = -1$ and $2r = 7$ mm; $c = -4$ and $2r = 3.25$ mm; $c = -3$ and $2r = 3.25$ mm, $c = -1$ and $2r = 3.25$ mm; $c = -1$ and $2r = 1$ mm.

These 5 flower populations do not sample the range of flower shape space that was physically available to me (see Fig. 3.3) uniformly. Referring to Figure 3.3, there is a large empty patch between 3.25 and 7 mm of nectary diameter and also toward the right side of the shape space (positive corolla curvatures parameter values). The reason for not sampling heavily in the 3.25-7 mm nectary diameter range was that Manduca's exploitation ability response seemed to plateau at 3.25 mm and not change at 7 mm, so it was expected to remain the same in the intervening nectary diameter range. With limited time and resources, I had to make strategic decisions about which flower morphs to sample for foraging trials and which to leave out. Given more time and resources, I would want to achieve a more uniform sampling to remove doubt about the presumed response in this region of shape space. However, for positive corolla curvature values above nectary diameters of 2.5 mm, the physical construction of the 3D printed bracket that holds the artificial flowers in place presented complications for accommodating artificial flowers in said parameter range. A redesign of the flower holder bracket may be required for future incorporation of artificial flowers that occupy the upper right portion of the shape space in Figure 3.3.

Exploitation and visitation data were collected for 25 foraging trials in each of the five artificial flower populations. The statistical unit of replication is the foraging trial (or, equivalently, the individual moth).

3.3.6 Statistical Analysis

All statistical tests were performed in MATLAB.

One of the main goals of this study was to construct a foraging performance landscape for naïve hawkmoths as a function of variation in flower corolla curvature and nectary diameter. Armed with such a landscape, I wanted to draw conclusions regarding the way in which corolla curvature and nectary diameter influence Manduca's naïve foraging performance, and whether these two aspects of floral shape interact to influence Manduca's foraging performance. In order to sample flower shape space in these two parameters, I had to choose pairs of these parameters to turn into artificial flowers to include in my experimental foraging arena. The arena was designed to accommodate 36 flowers in a symmetrical 6 by 6 square layout (square to eliminate spatial polarity effects as much as possible). With 36 flowers able to fit into the flower array, the decision was made to populate it with 6 distinct flower morphs, where a flower morph constitutes a combination of corolla curvature parameter and nectary diameter from the flower shape equation. With 36 available positions in the flower array, I was able to include 6 copies of each morph into the flower array. With just 6 distinct flower morphs present in the flower array, I would have to run foraging trials on multiple sets of 6 flower morphs in order to sample enough of flower shape space to construct a meaningful foraging performance landscape. However, this creates a concern regarding the appropriate application to statistical tests.

Concern: my foraging landscape dataset consists of flower morphs that were not all exposed to Manduca foraging *at the same time*. Instead, the flower morphs were exposed to Manduca foraging six at a time. Given this, the question must be asked about whether or not the context of which flower morphs were present in the flower array during any particular set of foraging trials affected the performance response of the hawkmoths. An analogy to sports might be in trying to compare the teams from each of the association football (soccer) leagues of Europe. For example, does the 4th place team from the English league merit more plaudits and prestige than the 5th place team from the Spanish league? If these two teams never actually competed against each other directly, then any conclusions would be highly speculative.

To get around this issue of context, I deliberately included some flower morphs into multiple groups of six morphs to be subjected to foraging by Manduca. I could then compare the foraging performance that hawkmoths achieved at such a flower morph when present in one group of six flower morphs vs. another group (same flower morph in different group contexts). If there is no statistically significant difference in the performance response achieved from the same flower morph when subjected to Manduca foraging in two different groups of six artificial flower morphs, then that would be taken as evidence that Manduca’s foraging ability on flowers of different shapes is independent of which combination of six flower morphs the hawkmoths are attempting to feed from. In other words, context does not matter. And if context does not matter, then I take that as a reasonable justification to perform statistical analyses on all of the foraging data from all flower morphs simultaneously (pooling all the data), regardless of the group context in which each individual flower morph was presented to Manduca hawkmoths for foraging trials.

However, even if data pooling were justified according to the technique described in the preceding paragraph, it must still be acknowledged that I cannot fully rule out the possibility that flower morph context influenced the results. This is because while the common flower morphs that I chose to include in multiple flower array populations might show no influence of context, it may be that other flower morphs in the pooled data would indeed have shown a strong influence of flower context. Such an influence, if it exists, would be unknowable from the data I have available to me.

Before asking the question of whether flower shape affects Manduca's innate foraging performance, I wanted to see whether other factors could potentially confound the situation. The biggest potential confounding factor that I could identify was the possibility of an innate preference for some flower shapes over others among Manduca hawkmoths. For example, it might be possible that Manduca feeds from significantly more flowers of a hypothetical "flower morph A" compared to a "flower morph B" not because morph A has physical features that are easier to exploit, but rather simply because Manduca hawkmoths have an innate bias for an unknown visual feature of morph A and consequently gives a greater search effort on morph A, perhaps by paying more visits to morph A than to morph B. To test for such a bias, I used one-way analysis of variance (ANOVA) to test for differences in visitation frequency within each artificial flower population that I collected foraging data from. A significance level of $\alpha=0.05$ was used.

Next, I wanted to compare moth foraging performance among flower types present in the five different experimental populations. In order to justify these direct comparisons, each flower population had at least one flower in common with at least one other population. For ease of description, these flowers will be referred to as "common garden" flowers later in the chapter. For every flower morph that was present in two or more flower populations, I performed a one-way ANOVA on the foraging performance data of that flower morph in all flower populations in which it was present. The lack of significant differences would be taken as justification in pooling data across flower populations. In order to reduce the probability of committing a Type I error due to the problem of multiple comparisons, I used a sequential Bonferroni correction to adjust the α-level of each test.

After data pooling was justified (see below in section 3.3.7), I performed a 2-way ANOVA on all of the flower exploitation data to test for effects of nectary diameter alone, corolla curvature alone, and the interaction between nectary diameter and corolla curvature on flower exploitation performance by Manduca.

3.3.7 Data Pooling and Justifications

There were no statistically significant differences in exploitation performance by hawkmoths between common garden flowers that were present in different experimental flower populations (Fig. 3.4). The trumpet-shaped flower with $c = -1$ and $2r = 1$ mm (Fig. 3.4C) was notable in that it did exhibit a marked drop in exploitation in one of the populations that it was in (P5) relative to the other populations. The p-value of 0.042 suggests that I should reject the null hypothesis of no differences between the foraging performances that this flower received when present in these three populations. However, the probability of making a Type I error is higher when attempting to make a statistical inference from multiple simultaneous comparisons than when making an inference from a single statistical test. To counteract this problem of multiple comparisons, I adjusted my α-level using a sequential Bonferroni correction, also known as the Holm-Bonferroni method. When this is done, the adjusted α-level of the comparison being made in Figure 3.4C becomes 0.017 which is lower than the p-value of 0.042.

This result supports the pooling of the flower exploitation data from the five experimental populations that I collected data from.

3.4 Results and Discussion

The main goals of this study were to ask whether variation in flower shape, specifically the curvature of the corolla and the diameter of the flower's nectary, could affect the foraging performance of a representative of the nocturnal/crupuscular guild of pollinators. Manduca hawkmoths are an excellent model organism for this, and I anticipate that many of the results

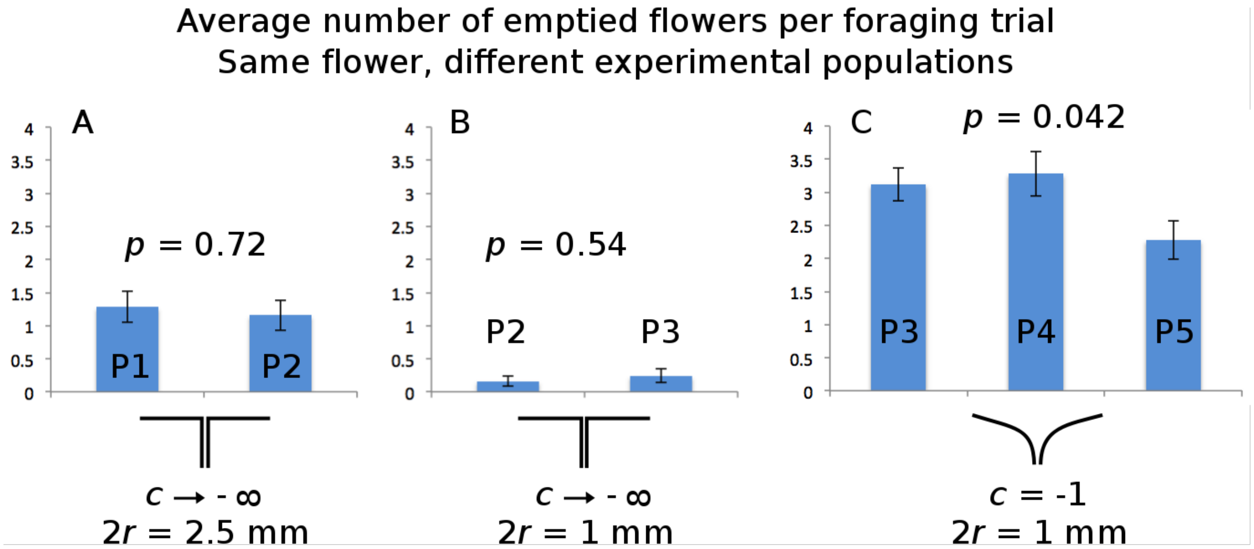

Figure 3.4
Comparisons of exploitation frequencies between the same flower morph when present in different populations of experimental flowers. In each case, there was no significant difference in exploitation of each "common garden" flower depending on what flower population it was included in. This result allowed me to aggregate all of the flower exploitation data from all flower populations into a single analysis. Although the p-value in C is below 0.05, the result is not considered statistically significant after applying a Holm-Bonferroni correction to the alpha value, in this case is 0.05/3 which equals 0.017.

drawn from this study can be applied to other members of this plentiful and widespread ecological guild. Generally speaking, it is seems clear from my data that corolla curvature and nectary diameter do indeed play a large role in Manduca's ability to discover the nectar source of a flower. Some combinations of these floral traits can effectively disrupt Manduca's ability to feed from the flower, despite strong efforts to do so. In contrast, other combinations of corolla curvature and nectary diameter, as defined in the mathematical model of flower shape employed in this study, allow Manduca to feed almost at-will, if they actually encounter and visit the flower. Most significantly, the data suggest that Manduca are extremely reliant on taking advantage of the physical features of a flower's three-dimensional morphology in order to successfully exploit a flower's energetic and nutritive resources. This broad conclusion can also probably be generalized to other members of Manduca's pollinator guild, which is characterized by probing at flowers with a flexible proboscis in low-intensity light conditions.

Plainly said, it appears as though the conclusion (from Chapter 2) that Manduca makes extensive use of what may be described as "morphological nectar guides" is now even more strongly supported. Visual and olfactory signals given off by the flower are not sufficient to account for the entire success of Manduca's foraging efforts. Instead, physical contact between the moth's proboscis and the flower's surface seems critical for nectar discovery, with the flower's contours at least passively providing a reliable guide into the nectar reservoir. But beyond implications for the proximate details of pollinator feeding ecology, there is also a more far-reaching implication for plant-pollinator interactions in an evolutionary context: it appears that flower shape can influence pollinator fitness, insofar as the ability to find and subsequently consume the nectar resource of flowers represents a significant determinant of individual fitness for a pollinating animal. The methodology presented in this study shows promise for facilitating experimental investigations of plant-pollinator evolutionary dynamics that have been prohibitively difficult or even impossible up to now. Below, I present and discuss the details of the results from this study.

3.4.1 Moths exhibit no visitation preference for flowers based on shape

There were no statistically significant differences in visitation frequency between flower morphs. In other words, moths always visited all flowers equally, but their ability to exploit the nectar resource varied substantially (see next section).

