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Cognitive Ecology of Color Vision in Orchid Bees

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Abstract

Animals interact with their environment and acquire information from it. Information can be processed by their sensory systems and influence behavior, often mediated through mechanisms of decision-making and learning. Animal pollinators use their sensory systems to acquire information from floral traits and use this information to make decisions about the flowers they visit. My dissertation research aimed to understand the role of color vision in a tropical pollinator, the orchid bee *Euglossa dilemma*. Chapter 1 is a review that explores pollination through the lens of prepared learning. Prepared learning proposes that animals learn some associations better than others due to an evolved match with the environment. I offer a brief history of the concept, build a conceptual framework for field and laboratory studies, explore examples of prepared learning in pollination, and suggest future directions for the field. Chapter 2 characterizes color vision in the orchid bee *Euglossa dilemma*. I compare *E. dilemma's* color vision to other related bees by comparing their spectral sensitivity curves and opsin protein amino acid sequences. My results show that *E. dilemma* is a trichromat, with peaks of Green, Blue, and Ultraviolet in similar regions to other bees. Ultraviolet photoreceptors are the most conserved among the compared bees, while blue photoreceptors and opsin proteins are the least conserved. Chapters 3 explores orchid bee color vision use, focusing on color choice and preference. Color choice was affected by time of day and humidity, and individual orchid bees show variability in their color preferences in our tested colors. Color preference was not affected by the abiotic or biotic factors measured nor predicted by a bee's first choice and there were no detected differences between male and female color choices. Chapter 4 tests whether the presence of scent affects the bees’ choices in color preference trials. Scent affected motivation to engage, but not participation (number of choices) or color preference. I also tested for the ability to condition a sugar reward to a scent cue but did not detect scent learning. My results show that male orchid bees attend to scent cues, delaying their choices about color cues when scent is present. This might indicate that male orchid bees are evolutionarily prepared to attend to scent cues. The results from this dissertation add to our knowledge of tropical bee decision-making, and the techniques and methodologies developed and implemented here can be used in other populations of wild bees.

Keywords: color vision, orchid bees, tropical bees, Euglossini, prepared learning, spectral sensitivity, opsin, corbiculate bee, decision making, color choice, color preference, scent learning.

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Introduction

Animals are constantly interacting with their environment and acquiring information from it. Available information can be processed by their sensory systems and influence behavior (Dall et al. 2005). The field of cognitive ecology studies how animal cognitive processes (acquiring and using information) evolved in an ecological context. One essential cognitive process is learning, which involves using previously acquired information to adjust behavior to the present environmental conditions, allowing animals to "fine-tune" their behavior (Shettleworth 2010). Learning is ubiquitous in the animal kingdom (Shettleworth 2010; Dukas 2013), and it plays a major role in pollination ecology: pollinators gather information about their environment in the form of floral cues and use this information to make decisions (Raguso 2004; Leonard, Dornhaus, and Papaj 2011; Latty and Trueblood 2020), which affect plant reproduction and animal-plant co-evolutionary relationships (Gegear and Burns 2007). Floral traits can be interpreted as cues to pollinators, for example, color and scent (Briscoe and Chittka 2001; Raguso 2008), and rewards, for example, nectar (Parachnowitsch, Manson, and Sletvold 2018). Rewards enhance or decrease pollinator visits and promote learning. The goal of my dissertation research was to understand the role of color vision in a tropical pollinator, the orchid bee *Euglossa dilemma*. This dissertation's structure is as follows: a review chapter on learning and pollination, a chapter describing the visual system of *E. dilemma*, and two chapters focused on aspects of color use and scent learning of *E. dilemma*.