3.4.2 Hawkmoth foraging success increases linearly with nectary diameter

The simplest pattern in the foraging performance data to interpret is for variation in nectary diameter, from Flower Population 2 (Fig. 3.3). Foraging performance increases linearly ($r^2 = 0.92$) as nectary diameter increases from 1 mm to 7 mm (Fig. 3.5). All of the flowers in this population were perfectly flat, so the flowers' surfaces provided no reliable tactile cues as to the location of the nectary. A linear relationship is consistent with a search process in which the proboscis of the moth touches down on the surface of a flower and then is dragged along the surface in a random walk-like manner until successful insertion into the nectary takes place. Under such a scenario, success would occur the moment that the proboscis reaches the edge of the nectary. The edge of the nectary is the circumference of a circle in these flat artificial flowers, and the circumference of a circle grows linearly with respect to its diameter. Video recordings of hawkmoths probing at artificial flowers with their proboscides are consistent with the proboscis search strategy described above (Campos *et al*. 2015), and so too are the results

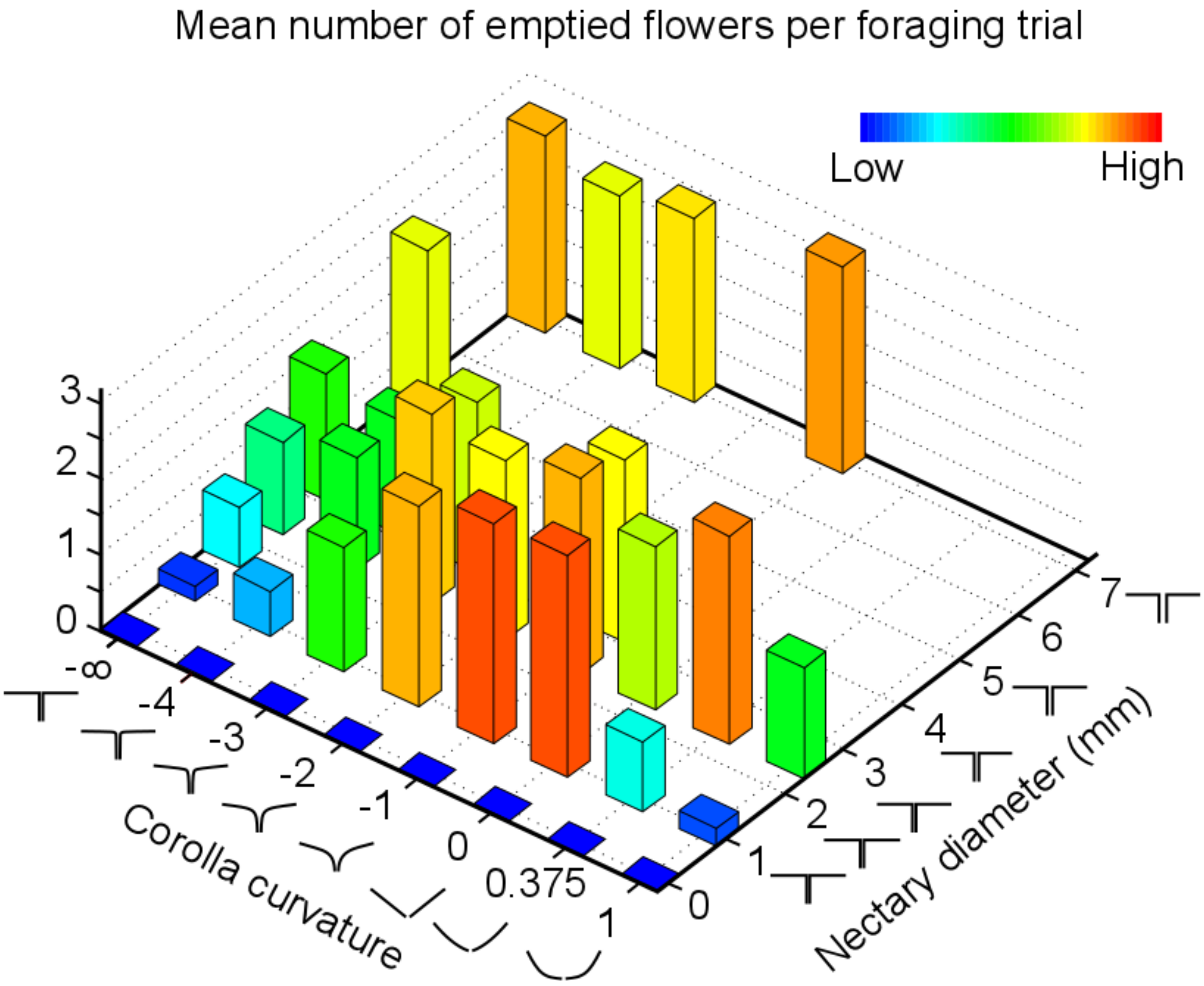

Figure 3.5
The full 3D foraging performance landscape based on foraging trials on five experimental flower populations. Bars are color coded according to relative height, with hotter colors representing higher foraging performance. Notice a row of zero-height bars at nectary diameter 0 mm, representing a known lower bound on foraging performance.

from other studies using nocturnal/crepuscular hawkmoths (Goyret & Raguso 2006; Goyret 2010) and diurnal hawkmoths (Goyret & Kelber 2011).

The linear trend in these data are also consistent with a search process in which vision plays a small role, either because of the low light intensity during the foraging trials, or because the movement of the tip of the moth's proboscis is not precise enough to allow for reliable placement at a known location, even if sufficient visual information is present.

3.4.3 Hawkmoth foraging success is maximized in trumpet-shaped flowers

There are two "series" of data that depict foraging performance as a function of variation in corolla curvature: Populations 1+4 and Populations 3+4. Each series is composed of data from two different flower populations. Two patterns are noteworthy.

1.) Both data series suggest that a peak in foraging performance occurs at intermediate corolla curvature parameter values. These can be qualitatively described as trumpet-shaped flowers (corolla curvature parameters of -2, -1, and 0) and stand in stark contrast to the two extremes of corolla curvature that are possible given my mathematical model of flower shape, which are flat and bowl-shaped corollas.

2.) The difference in foraging performance between trumpet-shaped flowers and the extremes of flat and bowl-shaped flowers is magnified as nectary diameter decreases.

These patterns corroborate the finding (from Population 2) that larger nectary diameters facilitate nectary discovery. Furthermore, trumpet-shaped flowers, particularly the c = -1 flower, are highly exploited by hawkmoths, while flat and bowl-shaped flowers receive much lower exploitation at nectary diameter of 1 mm (Fig. 3.5 and 3.6). This suggests that the steep, nectary directed curvature of the generic trumpet-like morphology plays a vital role in nectar discovery in nocturnal hawkmoths, as it is sufficient to rescue maximal foraging performance, so much so that the benefits seem to extend to the limits of physical exploitability. The average proximal width of the proboscis of Manduca is 0.84 mm (± 0.008 SEM; N = 30 moths), while the flowers that received maximum exploitation had a nectary diameter of 1 mm. As long as the flower remains trumpet-shaped, I expect exploitation performance to remain high if nectary diameter were to be continually and gradually decreased, up until the point where the nectary is so narrow that the proboscis would simply get stuck moving through it. If this is true, then this portion of the performance landscape (Fig. 3.5 and 3.6) will be extremely steep.

3.4.4 Hawkmoth foraging performance is sensitive to curvature only when nectary diameter is small

When the moth foraging performance data from the five experimental flower populations are plotted together, they form a performance landscape (Fig. 3.5 and 3.6). This landscape is not sampled uniformly in flower shape space (Fig. 3.3). Two-way ANOVA of these pooled data show that there is a significant effect on moth foraging performance due to nectary diameter ($p = 3.8 \times 10^{-6}$), corolla curvature ($p = 1.09 \times 10^{-15}$), and the interaction between nectary diameter and corolla curvature ($p = 4.7 \times 10^{-12}$). Of the 6 flowers of each morph that were present in the array,

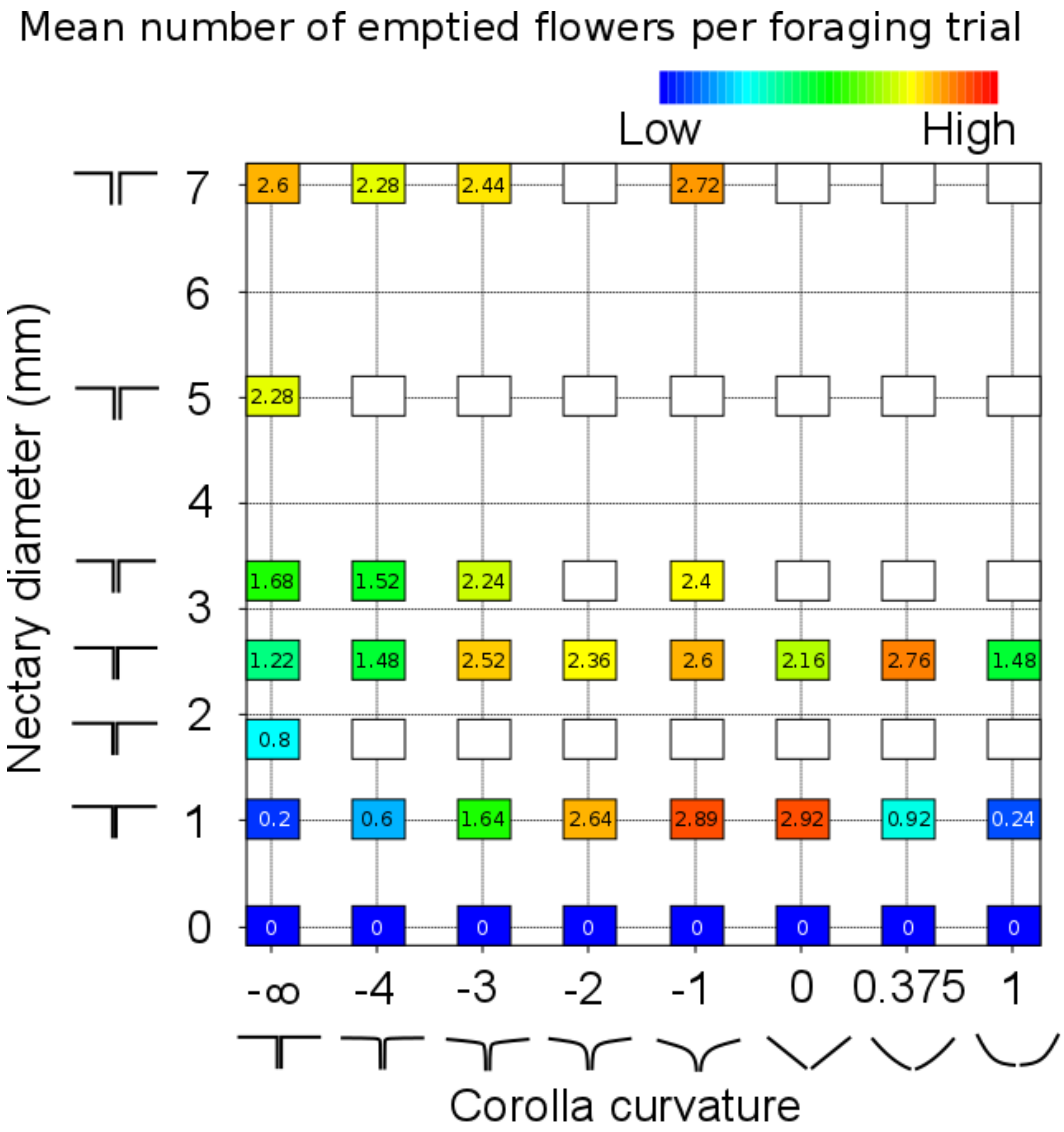

Figure 3.6
This is the same foraging performance data from Figure 3.5, but with numbers indicating the magnitude of performance instead of the height of each bar. The color code is the same as before. The heights of some bars in Figure 3.5 might be difficult to read or interpret due to occlusion from other bars. The orientation of the axes has been rotated so that the "bars" would be coming out of the page (or screen). This view might make it easier to see how much of the landscape was sampled and exactly where it was sampled. Together, figures 3.5 and 3.6 should provide a complete graphical depiction of the foraging landscape data.

the most-exploited flower was fed from at an average rate of 2.92 emptied flowers per foraging trial (± 0.28 SEM) while the least-exploited flower was emptied at an average rate of 0.16 flowers per foraging trial (± 0.07 SEM).

Individually, large nectary diameter and trumpet-shaped corolla curvature yield similarly high relative exploitation rates, at just under 3 exploited flowers per foraging trial, on average. If their individual effects were additive, then the simultaneous presence of both of these features in a single flower morph should yield an approximate doubling of this exploitation rate, which would be theoretically possible under the current experimental setup, as there were a total of six flowers of each flower morph present in the artificial flower array. But this was not the case. At large nectary diameters (greater than 2.5 mm), foraging performance seems to "max out." Foraging performance decreases steadily as nectary diameter decreases. However, at nectary diameters at or below 2.5 mm, trumpet-like curvature is able to rescue maximal foraging performance.

Interestingly, preliminary measurements of some species of flowers with the nocturnal hawkmoth pollination syndrome, such as *Petunia axillaris*, *Datura wrightii*, and various *Nicotiana* species, suggest that real hawkmoth-pollinated flowers have nectary diameters between 1 and 2.5 mm, which is the part of flower morphospace that my data suggest is acutely sensitive to corolla curvature. Indeed, all of these plant species produce flowers that can qualitatively described as trumpet-shaped. However, it would be insightful to actually fit shape parameters from the flower shape equation to real flowers from the hawkmoth pollination syndrome. Although the $c = -1$ curvature flower seems to be easiest for Manduca to exploit at small nectary diameters, this is no reason to suspect that real flowers should be under selection for that particular corolla curvature. This is because a flower's fitness is not directly related to how easy it is to exploit, but rather to how effectively an animal pollen vector makes contact with the reproductive structures of the flower, thereby allowing for outcrossing and the production of a seed set in the flower.

Taken together, these results support the conclusion that corolla curvature can be considered a morphological nectar guide in flowers (Campos *et al*. 2015).

3.5 Concluding Remarks and Future Directions

I have shown that trumpet-shaped corolla curvature significantly improves Manduca’s ability to find the nectar source of a flower. But the data presented here do not allow me to distinguish whether the moth is actively making use of mechanoreception through its proboscis by following steeper corolla slopes, or whether nectar discovery is a passive mechanical process in which converging petal slope “funnels” the tip of the moth’s proboscis toward the nectary. One way to test whether a passive process is sufficient to explain the observations presented in this study is to build a physical model of a moth’s proboscis whose proboscis tip moves similarly to a real moth during initial flower-proboscis contact. This physical model should not include any active feedback mechanisms. The investigator would then slowly bring artificial flowers of different morphologies into contact with the mechanical proboscis and observe the nectary insertion rate. Behavioral experiments that directly test the hypothesis of active sensing would likely take a control theory approach.

In addition, this study only examines plant-pollinator interactions from the ecological perspective of the pollinator, specifically with regard to foraging ability. But this system could also address questions of plant fitness and flower evolution in response to visitation and pollination by animals. To do this, a clear metric of plant fitness must be devised. One promising potential approach is to add a sensor to the artificial flowers that detects when a visiting animal would have made contact with a real flower's reproductive parts. An accelerometer mounted to a stigma/anther physical model analog is one possible implementation of such a sensor.