Chapter 1, titled "Prepared learning in Plant-Pollinator Interactions" is a review that explores the process of pollination through the lenses of prepared learning. Prepared learning was first proposed in 1970 by the psychologist Martin Seligman who hypothesized that a species' evolutionary history could make animals more prepared to associate a given stimulus with a specific response. Research in pollination shows that not every component of a floral display is important for learning, and not all floral traits are learned equally. A unifying framework for thinking about these disparate results is preparedness. By typifying the environment, we can make explicit predictions about the learning we expect to see with evolutionary preparedness and avoid the post hoc "just-so stories" that are prevalent for explaining anomalous results in the pollinator literature.

The remaining chapters focus on color vision and the cognitive ecology of the orchid bee *Euglossa dilemma*. Bees have been long studied for their contribution to pollination services and for their fascinating behaviors. The first studies on honey bee color learning date back over 100 years (Menzel 2012), and since then, honey bees and bumble bees have become learning models (Dukas 1995; Leadbeater and Chittka 2007; Menzel 2012), and results from these two groups are usually generalized to other pollinators, despite often significant differences in natural history. Orchid bees are closely related to honey bees and bumble bees. Together with stingless bees, they form the monophyletic clade Corbiculate bees (Michener 2007). Despite having relatively well-described behavior for some aspects of their natural history, to date, only one published paper has explored orchid bee behavior from a cognitive point of view (Eltz, Roubik, and Lunau 2005). This is surprising given the importance of orchid bees in the tropics; they are major pollinators in tropical systems, comprising approximately one-fourth of bee species in Neotropical forests (Roubik and Hanson 2004). Orchid bees likely share cognitive similarities with honey bees and bumble bees due to their shared evolutionary history. However, there is a remarkable difference between orchid bees compared to their close relatives: orchid bees never evolved true eusocial behavior (which is defined by true division of labor – workers, soldiers, and queen - and the presence of a reproductive caste – the queen is the only one allowed to reproduce and lay eggs) (Roubik and Hanson 2004; Freiria, Garófalo, and Del Lama 2017; Saleh and Ramirez 2019). This suggests that orchid bees face different challenges and may handle and use information differently. While a social bee can be specialized on a specific task, such as foraging, tending for the young, or protecting the colony, an orchid bee needs to perform all tasks by itself, which may require different cognitive abilities.

Orchid bee pollination is intimately linked to foraging behavior. Male orchid bees range daily on areas over a few kilometers while females are very local (Roubik and Hanson 2004); both sexes forage for nectar (energy source), but males actively search for and collect scents (possibly to attract females) while females collect resin (used to build a nest) and pollen (used to feed larvae) (Roubik and Hanson 2004). This foraging pattern leads to individual bees visiting different species of flowers in search of different resources. This results in orchid bees acting as pollinators for multiple species of plants (Armbruster 2017) across a large area (Roubik and Hanson 2004; Gilbert 1980). A recent study of a species of orchid bee brain (Brand, Larcher, Couto, Sandoz, and Ramirez 2018) suggests sexual dimorphism in brain anatomy that may be due to color vision. Male orchid bees have larger eyes and medulla (the brain region associated with color vision), suggesting that they invest more in their visual systems than females. These differences might affect color perception and choice in male and female orchid bees. Given the differences between orchid bee male and female ecology, color signals may be used differently in orchid bees. Foraging behavior may be a selective pressure driving sexual dimorphism in orchid bees' brains and color perception. Understanding how orchid bees learn about the flowers they pollinate is key for tropical conservation, but to this date, there has been no data on orchid bee color perception or color vision ecology.

The focal species, *Euglossa dilemma*, recently expanded its range into the United States to include south Florida (Skov and Wiley 2005; Eltz et al. 2011; Pascarella 2017). The presence of *E. dilemma* in the United States has opened a range of possible behavioral studies due to the ease of transporting nests and individuals to research institutions, such as universities and zoos. The population of *E. dilemma* in Florida has been the focus of detailed work on chemical ecology (Brand et al. 2015; Brand, Larcher, Couto, Sandoz, and Ramírez 2018), brain anatomy (Brand, Larcher, Couto, Sandoz, and Ramirez 2018), genetics (Zimmermann et al. 2011), social behavior (Saleh and Ramirez 2019; Saleh et al. 2021) and pollen diet (Villanueva-Gutierrez, Quezada-Euan, and Eltz 2013). The results from my experiments on the sensory ecology of these bees, combined with the growing understanding of other aspects of their biology cited above, provide a more robust framework for understanding pollination biology and bee natural history in a comprehensive way.