The data presented here center around only one aspect of the foraging process of a hovering crepuscular nectarivore: rate or frequency of nectar discovery. But my experimental setup actually included technologies and elements that allow me to characterize the final stages of the foraging process of Manduca in greater detail. For example, each artificial flower in the experimental array was attached to an infrared beam sensor that would record the occurrence of proboscis insertion into its nectary and the duration of the insertion event. I was also able to implement custom computer vision software to automatically track the location of each moth during its foraging bout. Such data can be combined to give handling times at different flowers and ask questions about whether some flower shapes enforce precise positioning of the (potentially) pollinating animal during a foraging visit. These questions are a start at considering the flower's ecological and evolutionary interests and form the basis of the next chapter of this dissertation.

Finally, it is interesting to note that the results presented here were obtained in the context of a dark-adapted hawkmoth, specifically a crepuscular one, in dim light conditions. Would a diurnal species of hawkmoth foraging in daylight conditions, such as the so-called hummingbird hawkmoth (*Macroglossum stellatarum*), exhibit a different pattern of visitation or exploitation frequency due to its presumed larger visual sensory bias? It is already known that initial proboscis placement on the surface of a flower by *M. stellatarum* is sensitive to chromaticity (Goyret and Kelber 2011) while Manduca uses achromatic contrast (Goyret 2010), but would the diurnal species show the same sensitivity to slope in its exploitation performance? Along the same vein, Manduca can in fact fly and feed from flowers during daylight conditions, albeit at lower frequency than when foraging in dim conditions (personal observation). It might be interesting to repeat these foraging trial experiments in daylight conditions to see if increased light availability would change Manduca's foraging performance. Many experimental manipulations are possible.

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3.7 Data Accessibility

Data are deposited in the University of Washington Research Works Archive.

http://hdl.handle.net/1773/39169

3.8 Supplemental Software Accessibility

Script for exploring shape parameter space of the artificial flowers described in this study:

https://github.com/eocampos/artificial_flower_shape_exploration

Script for shuffling positions of artificial flowers in the 36-flower array:

https://github.com/eocampos/random_flower_array_shuffler_36flowers

Script for data acquisition from IR sensors attached to each artificial flower through an Arduino Uno with an attached Mayhew Labs Mux Shield (http://mayhewlabs.com/products/arduino-mux-shield):

https://github.com/eocampos/arduino_mux_shield_and_LED_switch

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Chapter 4

Ambiguity Regarding Evolutionary Conflict or Harmony Between Hawkmoths and Sphingophilous Flowers: Insights from Experiments Using 3D-Printed Artificial Flowers

4.1 Abstract

I observed and measured various aspects of hawkmoth (*Manduca sexta*, hereafter Manduca) foraging behavior in a controlled laboratory setting using 3D printed artificial flowers whose shapes were mathematically specified. The goal was to explore proof-of-concept feasibility of utilizing lab-based experimental evolution toward the study of flower shape evolution in response to pollinator-mediated selection.

Hawkmoths were allowed to forage freely on an array of 36 artificial flowers containing 6 distinct morphologies present in equal numbers – with 5 'populations' of different flower morphologies (terminology defined in Materials and Methods). Each flower contained consumable artificial nectar to simulate the reward provided by flowers in nature. My experimental apparatus allowed me to observe or measure the number of visits that moths paid to each flower morph, the duration of visits, the number of proboscis insertions into the nectary of each flower, the duration of proboscis insertion events, and the distance of the moth's center of mass from the central axis of each flower during proboscis insertion.

Of those artificial flowers that had their nectar reservoir exploited, moths paid significantly more visits and spent more time visiting flowers with flat or bowl-shaped corollas than at trumpet-

shaped flowers. Thus, trumpet-shaped flowers maximize moth energy intake per unit of time and energy expenditure and therefore should be "preferred" by the moths in an evolutionary, fitness-optimizing sense. However, if increased effort translates into higher probability of contact between the moth and the reproductive structures of a flower, then flat and bowl-shaped corollas would be evolutionarily favored by flowers over trumpet-shaped corollas, suggesting a misaligning of the evolutionary interests of the moth and flower. But if number of proboscis insertions into the nectar reservoir and duration of time spent by moths with their proboscides inserted in the reservoir are considered as important determinants of pollination effectiveness, then the data suggest that moth and flower interests are aligned.

The scenarios of evolutionary conflict and harmony cannot be disambiguated using only the data available through the current experimental design. A more direct measure of flower fitness is required if experimental evolution of flower shape is to become a viable area of scientific inquiry. For this, I recommend the integration of a touch-sensitive physical sensor into an artificial flower, which would act as an analog for the flower's reproductive parts. If correctly designed and deployed, such a sensor could yield insights about what aspects of pollinator behavior result in effective pollination services to flowers.

4.2 Introduction

Land plants have a history spanning over 450 million years, beginning with the initial colonization of land by descendants of streptophyte algae in the Ordovician period (Becker &

Marin 2009; Sanderson *et al*. 2004). Ever since, they have comprised an important and ubiquitous part of terrestrial ecosystems, notably as primary producers (Running *et al*. 2004) and modifiers of their physical environment (Berner 1999; Scott & Glasspool 2006). Modern plant communities are dominated by a single lineage, the angiosperms or flowering plants. This dominance can be characterized in a variety of ways. They are represented by more than 250,000 known species and account for over 87% of extant plant species richness (Palmer *et al*. 2004; Moore *et al*. 2007). They also occupy every terrestrial habitat except the poles and high mountaintops (Soltis & Soltis 2004), where they are excluded by harsh (cold) physical conditions. Angiosperms owe their amazing species richness to an adaptive radiation in the Cretaceous which was likely made possible by the evolution of a key innovation, the flower (Friis *et al*. 2006). Although many flowers can be pollinated by abiotic vectors such as wind (Culley *et al*. 2002) and water (Cox 1988), the co-occurrence of the diversification of major lineages of modern flower-visiting insects with that of the angiosperms (Grimaldi 1999) suggests that the emergence of animals as pollen vectors played a significant role in the angiosperm adaptive radiation.

Plant-pollinator interactions likely predate the angiosperms. Fossil evidence suggests that mid-Mesozoic gymnosperms were involved in pollination mutualisms with scorpionflies that had evolved morphological adaptations for feeding on fluid secretions (Nepi *et al*. 2009) called pollination drops (Ren *et al*. 2009; Ollerton and Coulthard 2009). Recent evidence also suggests that mid-Mesozoic kalligrammatid lacewings had independently converged on Cenozoic butterfly morphology and used long tubular proboscides to consume gymnosperm pollination drops (Labandeira *et al*. 2016).

Just as the plant-pollinator interaction is not monopolized by angiosperms in evolutionary history, it is important to keep in mind that animals interact with angiosperms in a broad variety of ecologically and evolutionarily important ways beyond just as pollinators. Some of these interactions represent losses in plant fitness, e.g. animals consume plant biomass (Maron 1998) and propagules (Moles *et al*. 2003), and fell whole trees for procuring material to build shelters (Collen & Gibson 2001). Some interactions are complex regarding plant fitness effects. At face value, nectar robbing (the consumption of nectar through a perforation made in the side of a flower's corolla instead of through the flower's opening) should be expected to represent a loss in flower fitness. While there is some evidence of this (e.g. Irwin & Brody 1999; Richman *et al*. 2017), nectar robbing has also been shown to be neutral or even positive to whole plant fitness (Morris 1996; Maloof & Inouye 2000; Maloof 2001). Conversely, some interactions represent gains in plant fitness, e.g. distribution of propagules (Wenny 2001), often primed for germination by passage through the animal's gut (Traveset *et al*. 2008), consumption of animal biomass by carnivorous plants in nutrient-poor environments (Ellison & Gotelli 2009), defense from herbivory (Evans *et al*. 2013), efficient pollination, etc. Pollination interactions also sometimes feature cheating by the plants, as in the deployment of nectarless flowers (Thakar *et al*. 2003) that attract pollinating animals but offer no reward. Clearly, animals and plants interact in a rich variety of ways. This study concerns itself only with plant-pollinator interactions that are reciprocally legitimate, meaning that the plant's flower (or simply the flower for brevity) actually provides an energetic and/or nutritive reward for a visiting animal and the animal's serial flower-visiting behavior results in significant cross-pollination of the flowers.

Pollination of angiosperms by animal vectors is often cited as one of the classic examples of mutualism in nature (Santamaría & Rodríguez-Gironés 2015), an interaction between two different species in which both parties benefit. In an evolutionary context, these benefits manifest themselves as increases in reproductive fitness relative to conspecifics that do not engage in the mutualistic interaction (reviewed by Leigh 2010). Due to the reciprocal fitness benefits, mutualisms are often described as cooperative (Santamaría & Rodríguez-Gironés 2015). However, care must be taken in delimiting the scope of this cooperation. In doing so, it is helpful to distinguish pattern from process. Strictly speaking, the diagnosis of an interspecific association as a mutualism is only a statement about the result, i.e. the pattern, of the interaction for the involved parties. However, it can be easy to implicitly assume that the processes shaping mutualisms are in some way cooperative, i.e. that the evolutionary strategies that maximize fitness for one party also maximize fitness for the other party. Applied to pollination mutualisms, such an assumption about process might take the following narrative form: "Flower traits that maximize pollinator reward consumption also maximize pollination effectiveness." Or more simply, "What is best for the pollinator is also what is best for the flower."

The evolutionary interests of a flower and a pollinator may not be aligned. Despite being labeled as a "pollinator" by scientists, an animal that provides this ecological service only does so as an unintended consequence of its actual goal: to collect or consume the resources produced by the flower. While some pollinators, particularly bees, gather pollen in a targeted fashion as a nutritive resource (Hrassnigg & Crailsheim 1998), it is usually deposited on the pollinator's body as a result of incidental contact with a flower's stamen. And there is no evidence that subsequent deposition of the pollen on the stigma of another flower is the result of anything other than incidental contact as well. In other words, pollinators are oblivious to any pollination services they might provide. Likewise, flowers have no conscious realization that they "offer" an exploitable resource. Their only evolutionary concern is the deployment of their characters in a way that maximizes reproductive success. As nectar and pollen are energetically expensive to produce (Southwick 1984; Pyke 1991), flowers face significant opportunity costs if a visiting animal consumes large quantities of floral rewards without rendering effective pollination (Santamaría & Rodríguez-Gironés 2015). Framed in this way, it has even been suggested that flowers and pollinators should be depicted as agents in a biological market seeking to obtain maximal services from each other in complete disregard for the benefit of the other party, instead of as "cooperative partners" (Noë & Hammerstein 1995; Santamaría & Rodríguez-Gironés 2015). It may also be useful to think of mutualisms as “reciprocal parasitisms,” that is, interactions based on a foundation of mutual exploitation by each party in the interaction (Bronstein 1994).

This study makes use of an experimental system in which plants are represented by individual artificial flowers and the pollinator is represented by the crepuscular hawkmoth, Manduca sexta, hereafter referred to simply as Manduca. The artificial flowers were 3D printed and had variable shapes that were mathematically specified. Each flower also contained equal quantities of sugar water to represent the floral nectar resource that Manduca exploits in the wild.

In Chapter 3, I presented a foraging performance landscape (Ch3, Fig. 3.5) demonstrating that nectar discovery ability in Manduca depends on a tight interaction between floral corolla curvature and nectary diameter. As a first approximation, this foraging performance landscape could also be interpreted as a fitness landscape for Manduca with regard to flower morphologies that are likely to be energetically profitable to visit or not.

To document pollinator foraging behavior more fully, I have employed two additional features of my experimental apparatus that were not taken advantage of in Chapter 3.

First, variation in flower shape might lead to variation in a pollinator's positioning relative to the flower's reproductive structures, leading to variation in pollination efficiency. To address this, I have implemented a custom computer program to utilize the video footage recorded during foraging trials to track the position of hawkmoths relative to each visited flower. The large volume of video generated during my experimental procedure means that using a human agent to manually carry out the pollinator tracking is intractable. The tracking software processes video at approximately twice the frame rate of the video, further adding to the high-throughput characteristics of my experimental procedure.

Second, I have used infrared sensors attached to the nectary of each artificial flower in my flower array to monitor frequency and duration of proboscis insertion into each flower. It is possible that the phase of a pollinator visit in which the pollinator is actively engaging with the floral reward could be associated with high probability for cross-pollination, so the ability to sense when these events occur could be indicative of flower fitness.

In this study, my goal is to identify aspects of pollinator foraging behavior that have bearing on the relative fitness of the flowers and to identify how variation in flower shape affects flower fitness. Such knowledge could shed insight on whether pollination mutualisms are cooperative in process in addition to pattern. In addition, refinements of the experimental design described here could lead to feasible experimental evolution studies to explore how animal visitation influences the evolution of flower shape.

4.3 Materials and Methods

4.3.1 Animals

This study is based upon data collected from the same set of individual moths as in Chapter 3. Therefore, the information regarding pollinator supply, acquisition, and treatment for this study is identical to that of Chapter 3. This information is presented here again for the reader's convenience:

Individual Manduca were obtained from a colony maintained by the Department of Biology at the University of Washington (see Materials and Methods from Chapter 2 for relevant details). Moths were flower-naïve and unfed for 2-3 days post-eclosion. The moths used in foraging experiments were obtained haphazardly with respect to sex. The lack of any statistically significant differences between the foraging behavior of males and females in Chapter 2 informed my decision to pool the data from males and females. Any moth that failed to fly or feed from any artificial flowers within 5 minutes of being released into the experimental arena was excluded from the study. In total, I attempted to collect data from 190 moths. Of those, 125 moths (68 males, 57 females) successfully foraged from the artificial flowers and were included in the analysis.

4.3.2 Shape Parameters of Artificial Flowers

The artificial flowers used in this study were made using a 3D printer, using the same methods described in Chapter 2 (Campos *et al*. 2015) and in Chapter 3. This information is presented here again for the reader's convenience:

Artificial flowers were designed in a computer-aided design application based on an equation that allows for the manipulation of up to four aspects of flower shape: corolla curvature, nectary radius, flower length, and whole-flower radius. Of these, only corolla curvature and nectary radius were manipulated in this study, but other floral shape parameters may be manipulated in future experiments. The shape equation is:

$$z(r) = L\left(\frac{r - r_0}{R}\right)^{e^c}$$

where z represents the longitudinal (often vertical) axis of our flower model and r represents the radial distance of the corolla from the central z-axis. In addition, c is a curvature parameter determining the lateral profile of the corolla, r_0 is the nectary radius, L is the flower length, and R is the lateral extent of the corolla from edge of the nectary to the outer lip of the flower (note that whole-flower radius is equal to $r_0 + R$).

4.3.3 Experimental Apparatus and Flight Arena

The data that this study is based on originated from the same set of foraging trials as in Chapter 3. Consequently, much of the information regarding the features of the experimental apparatus is identical to Chapter 3, with the notable exception of how the data from the infrared sensors was used:

Artificial flowers were arranged in a square array that could hold 36 evenly spaced flowers. Flower centers were spaced approximately 30.5 cm apart. Modular T-slot extruded aluminum (80/20® Inc.) was used as the structural support of the flower array. The 36-flower array was populated with 6 distinct flower morphs, present at equal frequencies of 6 of each. At this point, it could be helpful to define a few terms within the context that they are used in this study:

Flight arena: the enclosure (4 walls and a roof) inside which foraging trials take place. The flower array exists inside the flight arena. The dimensions of the flight arena were 2.4 m width by 4.0 m length by 2.5 m height.

Flower array: The physical structure within the flight arena, which holds individual artificial flowers in place while hawkmoths attempt to forage during foraging trials. The flower array, as designed for this study, holds 36 flowers in a 6 by 6 square pattern.

Flower morph: an artificial flower with a distinct combination of flower shape parameters.

Flower population: A distinct group of 6 flower morphs and their duplicates, forming the 36 flowers that populate the flower array.

Only corolla curvature and nectary diameter were varied in this study. Flower positions were randomized with a custom Python script before each foraging trial. Each artificial flower's nectar reservoir was filled with 20 µL of 20% w/v sucrose solution. Two dim white LED lights illuminated the flight arena from above, calibrated to a combined illuminance of 0.1 lux at flower level to simulate moonlight conditions (Gossen Mavolux 5032C lux meter, Gossen Foto- und Lichtmesstechnik GmbH, Nuremberg, Germany). The flight arena was also illuminated from above with a single infrared light (Magnalight LEDLB-16E-IR; 790-880 nm flat emission peak, modified by removing the light’s focusing lenses to create even, diffuse lighting) to allow video recording of the moth's foraging trajectory (Fig. 4.1).

Each artificial flower was held in the flower array by a 3D-printed bracket that contained an infrared (IR) emitter-detector (Fig. 4.2), creating an infrared beam sensor. When a moth's proboscis enters the nectar reservoir of any artificial flower, the infrared beam is broken, and this event is recorded through a data acquisition system consisting of an Arduino® microcontroller and a laptop computer (Fig. 4.1). The IR beam sensor data were used in this study to identify the number of proboscis insertion events that occurred at each flower and to calculate the duration of each proboscis insertion event.

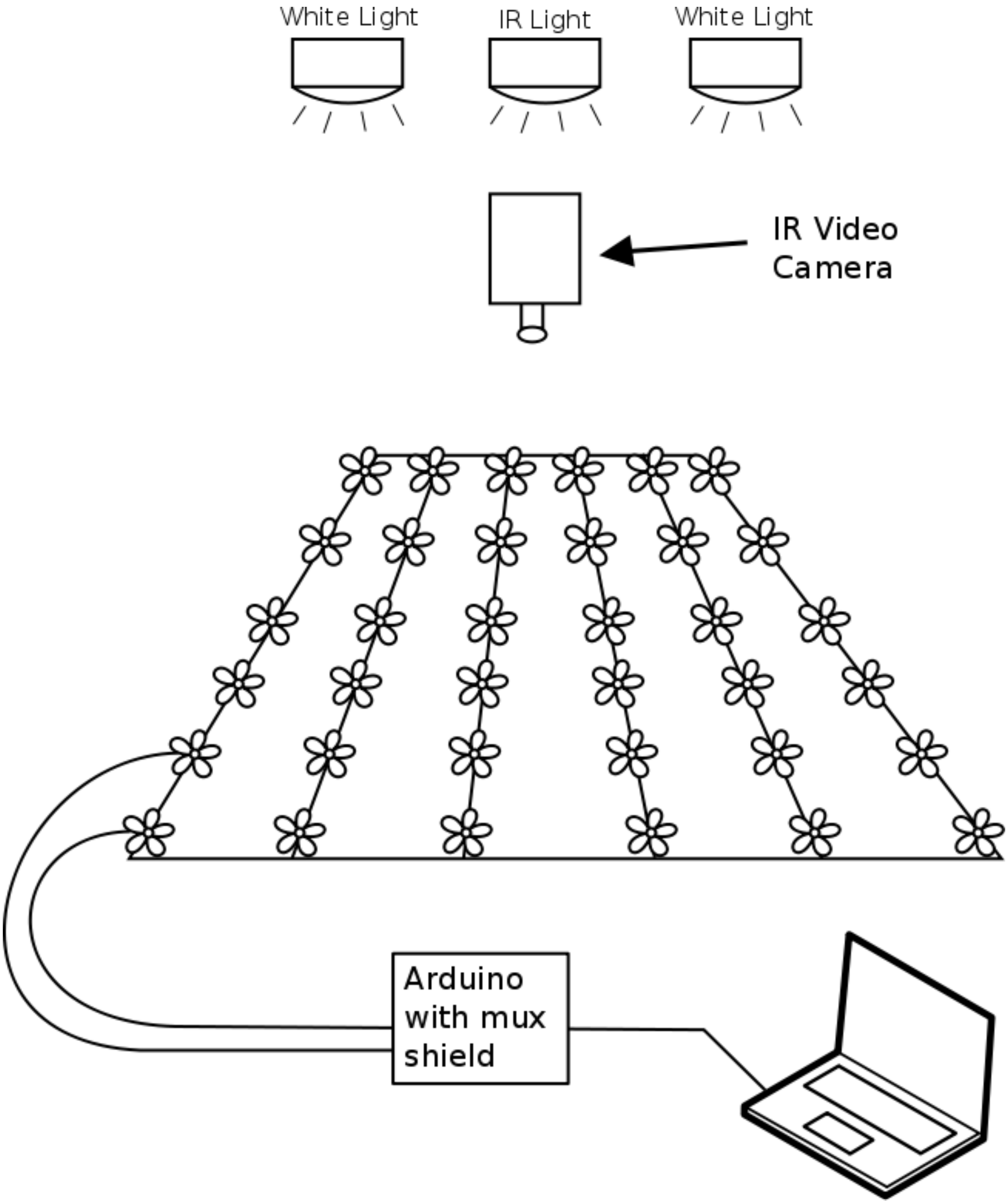


Figure 4.1 Line drawing depicting major elements of my experimental arena. The morphological disparity of the artificial flowers populating the array is not reflected here. Each artificial flower was attached to an infrared sensor to detect when proboscis insertion into the nectary occurred at each flower, and to calculate the duration of each probing event. The signal from each flower was routed to an Arduino® microcontroller and then relayed to a laptop via USB. For simplicity, only two of the flowers are shown "wired up" in this graphic.

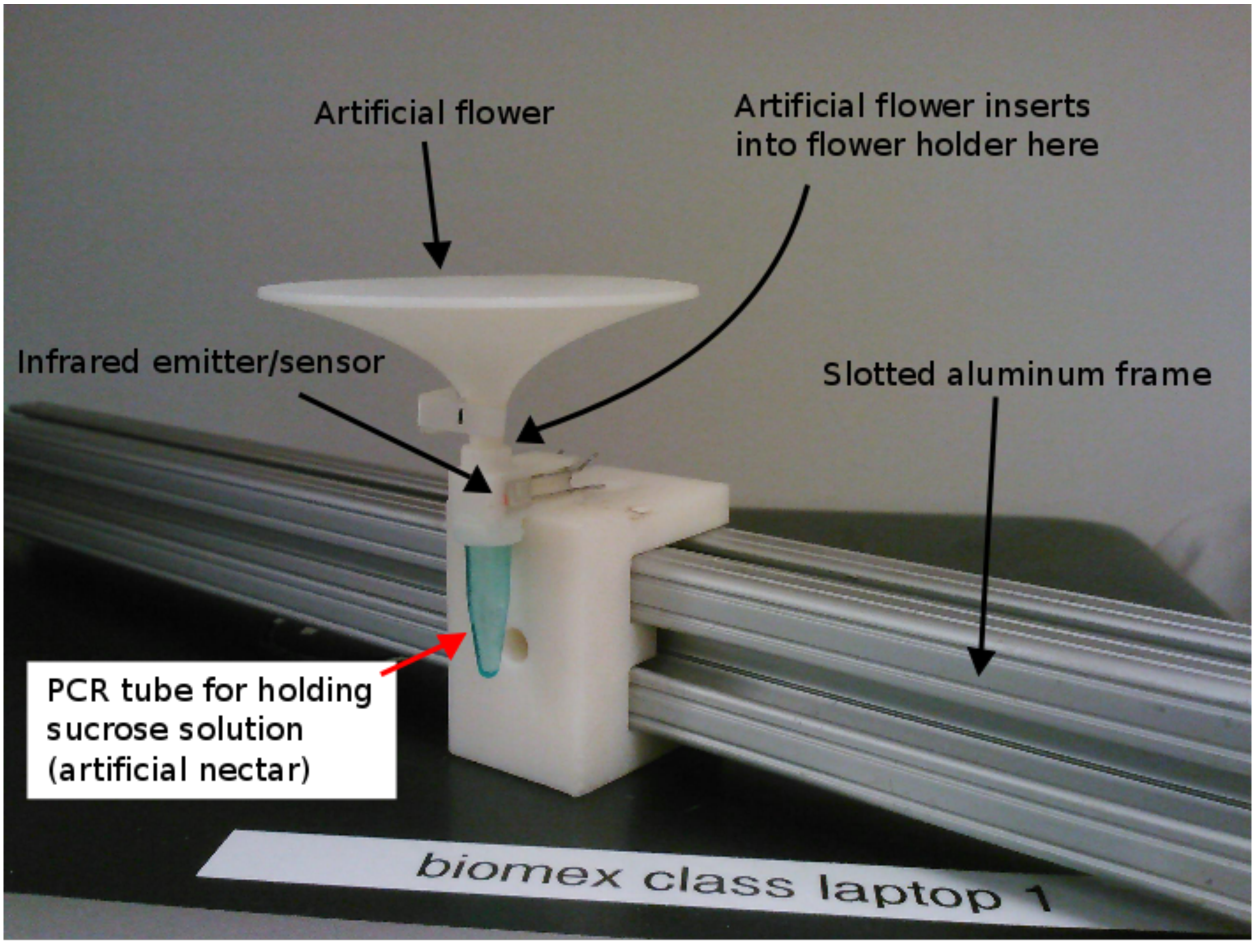


Figure 4.2
Photograph depicting the custom 3D-printed bracket used to hold artificial flowers in the 36-flower array with integrated IR emitter/detector diodes. Note the electrical leads of this demonstration model are not connected to anything.

An infrared-sensitive USB video camera (Point Grey Firefly® MV, model FMVU-03MTM-CS, 640x480 resolution) was mounted above the flower array looking straight down, to capture a bird's eye view of the moth's flight trajectory as it foraged on artificial flowers. In preparation for a foraging trial, the IR sensors/flower holders were covered with a sheet of black felt to provide a low-glare, high-contrast background against which the moth could easily be seen in the video footage of each foraging trial. All IR sensor wires and the video camera cable were routed under the door of the flight arena and relayed to a laptop computer, allowing me to monitor the live video feed and IR sensor states from outside of the arena, so as to not disturb a moth during its exploration of the arena.

The air temperature of the flight arena was kept at a constant 24^{o}C using an electric space heater located under the flower array, out of view of the moth. The olfactory cue necessary for initiation of Manduca’s appetitive behavior was provided by the presence of a 7-component artificial flower mixture, as described in the Methods of Chapter 2 (Campos *et al*. 2015).

4.3.4 Behavioral Assays and Response Variables Measured

I exposed moths one at a time to my hexamorphic flower array. After a moth successfully probed the nectary of any flower, I allowed it to continue foraging on the artificial flower array for as long as it was willing to visit flowers. A foraging trial was ended when the moth left the flower array. Foraging trials typically lasted from 4 to 12 minutes. No moth participated in more than one foraging trial. As in Chapter 2 (Campos *et al*. 2015), flower nectaries were always either completely full or completely empty after a foraging trial, suggesting that a nectary was completely drained the first time it was successfully probed.

If a moth ever emptied all 6 of any particular flower morph during a foraging trial, I used the data from the IR sensors of the flowers to find the time at which the 6th flower of that morph was first probed, and considered this the end of the foraging trial. In this way, I could be sure that my exploitation data accurately reflected relative ease of exploitation among the various flower shapes present in the array, rather than a response to the absence of a nectar reward.

After each foraging trial, I replaced the nectar reservoir of any flower that was exploited and re-randomized the positions of the flower in the array in preparation for the next foraging trial.

In addition to flower exploitation data in the form of counts of emptied flower morphs (Which were used in Chapter 3), I also recorded or calculated the following moth behavioral responses using video footage and custom computer programs or scripts:

(a) I counted the number of visits paid to each flower by individual moths during each foraging trial.

(b) I calculated the duration of each visit to each flower.