Chapter 2, titled "Spectral sensitivity of the orchid bee *Euglossa dilemma* - Color is in the eye of the beeholder" describes the spectral sensitivity and characterize color vision on the orchid bee *Euglossa dilemma*. I compared *E. dilemma's* color vision to other closely related bees by comparing their spectral sensitivity curves and opsin protein amino acid sequence. Chapter 3, titled "Color choice and preference in the tropical orchid bee *Euglossa dilemma*" presents the first study of orchid bee color vision. Bees were tested in the field for their color choice and preference, and I also described an adaptation of a novel methodology for field behavioral testing of tropical bees. Chapter 4, titled "Scent and color cues in nectar foraging in the orchid bee *Euglossa dilemma*" builds upon the results of Chapter 3, exploring the role of scent on color choice, color preference, and scent learning.

The techniques and methodologies I have developed and implemented in this project can be applied in future studies of orchid bees in Central and South America, where the diversity of bees is higher and less studied, as well as in future studies with other species of bees in their natural environment. The field of sensory ecology in insects has traditionally been limited to studies with species in captivity. As a result, most of what we know about bee cognition is generalized from tested in laboratories or very controlled field situations (Muth et al. 2017; Amaya-Márquez et al. 2019). By studying sensory systems in the field, we can expand the number of species that can be tested and gain a more comprehensive understanding of how natural populations use information from their natural surroundings. The methodologies proposed here will help bridge the gap between laboratory insect cognition and natural sensory processes in an ecological context.

Human activity has caused rapid environmental change, which is responsible for a drastic decline of pollinator populations globally (Lebuhn et al. 2013). Recent years have seen increased numbers of studies linking animal cognition and population stability to human-induced rapid environmental change situations. The genetic diversity of *E. dilemma* appears to be stable even in areas affected by human activity (Soro et al. 2016), and there might be behavioral and cognitive reasons why *E. dilemma* can cope with modified environments. Data from *E. dilemma* presented in this dissertation can be compared with other populations of orchid bees and pollinators in general to identify species that might be more vulnerable to environmental change. A better understanding of how orchid bees react to rapid environmental change and how their sensory ecology helps them cope with deforestation and irregular climate patterns is key to planning and executing policy and conservation efforts.

Regarding conservation policy, orchid bees are already considered necessary in conservation efforts. The Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) places orchid bees as key organisms for environmental assessment (McCravy et al. 2016; Nemesio and Vasconcelos 2014), meaning that the presence or absence of orchid bees in an area is informative of the quality of the area for other organisms, highlighting the importance of conserving this group. By understanding how bees see color and how they use color information to make decisions, I am providing the building blocks for investigating how orchid bee color vision affects flower visitation, which directly impacts plant reproduction, thus affecting the whole ecosystem.

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Chapter I: Prepared learning in Plant-Pollinator Interactions

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Abstract

Animal pollinators gather information about their environment in the form of floral cues and use this information to make decisions. Plant traits can condition pollinators' behavior, leading to the development of preferences, biases, and the evolution of learning, but also to aversion of colors, scents, shapes, among other plant traits. Therefore, a pollinator's sensory and cognitive system and behavior are evolving in response to plant traits, which are evolving in response to pollinator behavior and sensory abilities. In this review, we propose prepared learning as a framework for the study of pollination systems. Prepared learning is a theory that proposes that animals learn some associations better than other associations due to an evolved match with the environment. We offer a brief history of the concept, build a conceptual framework for field and laboratory studies, explore examples of prepared learning in pollination, and suggest future directions for the field.

Keywords: *preparedness, pollination, evolution of learning, animal learning.*

Introduction

In recent years, we have seen an increase in the number of studies on animal learning in pollination systems. Animal pollinators gather information about their environment in the form of floral cues and use this information to make decisions (Latty and Trueblood 2020; Chittka and Thomson 2001). Plant traits act as stimuli. They can condition pollinator's behavior, leading to the development of preferences, biases, and the evolution of learning (Schiestl and Johnson 2013), but also aversion of colors, scents, shapes, among other plant traits. Therefore, a pollinator's sensory and cognitive system and behavior are evolving in response to plant traits, which are evolving in response to pollinator behavior and sensory abilities.

Pollination is a multimodal and complex interaction where a single floral trait may be sending different signals to different animal pollinators, with many potential floral traits adding even more complexity (Leonard, Dornhaus, and Papaj 2011). However, it is unlikely that animal pollinators are responding to all traits produced by a plant. One common thread of the growing research in pollinator learning is that not all plant traits are cues for every pollinator, and not all cues are treated or learned the same by pollinators. A question at the heart of animal pollinator learning is *why do animals learn some cues better than others*? This is not a new question in the broader field of animal learning, with researchers posing this question since the 1960s (Garcia and Koelling 1966; reviewed in Dunlap 2018). A handful of approaches, such as constraints on the learning mechanisms (Shettleworth 1972), the concept of adaptive specializations (Shettleworth 2010), and the phenomenon of selective associations (LoLordo 1979), have been proposed as answers. The most flexible of all frameworks for answering this question is prepared learning (Seligman 1970).

In this review, we propose prepared learning as a framework for the study of pollination systems. We offer a brief history of the concept, build a conceptual framework for field and laboratory studies, explore examples of prepared learning in pollination, and suggest future directions for the field. We propose that by using an interdisciplinary approach and applying deep knowledge from over a century of learning theory to the ecology and evolution of pollination, we can find much-needed answers to the question of why animals learn some cues better than others. This review is timely because it merges our robust and long-term understanding of the natural history of pollination systems with recent developments that allow for a much better understanding of behavior and neurobiology mechanisms in animal pollinators.

What is prepared learning?

Learning is a basic and pervasive behavior and is crucial for animals, ranging from foraging to communication to mate choice. In the past, psychologists typically considered learning a general process that enabled animals to form arbitrary associations. Most traditional learning theories (e.g., Thorndike, Skinner, Hull, Watson) assumed an "empirical principle of equipotentiality" whereby the laws of learning applied equally across stimuli and responses (Domjan 1997). This was a convenient assumption because it enabled scientists to work with a few model organisms from which all learning could be generalized across species. However, this assumption was not to last as researchers expanded their work into different species and contexts. Breland and Breland (1961) challenged B.F. Skinner's radical behaviorism approach with *The Misbehavior of Organisms*. A quote from this book sums up what others also were discovering, "*After 14 years of continuous conditioning and observation of thousands of animals, it is our reluctant conclusion that the behavior of any species cannot be adequately understood, predicted, or controlled without knowledge of its instinctive patterns, evolutionary history, and ecological niche*." This observation describes the challenges over the next 50 years of analyzing learning from an evolutionary and ecological view.

Around the same time, Garcia and colleagues (beginning with Garcia and Koelling 1966) presented a series of experiments suggesting significant biological constraints on the kinds of associations that animals can and cannot learn. These, and many other studies, focused attention on the role of constraints (e.g., Shettleworth 1972, Domjan & Galef, 1983). The term preparedness first emerged in 1970, when Seligman hypothesized that a species' evolutionary history could make animals more prepared to associate a stimulus with a response (Seligman 1970). Likewise, evolution could function to make an animal contraprepared for a given association that was very unlikely to occur. Moreover, many relationships would be simply neutral or unprepared. Numerous studies in the coming decades demonstrated that animals are better prepared to learn some associations and that prepared learning is often consistent with biological expectations. In a now-classic series of studies, Cook, Mineka, and colleagues showed preparedness of fear conditioning for biologically relevant stimuli (Mineka et al. 1984; Cook and Mineka 1990), developing a framework for understanding the evolution of phobias (e.g., Öhman and Mineka (2001).