(c) I counted the number of proboscis insertion events that occurred at each flower using a custom MATLAB script (GitHub repository: https://github.com/eocampos/ir_sensor_filtering) that filtered the IR beam sensor data from each flower (Fig. 4.3). The MATLAB script implemented a signal threshold and 5th-order low-pass Butterworth filter with a cutoff frequency of 0.15 Hz to identify the location in the sensor signal at which proboscis insertion began and ended. The number of "insertion beginning" events recorded for each flower were summed and taken as the number of insertion events that occurred at each flower.

(d) I calculated the duration of each proboscis insertion event using the filtered data described in (c) above. Subtracting the location at which an insertion event began from the location at which it ended in the sensor signal and multiplying by the sampling period yielded a time, the duration of the insertion event (Fig. 4.3B).

(e) I calculated the average distance of the moth from the central axis of a flower during proboscis insertion. To do this, I used a custom automated object-tracking program (GitHub repository: https://github.com/SpaceKitKat/Tracker-moth) to record the position of the moth during each video frame of each foraging trial. Details regarding the tracking program are given in part (4.3.5) of the Materials and Methods, below.

4.3.5 Automated Moth Position Tracking

Calculating the distance of a moth from the center of a flower requires recording the position of the moth during its foraging efforts. The bird's eye view camera that I equipped in my flight arena was ideally positioned to provide the required 2-dimentional perspective of the moth's flight path. The 2-dimensional position in Cartesian coordinates of an objects can be tracked in a

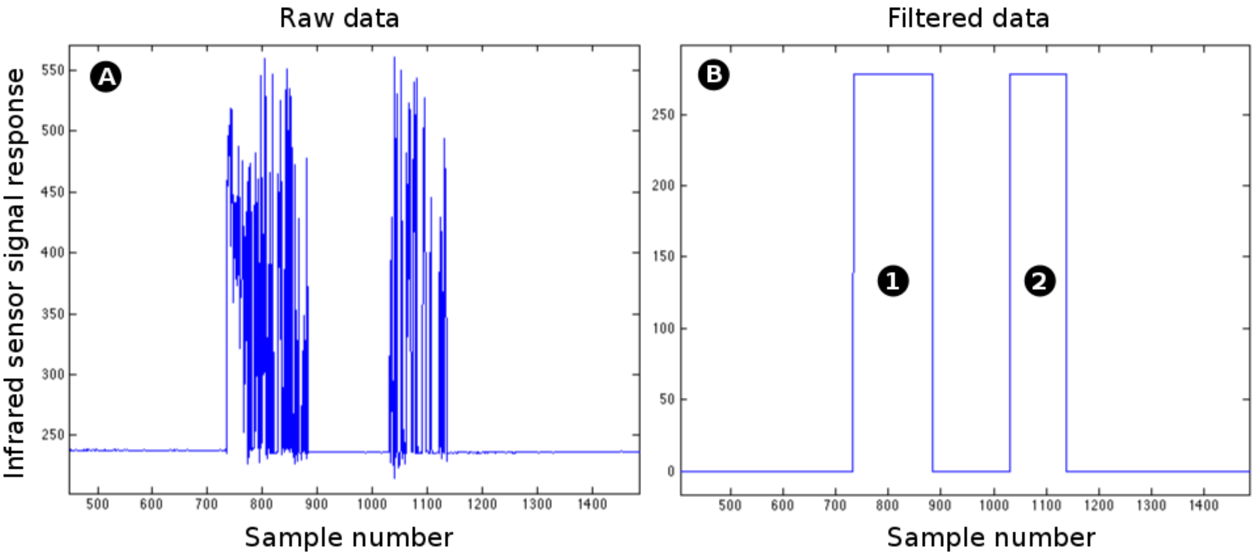


Figure 4.3
(A) Representative example of the raw signal coming from the infrared proboscis sensor attached to one of the flowers in the flower array. Interruption of the infrared beam causes the sensor signal to increase in proportion to the level of beam occlusion. The raw signal had to be interpreted to yield the number of proboscis insertion events and the duration of each insertion event. (B) The processed data after passing it through a low-pass Butterworth filter followed by optimized thresholding to create an easily interpretable square wave-like signal. Each individual plateau (numbered 1 and 2) is interpreted as a single proboscis insertion event. The width of the plateau at its base is used to calculate the duration of the insertion event by dividing by the sampling frequency. The data filtering and accounting of number and duration of proboscis insertion events at each flower for each foraging trial was carried out by a custom MATLAB script.

single video stream by a human agent with high accuracy following a minimal period of training with the aid of certain video and image-processing software packages, some of them open source or even in the public domain (e.g. NIH's ImageJ). However, using a human agent was deemed to be impractical in this case because of the large numbers of video files and the large numbers of frames present in each video. The decision was made to explore options in the realm of computer vision to implement an entirely software-based automated object tracking method. As there were no such commercial software packages that were readily available or affordable to me, I recruited the help of a research assistant who was well versed in computer science to develop a custom piece of automated object tracking software.

The software was written in C++ and employed OpenCV, an open source library of programming functions optimized for real-time computer vision.

In order for the target object (the flying moth) to be tracked, it must be detected. Thanks to the experimental setup involving a single object moving in a relatively static scene, background subtraction could be used to filter each video frame to detect the moth. Background subtraction involves subtracting the intensity values of each pixel in a frame that is deemed to represent the static "background" of the scene from the intensity values of each corresponding pixel in the video's frames. Areas of the video frame where no object was present should yield a difference of or very nearly zero intensity, while areas that contain the flying moth should yield differences of relatively larger absolute values. To create the most consistently performing results, the tracking program constructs the reference background by taking a weighted average of all frames in an input video. This approximates the scene without any moving objects.

After background subtraction, a video frame is passed through a threshold filter that assigns the value of zero (designates as “background”) to pixels with absolute intensity values below the threshold and assigns an arbitrarily high intensity values (designates as “object,” or in this case, “moth”) to any pixels above the threshold. The resulting frame will be composed of a number of blobs of connected pixels that presumably represent the moth. A size threshold is used to eliminate noise so that only the true contours of the moth are ultimately identified as the target object.

Once the contours of the moth are identified, the location of the moth is calculated as the x,y coordinates of the centroid of the surviving contour in that video frame. In cases where the moth had flown out of the camera’s view or blended into the background too much to be identified by background subtraction, the algorithm returned NaN, a placeholder for missing data. All location data were then written to a text file along with frame identity information.

The wide-angle lens used on the video camera inside the flight arena introduced significant fisheye image distortion (Fig. 4.4A). This distortion needed to be eliminated or minimized as much as possible in order to have reliable position and distance measurements between moths and flowers. In order to remap the raw video images into rectilinear format, MATLAB’s Camera Calibration Toolbox was used to compute equipment and image properties such as focal length, image center, and radial and tangential distortion coefficients. These parameters were then fed into OpenCV’s Image Processing package in the tracking program to ultimately remove distortion from the video frames (Fig. 4.4B).

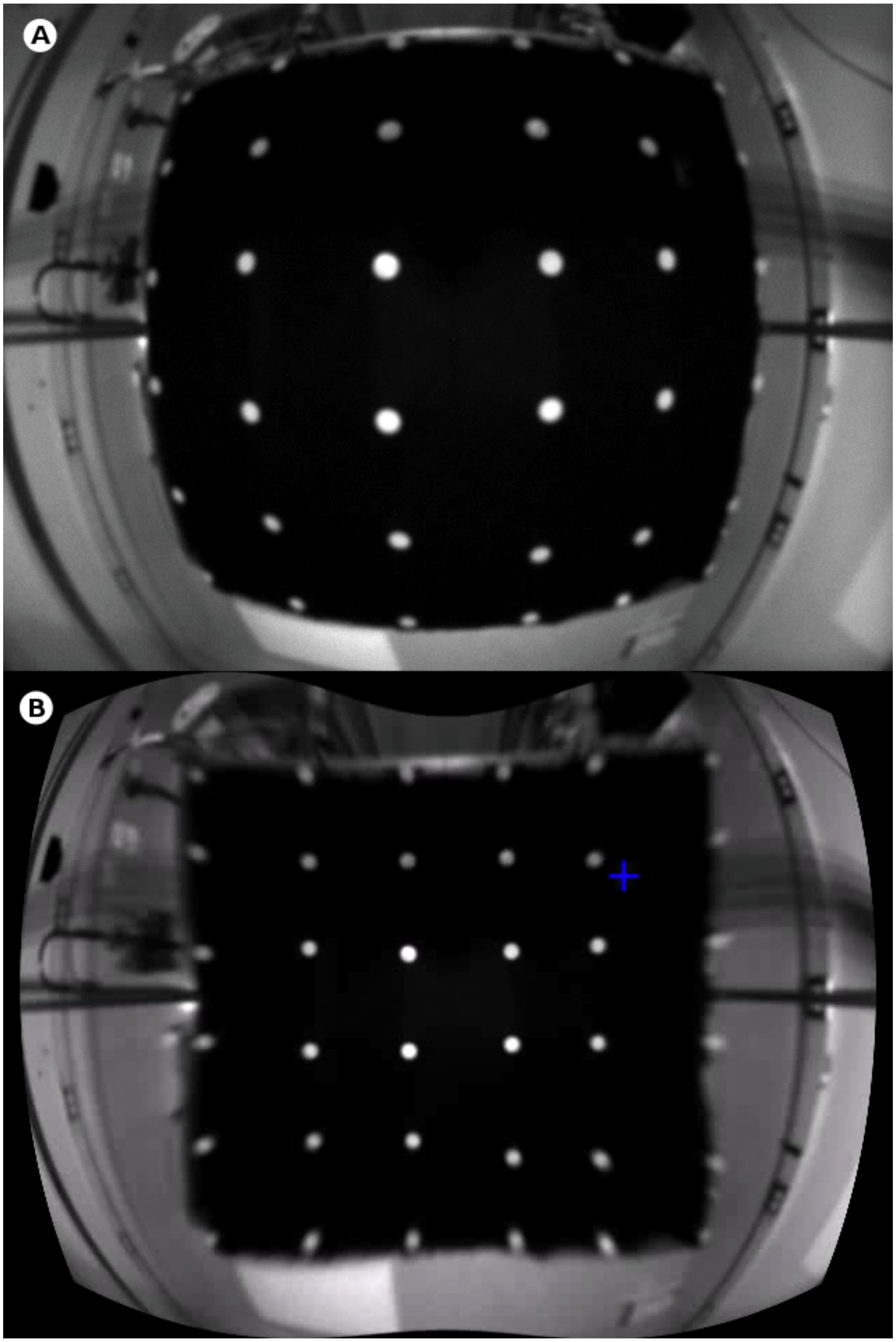


Figure 4.4 (A) A video frame from one of the foraging trials recorded in this study. Significant barrel distortion due to the use of a wide-angle fisheye lens is apparent. (B) The same video frame (with blue cross marking the position of the moth) after removal of the majority of the distortion within the confines of the 36-flower array, as implemented in the automated object tracking program developed for this study.

4.3.6 Experimental Treatments

I exposed moths to 5 different artificial flower populations. All flowers had a length of 20 mm. The flower holder that each flower plugged into added 10 mm of overall length from the top of the flower to the beginning of the nectar tube, making overall flower length 30 mm, the same as for the artificial flowers used in Chapter 2. All flowers also had overall diameters of 55 mm, the same as in the artificial flowers used in Chapter 2. I varied the nectary diameters and corolla curvatures of the flowers in my 4 experimental populations as follows:

Population 1: Nectary diameter ($2*r_o$) of all flowers held constant at 2.5 mm; corolla curvature parameters, $c = -\infty, -4, -3, -2, -1$, and 0. With this population, I explored the effects of variation in corolla curvature on hawkmoth foraging performance with flowers ranging from perfectly flat disks ($c \rightarrow -\infty$), through various degrees of trumpet-like curvature, and finally ending in a perfect cone ($c = 0$).

Population 2: Corolla curvature of all flowers held constant at $c \rightarrow -\infty$; nectary diameters $2*r_o=$ 1, 1.75, 2.5, 3.25, 5, and 7 mm. With this population, I explored the effects of variation in nectary diameter on a flat disk floral surface.

Population 3: Same as Population 1, except nectary diameter (2r) held constant at 1 mm instead of 2.5 mm. With this population, I explored the effects of corolla curvature on foraging performance in a set of flowers whose nectary opening barely larger than the moth's proboscis (mean proboscis width proximal to the head = 0.84 mm± 0.008mm SEM, N = 30).

Population 4: A mix of curvatures and nectary diameters mostly intended to sample the bowl-shaped part (c>0) of flower morphospace. c = -1 and 2r = 1 mm; c = 0.375 and 2r = 1 mm; c = 1 and 2r = 1 mm; c = 0.375 and 2r = 2.5 mm; c = 1 and 2r = 2.5; c = -3 and 2r = 7 mm.

Population 5: Another mix of curvatures and nectary diameters, this time intended to sample regions of the “large nectary diameter” (3.25-7 mm) parts of the flower morphospace. C = -4 and 2r = 7 mm; c = -1 and 2r = 7 mm; c = -4 and 2r = 3.25 mm; c = -3 and 2r = 3.25 mm, c = -1 and 2r = 3.25 mm; c = -1 and 2r = 1 mm.

These 5 flower populations do not sample the range of flower shape space that was physically available to me (see Fig. 4.5) uniformly. Referring to Figure 4.5, there is a large empty patch between 3.25 and 7 mm of nectary diameter and also toward the right side of the shape space (positive corolla curvatures parameter values). The reason for not sampling heavily in the 3.25-7 mm nectary diameter range was that Manduca’s exploitation ability response seemed to plateau at 3.25 mm and not change at 7 mm, so it was expected to remain the same in the intervening nectary diameter range. With limited time and resources, I had to make strategic decisions about which flower morphs to sample for foraging trials and which to leave out. Given more time and resources, I would want to achieve a more uniform sampling to remove doubt about the presumed response in this region of shape space. However, for positive corolla curvature values above nectary diameters of 2.5 mm, the physical construction of the 3D printed bracket that holds the artificial flowers in place presented complications for accommodating artificial flowers in said parameter range. A redesign of the flower holder bracket may be required for future incorporation of artificial flowers that occupy the upper right portion of the shape space in Figure 4.5.

Exploitation and visitation data were collected for 25 foraging trials in each of the five artificial flower populations.

4.3.7 Statistical Analysis

All statistical tests were performed in MATLAB.

For each moth behavioral response that I observed or calculated (see Methods, Section 4.3.4), I performed a 2-way ANOVA with nectary diameter and corolla curvature as factors in order to test for interactions between these two aspects of flower shape.

In addition to the 2-way ANOVAs, I also performed three 1-way ANOVAs per response variable:

(a) One on all the data

(b) One on just the data representing flowers with nectary diameters of 1 mm

(c) One on just the data representing flowers with nectary diameters of 2.5 mm

The reason for performing 1-way ANOVAs was to ask the simpler question in the particular situations mentioned in parts (a-c) above of whether or not Manduca’s behavioral response was higher for any particular flower morphology regardless of which factor might be explaining the difference. Since I am not looking for interactions between factors in these cases, the 2-way ANOVA was deemed to not be necessary for these cases.

A significance level of $\alpha=0.05$ was used for each test. Pooling of data from different artificial flower populations was justified as in Chapter 3.

Note: the data set for the response variable of mean distance of the moth from the center of a flower during proboscis insertion lacks series data for flowers of nectary diameter 3.25 mm and 7 mm.

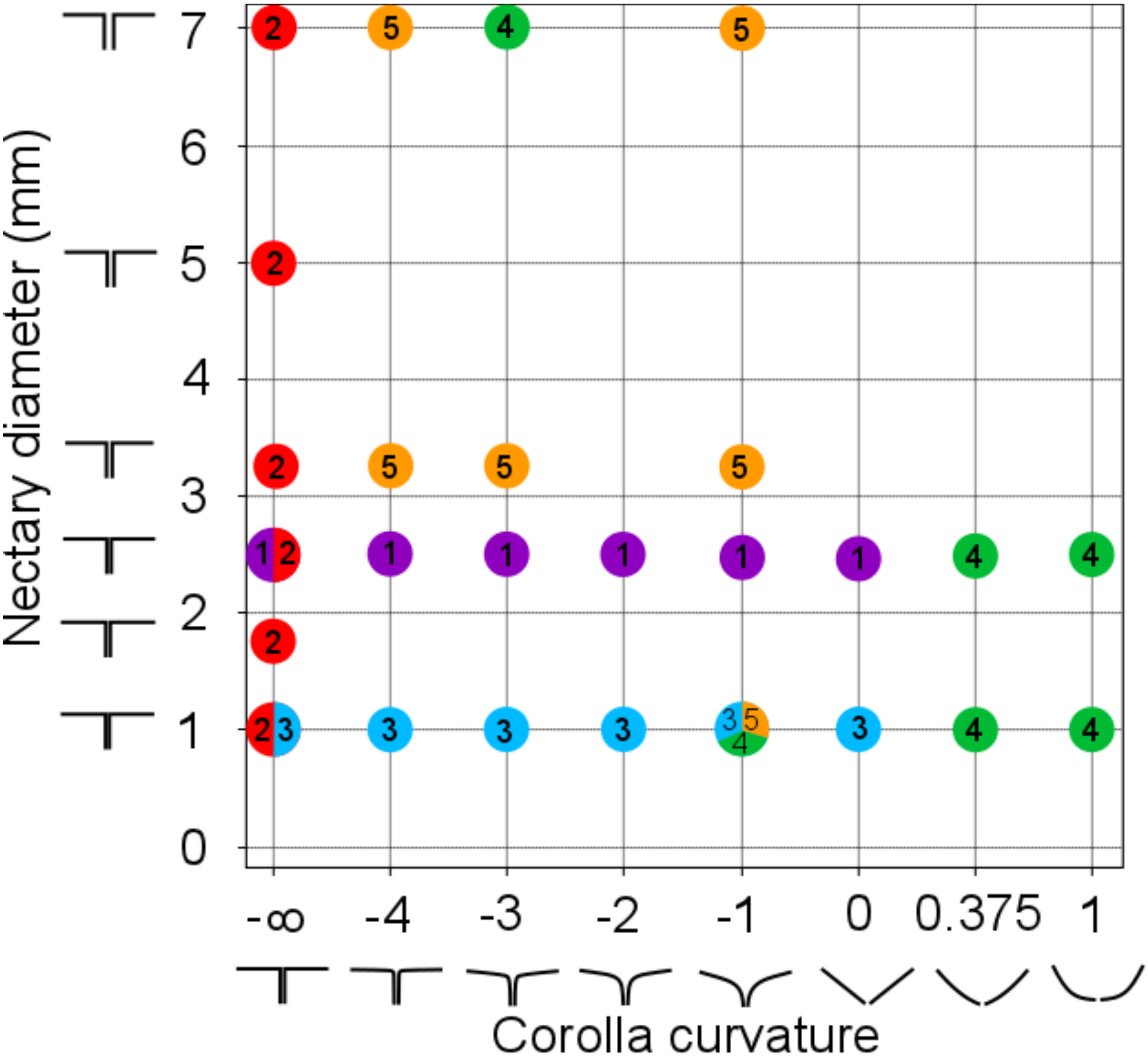


Figure 4.5
Graphic depicting the locations (colored circles) in the theoretical flower morphospace of the flowers used in each of my 5 experimental artificial flower populations. Numbers inside colored circles represent the population (numbered 1 through 5 in the main text) that each flower was used in. Exact values of the shape parameters are given in the Methods, Section 4.3.6 of the main text. Three specific flower morphs were used in two or more populations each, represented by the chimeric colored circles and multi-digit numerical codes. Example: the flower location coded 12 was used in populations 1 and 2.

4.3.8 Data Pooling and Justification

In performing the statistical tests described in section 4.3.7 above, I was pooling data obtained from different flower populations. This meant that I was comparing behavioral responses obtained from artificial flower morphs that had never been foraged upon simultaneously. This presents a potential problem, described in section 3.3.6 of Chapter 3. Following, I repeat the nature of this data pooling concern and the steps taken to ultimately justify it, while acknowledging the inherent limitation of the justification itself:

Concern: my foraging landscape dataset consists of flower morphs that were not all exposed to Manduca foraging *at the same time*. Instead, the flower morphs were exposed to Manduca foraging six at a time. Given this, the question must be asked about whether or not the context of which flower morphs were present in the flower array during any particular set of foraging trials affected the performance response of the hawkmoths. An analogy to sports might be in trying to compare the teams from each of the association football (soccer) leagues of Europe. For example, does the 4th place team from the English league merit more plaudits and prestige than the 5th place team from the Spanish league? If these two teams never actually competed against each other directly, then any conclusions would be highly speculative.

To get around this issue of context, I deliberately included some flower morphs into multiple groups of six morphs to be subjected to foraging by Manduca. I could then compare the foraging performance that hawkmoths achieved at such a flower morph when present in one group of six flower morphs vs. another group (same flower morph in different group contexts). If there is no statistically significant difference in the performance response achieved from the same flower morph when subjected to Manduca foraging in two different groups of six artificial flower morphs, then that would be taken as evidence that Manduca's foraging ability (or any other behavioral response associated with foraging effort) on flowers of different shapes is independent of which combination of six flower morphs the hawkmoths are attempting to feed from. In other words, context does not matter. And if context does not matter, then I take that as a reasonable justification to perform statistical analyses on all of the foraging data from all flower morphs simultaneously (pooling all the data), regardless of the group context in which each individual flower morph was presented to Manduca hawkmoths for foraging trials.

However, even if data pooling were justified according to the technique described in the preceding paragraph, it must still be acknowledged that I cannot fully rule out the possibility that flower morph context influenced the results. This is because while the common flower morphs that I chose to include in multiple flower array populations might show no influence of context, it may be that other flower morphs in the pooled data would indeed have shown a strong influence of flower context. Such an influence, if it exists, would be unknowable from the data I have available to me.

4.4 Results and Discussion

The main goal of this study was to identify was to identify foraging behaviors in Manduca hawkmoths that could be used as proxies of plant relative fitness. If such proxies of plant fitness can be identified, it sets the stage for using the concept of the experimental design and apparatus introduced in this and previous chapters of this dissertation to conduct experimental tests of the long-standing pollination syndrome framework.

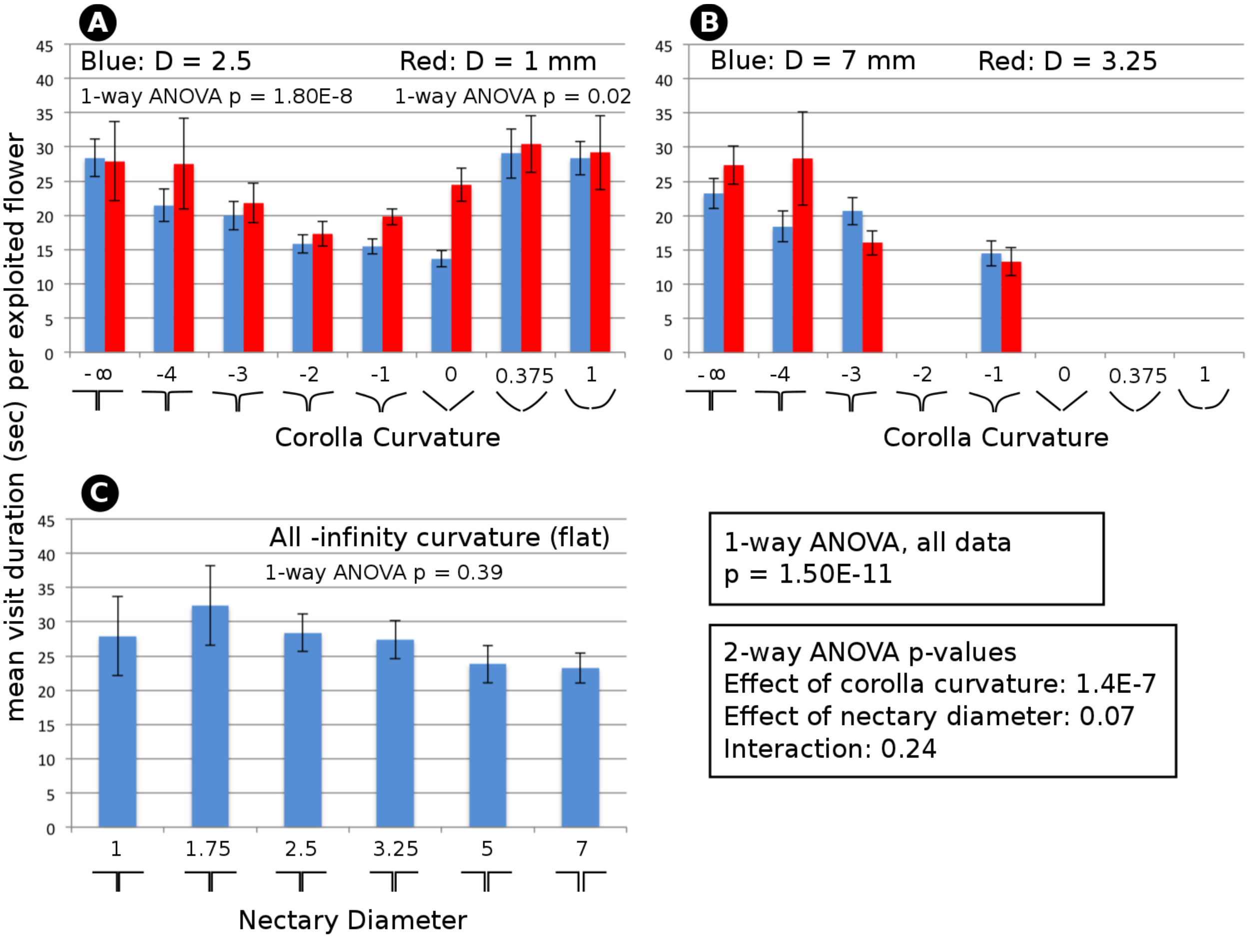
Unfortunately, the data available through this study are inconclusive regarding the existence of reliable indirect proxies of plant fitness from pollinator behavioral responses to foraging. Two of the proposed proxies of plant fitness indicate trumpet-shaped (intermediate corolla curvature values) flowers are more fit than flat and bowl-shaped flowers (the extremes of corolla curvature). In contrast, two other of the proposed proxies of plant fitness indicate that the complete opposite is true. The fifth (final) proposed fitness proxy is uninformative. As such, a reliable indirect proxy of plant fitness is, as of yet, unavailable. What this also means is that I cannot make strong conclusions regarding whether or not the mutualistic relationship of nocturnal/crepuscular hawkmoths and the flowers that they visit in nature is cooperative in process in addition to pattern. In order to better address these questions in pollination biology, it appears that the experimental design introduced in this dissertation must go through a specific iterative improvement: I propose that a physical touch sensor be included into each artificial flower in the flower array to act as a surrogate for the anthers and stigma (the reproductive structures) of a real flower. Such a touch sensor could be interpreted as a more direct proxy for flower/plant fitness than the ones discussed in this study, thereby making evolutionary conclusions drawn from the subsequent foraging data more relevant to the biological processes of cross-pollination and sexual reproduction in real flowers. Below, I present and discuss the details of the results of this study and expand on the proposed beneficial iterative improvements to the current experimental design.