The idea that animals learn some associations better than other associations due to an evolved match with the environment is an underlying assumption for many biologists, without necessarily making specific predictions. All stimuli are not equally associable, and prepared learning is one of the explanations for why this might evolve. For pollinators, their coevolutionary history with plants shapes their learning abilities and is key to understanding how and why some plant cues are learned better than others.

Calls for cognitive traits to be studied with the natural history of the animal at the forefront of experimental design have been answered in recent years (Balda, Pepperberg, and Kamil 1998; Morand-Ferron, Cole, and Quinn 2016). This, as it was pointed by Seligman (1970), is a call for experiments in conditions in which animals were biologically prepared to respond. Numerous studies test learning and memory in wild populations (Morand-Ferron, Cole, and Quinn 2016; Roth, LaDage, and Pravosudov 2010). Although studies of cognition in the wild are a somewhat recent development in the field of cognitive ecology, pollination ecology has a long history of studying pollinator behavior, taking into consideration their natural history.

Pollination systems provide an excellent path to the study of preparedness. From the plant's perspective, floral traits may have evolved in the context of pollination as a signal for the animal pollinator. Alternatively, floral traits may have evolved for many other reasons and, once present in the population, be "co-opted" to act as signals for pollinators (e,.g. Hanley, Lamont, and Armbruster 2009; Armbruster 1997). Prepared learning could benefit plant fitness by increasing the chances of effective pollen transfer, for instance, through increased constancy of visits. Since there are costs associated with making signals and a high cost with missing reproductive opportunities, prepared learning could make for a surer bet for the plant. From the pollinators' perspective, animals can be evolutionarily prepared, unprepared, or contraprepared to learn floral traits. If an animal pollinator is prepared to learn a floral trait, learning will happen faster, and that association will be harder to forget. Most floral traits are expected to be neutral, meaning that pollinators are not evolutionarily prepared or contraprepared to learn such traits. It is worth mentioning that learning can still happen under neutral conditions, but it will be slower than for prepared traits. Finally, animal pollinators can be contraprepared to learn a floral trait. In such cases, learning will happen much slower or will not happen at all, despite other conditions for learning being met. Prepared learning benefits the animal's fitness by increasing the success of foraging for resources, which are often directly related to essential aspects of an animal's life, such as feeding, securing mates, and providing for their young.

The role of reliability and the evolution of prepared learning

The most critical variable in the evolutionary ecology of animal information use is the role of change and reliability. Reliability in the context of learning can be defined as the conditional probability that a cue available for learning reliably predicts the best action for an animal to take. In nature, pollinators often experience unreliable resources due to competition, misinformation or mistakes from the animal, floral constraints, or floral deception. Stimuli, reinforcers, rewards, and responses that have been reliably paired over evolutionary time may eventually result in the evolution of preparedness (Dunlap and Stephens 2014, 2016). For example, experimental evolution in fruit flies has shown that statistical patterns of reliability predict when preparedness will evolve (Dunlap and Stephens 2014). Reliability of the stimulus-reward contingency has strong effects across experimental contexts. However, it is rarely manipulated in pollination studies and in learning experiments in general, where stimuli are generally completely reliable or completely unreliable. Moreover, an experimental evolution approach would not be feasible for most pollination systems due to long generation times and practical constraints of husbandry and horticulture in combination for experimental co-evolution.