4.4.1 Visiting trumpet-shaped flowers maximizes Manduca (pollinator) fitness

Manduca commonly hovers in front of flowers that it visits (Sprayberry and Daniel 2007). As hovering flight is energetically demanding (Norber 1990; Vogel 1994), flowers at which a hovering pollinator can extract large amounts of nectar per unit of invested time should yield the largest fitness payoff for the pollinator. Among artificial flowers that were exploited during foraging trials, corolla curvature had a statistically significant effect on visit duration (Fig. 4.6A; 2-way ANOVA $p = 1.4 \times 10^{-7}$). At flowers with nectary diameters of 1 mm and 2.5 mm, visit duration follows a U-shaped pattern as a function of corolla curvature, with the extremes of flat and bowl-shaped flowers yielding values that approach twice that (Fig. 4.6A) of trumpet-shaped flowers (corresponding to flowers with corolla curvature parameters of -2, -1, and 0). Since an exploited flower had all of its nectar removed and all flowers contained equal amounts of nectar at the start of a foraging trial, effective rate of energy intake per unit time is the inverse of the relative patterns shown in Figure 4.6. Thus, it seems as though foraging on trumpet-shaped flowers maximizes Manduca's rate of energy intake and consequently its average fitness. This means that from a fitness perspective, it is in Manduca's evolutionary interest for flowers to be trumpet-shaped as opposed to flat or bowl-shaped. This is concordant with the foraging performance landscape presented in Chapter 3 (Fig. 3.5).

4.4.2 Visit durations and visit frequencies suggest evolutionary conflict between flower and pollinator

While it is in the pollinator's evolutionary interest to reduce foraging effort, the opposite is expected to be true for flowers. All else being equal, larger visit durations and number of visits should represent greater opportunities for contact between a pollinator and the reproductive



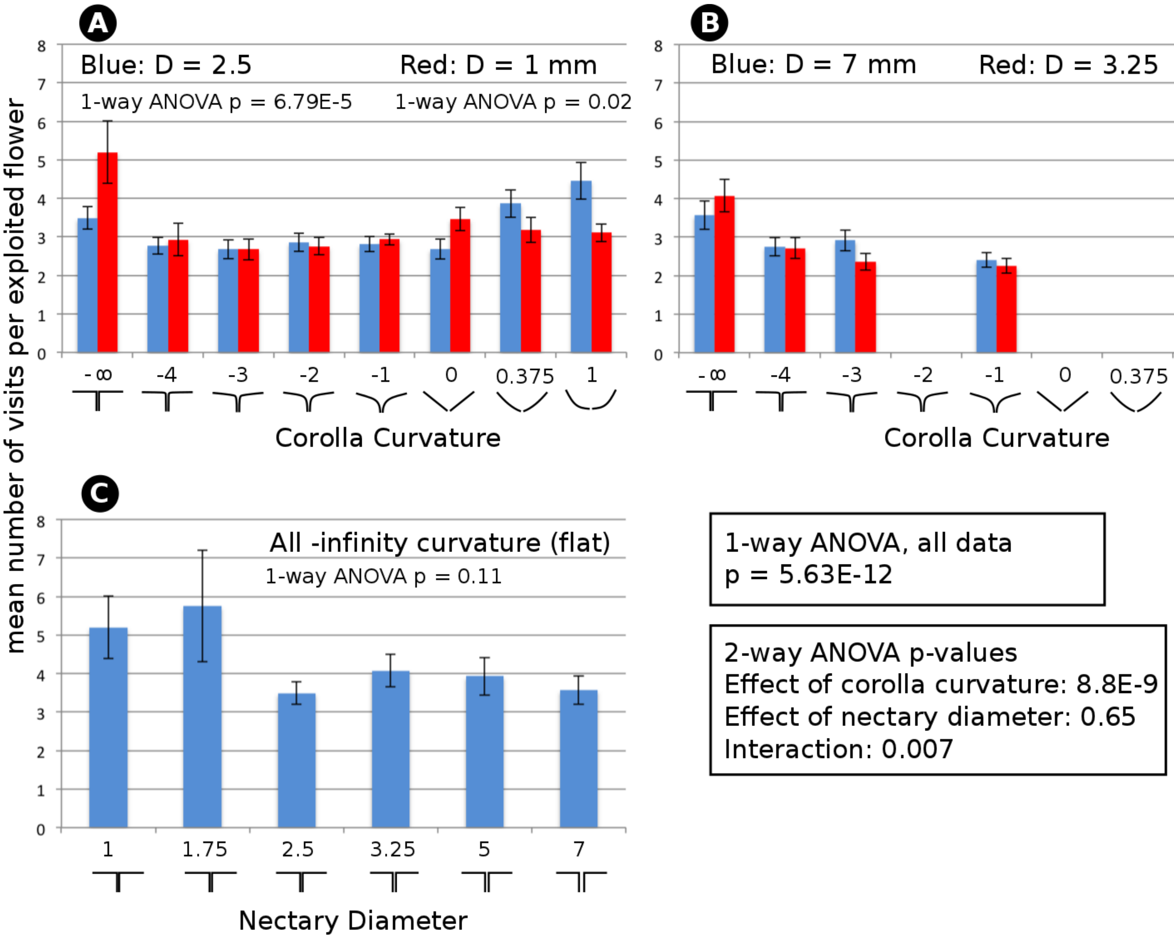
1-way ANOVA, all data
p = 1.50E-11

2-way ANOVA p-values
Effect of corolla curvature: 1.4E-7
Effect of nectary diameter: 0.07
Interaction: 0.24

Figure 4.6
Visit duration per exploited flower, another measure of foraging cost for a hovering nectarivore. If more time spent visiting also represents increased opportunity and probability of cross-pollination for a flower, then the U-shaped pattern associated with variation in corolla curvature (A, B) again is suggestive of a misalignment of the evolutionary interests of the pollinator and the flower.

structures of the flower, which in turn would be positively correlated with seed set (e.g. Manetas & Petropoulou, 2000). Thus, the U-shaped pattern in visit duration as a function of corolla curvature (Fig. 4.6A) suggests that flat and bowl-shaped flowers might be expected to experience higher relative fitness than trumpet-shaped flowers. Under such a scenario, flower shapes that yield the highest flower fitness yield the lowest pollinator fitness, and flower shapes that yield the highest pollinator fitness yield the lowest flower fitness.

Similar to visit duration, corolla curvature had a statistically significant effect on visit frequency (Fig. 4.7A; 2-way ANOVA $p = 8.8x10^{-9}$). Although the U-shaped patterns are not as visually pronounced and as symmetrical as for visit duration, flat and bowl-shaped flowers still represented the maxima while intermediate corolla curvature parameter values contained the minima. Thus, flat and bowl-shaped flowers that were exploited received more visits than intermediate trumpet-shaped flowers. If each visit by a pollinator to a flower represents a discrete opportunity for pollen transfer, then trumpet and bowl-shaped flowers would be expected to be more fit than trumpet-shaped flowers, again leading to a situation of evolutionary conflict between flower and pollinator.



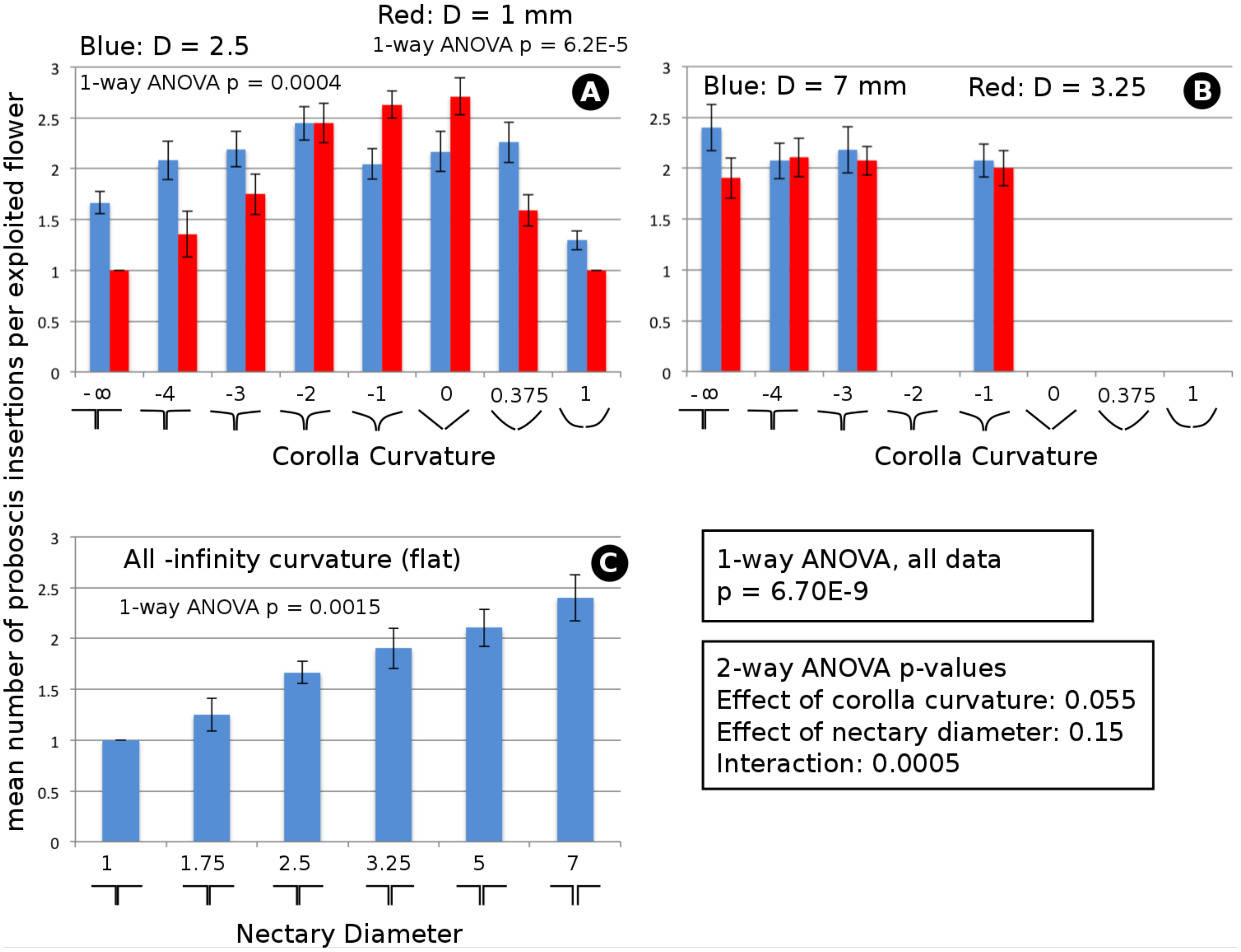
1-way ANOVA, all data
p = 5.63E-12

2-way ANOVA p-values
Effect of corolla curvature: 8.8E-9
Effect of nectary diameter: 0.65
Interaction: 0.007

Figure 4.7
Visit frequencies to flowers that were exploited during a foraging trial according to flower morphology. (A, B) Trumpet-shaped flowers tended to be visited fewer times than flat and bowl-shaped flowers. If number of visits represents a unit cost of foraging by hawkmoths, then hawkmoth fitness would be maximized by visiting trumpet-shaped flowers over either extreme in corolla curvature. However, if each visit by a moth represents an opportunity for a flower achieve cross-pollination, then flower fitness would be maximized by being “difficult to exploit,” represented by the extremes in corolla curvature, flat and bowl-shaped. This would represent a situation of evolutionary conflict between the fitness interests of the pollinator and the flower.

4.4.3 Proboscis insertion frequency and insertion duration suggest evolutionary harmony between flower and pollinator

A pollinator may visit a flower without exploiting the floral reward or making contact with the flower's reproductive structures (Muchhala 2003). Some flowers, especially those that are habitually visited by bats and birds, deploy their reproductive parts in the same physical space that the pollinator must occupy during its nectar extraction efforts (e.g. Caruso *et al*. 2003; Muchhala 2007; Muchhala & Potts 2007; Wester & Claßen-Bockhoff 2007; Muchhala & Thomson 2009). Thus, it is possible that the frequency of proboscis insertion events into a flower's nectar reservoir or the cumulative duration of such events might represent an accurate measure of a flower's fitness. Both of these metrics of pollinator foraging behavior exhibited similar patterns in my data. With regard to corolla curvature, both metrics exhibit mound-shaped distributions with extremely low 1-way ANOVA p-values (Fig. 4.8A and 4.9A). Exploited trumpet-shaped flowers receive more proboscis insertions and are probed for more time than flat or bowl-shaped exploited flowers. If these metrics of foraging performance are indeed positively correlated with flower fitness, then they suggest a scenario of evolutionary harmony between the flower and the pollinator: flower shapes that lead to maximal rates of energy gain for the pollinators also lead to pollinator behaviors that maximize opportunities for cross pollination and increased seed set in the flowers.



1-way ANOVA, all data
p = 6.70E-9

2-way ANOVA p-values
Effect of corolla curvature: 0.055
Effect of nectary diameter: 0.15
Interaction: 0.0005

Figure 4.8
The number of times that a moth inserts its proboscis into a flower's nectary might be positively correlated with flower fitness, if it places the moth in a physical position where contact with the flower's reproductive parts is more likely. Under such a scenario, the high insertion frequencies at trumpet-shaped flowers (A) suggest that the evolutionary interests of the moth and flower are aligned, as the flower morphs that would lead to the highest flower fitness are the same as what would lead to highest fitness in the pollinator.