One alternative way to study the evolution of prepared learning in pollination systems is to use information from a system's natural history to map the historical patterns of reward of a stimulus in the environment. This allows for predictions of where preparedness would be expected to evolve, and these predictions can then be tested in natural systems (Dunlap, Austin, and Figueiredo 2019). More specifically, we can take advantage of measurements of stimuli (flower traits) and rewards (nectar, pollen, resin, oils, scents) recorded over ecological time. These parameters can then be used to calculate patterns of change and reliability of resources a given group of pollinators has experienced over several generations. For example, Baude et al. (2016) compared 80 years of nectar quantity measurements with pollinator diversity in Great Britain, from 1930 to 2007. They found trends of loss and stabilization of nectar quantity corresponded to trends of decline and stabilization of pollinator diversity across different habitats of the region. One could use similar data on floral resources in a region over several years, interpose resource data with seasonality and phenology data on different plant species, and map out how reliably floral traits match resources over several generations of a pollinator population. By understanding which resources and floral traits were more likely to be reliably paired, we can develop hypotheses about the evolution of prepared learning and design specific experiments to test those predictions.

The evolution of prepared learning is not outside of the realm of evolution of behavior and plasticity. Theory on evolutionary change mediated by plasticity, such as the Baldwin effect, support the evolution of prepared learning. The Baldwin effect explains the evolution of learning through natural selection by proposing that individuals' plastic traits, such as learning, can increase fitness and be selected for, making plasticity a positive force driving the evolution of a population (Baldwin 1896; Crispo 2007; Scheiner, Barfield, and Holt 2017). Thus, individual learning plays an indirect role on natural selection (Crispo 2007). From this framework, we can think of prepared learning arising in a population as the plastic trait of associative learning. If the learned association increases fitness, there will be selective pressure that will increase the frequency of this behavior in several forms (anatomical, physiological, cognitive), which will result in more individuals in this population having the machinery for learning this association, as well as the evolution of potential preferences for specific cues or types of cues. Similarly, genetic accommodation, which can be defined as a change in gene frequency in response to selection of a novel trait (West-Eberhard 2005; Crispo 2007), is another mechanism that can work with the evolution of learning to result in the evolution of preferences.

Stimulus, reinforcer, response, and reward

Before we proceed, it is important to specifically define the stimulus, reinforcer, response, and rewards in a plant-pollinator interaction. These concepts, defined in Table 1 and used throughout the text, follow the concepts from animal psychology. Some of these concepts have been loosely used in the pollination literature, but it is important to define terminology. Figure 1 shows a depiction of the process of pollination from an animal learning perspective and where these terms apply. "Stimulus" refers to the conditioned stimulus (Figure 1). For pollination, a stimulus is almost always a floral trait: color, shape, size, odor, symmetry, electromagnetic field, etc. The "reinforcer" is a stimulus, but instead of conveying information about a resource, it enhances the pollinator's behavior. Reinforcers should invoke reward circuitry in the brain and, therefore, be empirically tested on a behavioral and physiological level. Current reinforcers, such as nectar, could have been stimuli that were conditioned and evolved to become unconditioned stimuli. The "response" is the behavior itself. Most pollination studies focus on the pollinator's response because that is the part of the interaction that is more readily available to the researcher since behaviors can be observed, quantified, and manipulated through an experiment. The response is limited by the pollinators own biology – their anatomy, physiology, sensory system, and motor abilities, in addition to temporal constraints (for example, a bee cannot buzz a flower and brush it at the same time) and constraints imposed by the flower itself (corolla length, the distance between different floral parts, etc.). All these internal and external constraints can result in specific motor patterns of responses, which help define pollinator functional groups [as defined by Armbruster (2017)] and also can be used to make predictions in paradigms such as pollination syndromes (Krakos and Austin 2020). Finally, the "reward" equates to an unconditioned stimulus (Figure 1). The reward is the resource the pollinator collects, such as nectar, pollen, resin, leaves, oil, scents, etc.