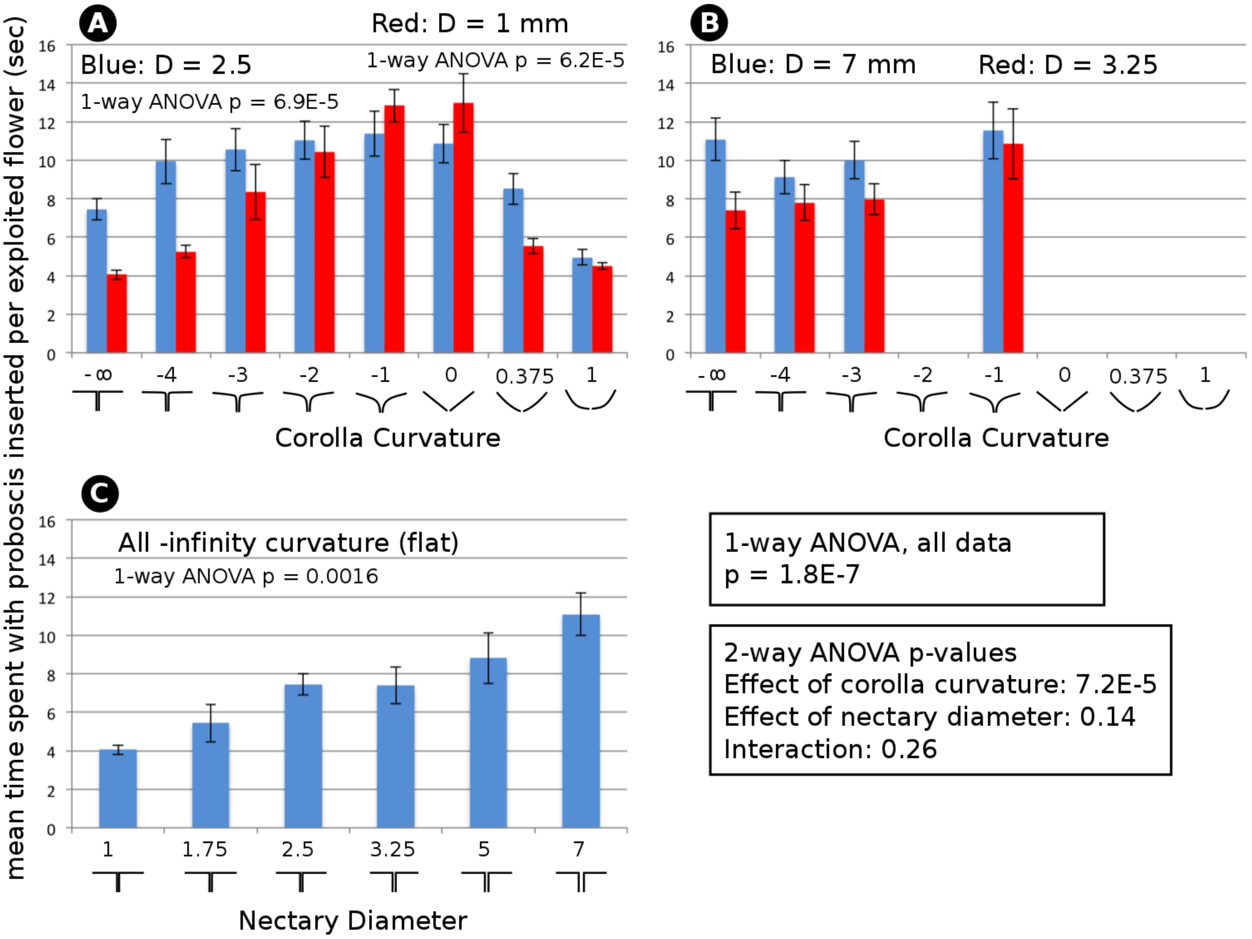
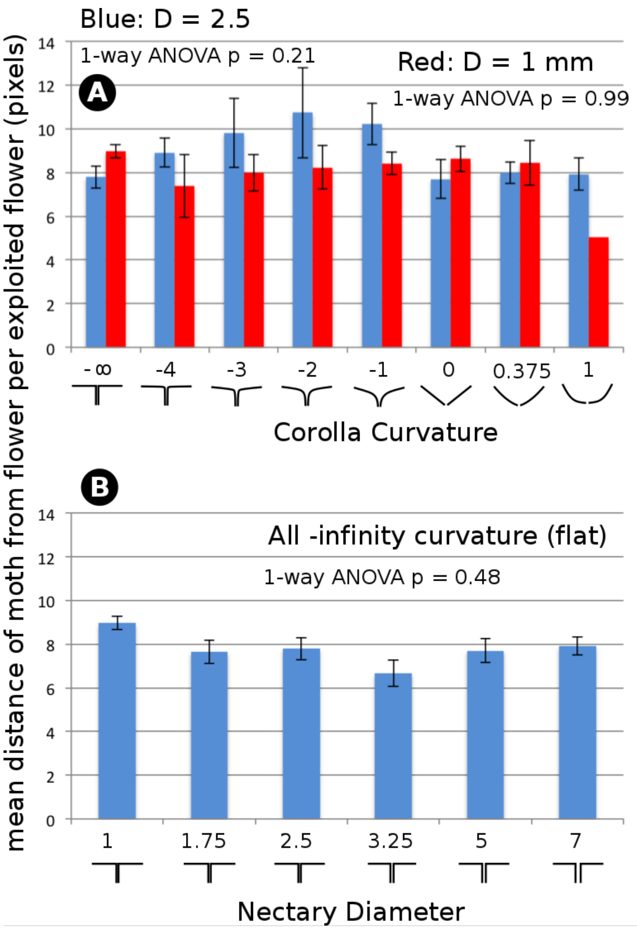


Figure 4.9
The amount of time spent by a moth with its proboscis inserted into a flower's nectary might also represent opportunity for gained fitness for a flower. If so, then this metric of pollinator foraging behavior is suggestive of evolutionary harmony between the pollinator and flower. Trumpet-shaped flowers (A) are probed for longer durations than flat or bowl-shaped flowers, and they also represent the corolla curvature that is least costly to exploit for the moths.

4.4.4 Manduca’s distance from flower central axis while feeding did not vary with flower morphology

If the position of a pollinator relative to a flower is an important predictor of probability of pollination success, then closer proximity to a flower’s central axis might be correlated with increased pollination effectiveness, assuming that the reproductive structures are deployed within close proximity of the flower’s central axis. A more constricted flower morphology, such as narrow nectary diameters or corollas that are relatively flat at their distal ends, might force pollinators to come closer to the central axis. However, Manduca’s mean distance from the flower’s central axis while the proboscis was inserted in the flower’s nectary showed no statistically significant differences as a function or either corolla curvature or nectary diameter (Fig. 4.10). As such, there is no strong evidence that this metric of pollinator behavior is useful as a proxy for flower fitness.



1-way ANOVA, all data
p = 0.46

2-way ANOVA p-values
Effect of corolla curvature: 0.45
Effect of nectary diameter: 0.18
Interaction: 0.63

Figure 4.10
The distance of a moth from the central axis of a flower during proboscis insertion into the nectary might be correlated with flower fitness if closer proximity leads to higher probability of contact with the flower's reproductive parts. However, moths did not exhibit statistically significant differences in this metric as a function of flower morphology. Therefore, there is no evidence that this metric is indicative of a difference in relative flower fitness.

4.5 Concluding Remarks and Future Directions

4.5.1 Advantageous features of this study's experimental design

The experimental approach to plant-pollinator interactions described in this study has several key features that I think make it an asset to biologists interested in studying pollination ecology.

> **(a) Flower morphology is mathematically determined.** As such, the investigator has the power to introduce a wide variety of morphological manipulations into their experiments without having to rely on the existence of such morphs in natural flower populations or laboratory-bred stocks. Also, the explicit mathematical quantification of flower shape could facilitate study reproducibility by allowing colleagues or other research teams to know exactly which flower morphs were tested and in what materials. It also means that discussions of qualitative aspects of flower shape, which can be immensely useful but also difficult to interpret in the literature, can be cast in terms of unambiguous quantitative parameters.
>
> **(b) Rapid prototyping technologies offer consistency, speed, and scalability.** Artificial flowers have a long history of use in studies of plant-pollinator interactions (reviewed in Campos *et al*. 2015), but have also been laborious and time-consuming to produce. Rapid prototyping technologies have become faster, more precise, less expensive, more diverse (in terms of methods and materials), and more user-friendly at a startling pace since 2010. Biologically realistic artificial flowers have recently been used to study plant-pollinator interactions by combining 3D scanning, 3D printing, and

molding techniques (Policha *et al*. 2016). Using a 3D printer to fabricate my artificial flower by way of additive manufacture was an excellent method of construction for this study. An entire population of 36 flowers could be prepared within one week's time with guarantee of fidelity to conceptual prototypes. Future researchers might be able to deploy more flowers for less or equal cost, greatly increasing the statistical power of such future studies.

(c) Infrared nectary sensors allow proboscis insertion and timing to be monitored. Without the infrared sensors attached to each artificial flower's nectary, it would have been impossible to determine from video exactly how many times a particular exploited flower was probed and for how long. The videos taken of the foraging trials in this study were not of sufficient spatial resolution to allow the moth's proboscis to be consistently identified. As such, proboscis insertion events could not be reliably monitored from video data alone.

(d) Automated object-tracking software made moth-to-flower distance tracking feasible. The tracking software developed specifically for this study made moth tracking logistically feasible. Software exists that allows for human-based object tracking. However, this is a slow and labor-intensive process. The automated tracking software led to at least a 20-fold increase in the speed at which video data could be processed for this study.

4.5.2 Disadvantages and aspects in need of improvement

Despite the various positive features of my experimental setup, there is still room for improvement. Some areas of improvement would make the setup more reliable, while others would extend the types of questions that can be addressed.

(a) Consistency of automated object tracking: While the custom automated tracking software used in this study made moth-to-flower distance calculations feasible given the large amount of data generated, the software's consistency limited the diversity of ways in which the data that could be analyzed. Accuracy was excellent for video frames in which the software was able to correctly identify the moth. However, for unknown reasons, the incidence of failure to track increased during the critical proboscis insertion phase of a floral visit. Although the large number of foraging trials and floral visits within each foraging trial led to some usable data, sample sizes related to moth-flower distances would at least triple if consistency were perfect (meaning that the software would always succeed in identifying the moth when it in fact was in the video frame). Computer vision is not a trivial matter and the software used in this study was developed with an extraordinary investment of time and effort. Increasing tracking consistency beyond current performance would not be an easy undertaking, but it is technically possible to do.

(b) Uncertainty in correlation between pollinator foraging behavior and flower fitness: The goal of this study was to investigate whether any aspects of pollinator foraging behavior could be used as a reliable proxy metric for flower fitness. If such a proxy for flower fitness could be found, it could be used in conjunction with the experimental design described here to experimentally test questions concerning the evolution of flower shape. For instance, to what degree does the classical notion of pollination syndromes (Fenster *et al*. 2004) accurately explain patterns of floral phenotype diversity and pollinator visitation (Hingston & McQuillan 2000; Ollerton *et al*. 2009)? For example, would a population of artificial flowers with an underlying modeled genetic system respond to visitation by specific ecological guilds of pollinators by evolving in the directions predicted by the pollination syndrome concept?

Since different metrics of pollinator foraging performance suggested opposing conclusions regarding the predicted evolutionary response by flowers to pollinator visitation, it is not possible to say with any certainty how the specific pollinator foraging behaviors presented here correlate with flower fitness. Does the number of proboscis insertions into a flower's nectary correlate with seed set, or is visit duration or visit frequency more important? It is impossible to say from the data presented here alone. What is needed is a more direct method of accounting for the flower's evolutionary interest. In order for a flower to produce a seed set, the pollinator must invariably make contact with the reproductive structures of the flower – the anthers and the stigma. A physical touch-sensitive sensor incorporated into the artificial flower system that I have developed could serve as an analog for the flower's reproductive parts. Just as the infrared proboscis sensors already unambiguously provide information regarding exploitation of a flower, a touch sensor could provide direct information regarding a flower's relative fitness. This touch sensor may take the form of an accelerometer attached to a thin stalk deployed in the same general location that a real flower's reproductive structures are expected to be. The physical arrangement of stigmas has already been shown to influence pollen exchange (Kudo 2003). Thus, the morphology of an anther sensor could become another informative morphological variable in the artificial flower system.

In conclusion, the indirect putative measures of flower fitness discussed in this study point to opposing conclusions regarding the existence of evolutionary conflict or harmony between angiosperms and crepuscular sphingid moths. Pollinator visit duration and visit frequency suggest evolutionary conflict while proboscis insertion duration and frequency suggest evolutionary harmony. Either scenario is theoretically possible, depending on which pollinator foraging behaviors are more correlated with contact between the pollinator and the reproductive structures of a flower. The inclusion of a touch-sensitive sensor to the artificial flower system described herein would do much to eliminate the uncertainty inherent with the use of indirect measures of organism fitness. Such a sensor would also greatly increase the feasibility of experimental evolution studies of flower morphological phenotype in response to visitation by putative pollinating animals. Rapid prototyping combined with mathematical modeling of floral shape has the potential to be a flexible and powerful experimental tool in the study of plant-pollinator interactions.

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4.7 Data Accessibility

Data are deposited in the University of Washington Research Works Archive.

http://hdl.handle.net/1773/39169

4.8 Supplemental Software Accessibility

Script for exploring shape parameter space of the artificial flowers described in this study:

https://github.com/eocampos/artificial_flower_shape_exploration

Script for shuffling positions of artificial flowers in the 36-flower array:

https://github.com/eocampos/random_flower_array_shuffler_36flowers

Script for filtering and processing data from infrared occlusion sensors for determining when and for how long a moth's proboscis entered the nectary of an artificial flower:

https://github.com/eocampos/ir_sensor_filtering

Source code for custom automated object-tracking program:

https://github.com/SpaceKitKat/Tracker-moth

Script for data acquisition from IR sensors attached to each artificial flower through an Arduino Uno with an attached Mayhew Labs Mux Shield (http://mayhewlabs.com/products/arduino-mux-shield):

https://github.com/eocampos/arduino_mux_shield_and_LED_switch

4.9 References

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