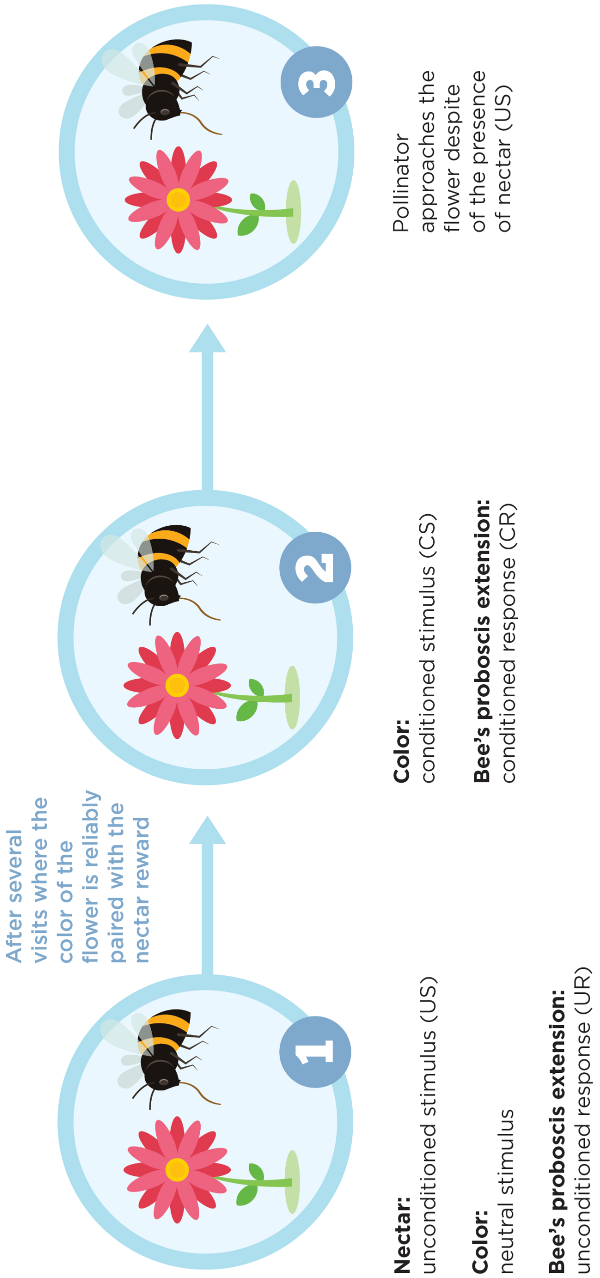

Figure 1 –Series of figures explaining unconditioned stimulus, unconditioned response, conditioned stimulus, and conditioned response in plant-pollinator interactions using a

bee as an example

Table 1 – Concepts from animal psychology applied to pollination systems

Concept	**Animal Psychology**	**Examples from pollination systems**
Cue	Feature of an animal or the environment that contains potential information about another animal and/or the environment	Plant traits, usually floral traits but also leaves, etc.
Signal	A cue that is used and modifies behavior, it is implied that it has evolved in a communication framework	Plant trait that sends a message, such as petal colors changing to indicate nectar availability.
Stimulus	Element from the environment that elicits a response. Can be natural (unconditioned stimulus) or learned through training (conditioned stimulus)	Plant traits, usually floral traits, such as petal color, flower symmetry, etc.
Reinforcer	Stimulus or reward that increases the frequency of the response. Primary reinforcers fulfill a biological need such as feeding; secondary reinforcers are associated with primary reinforcers.	Plant traits or plant resources. It is important to note that the behavior itself can also be reinforcing.
Response	Behavior that happens as a response to a stimulus. Can be a natural behavior (unconditioned response) or behavior learned through training (conditioned response)	Pollinator behavior itself, such as extending proboscis to drink nectar, brushing a petal, etc.
Reward	A resource that is accessed when the animal engages in the behavior (response).	Resource collected by a pollinator, such as nectar, pollen, oil, resin, etc.
Associative learning	Learning that is based on the pairing of a stimulus with a reward	Bees learning to associate a stimulus (flower color) with a reward (better nectar), thus increasing visitation to those flowers that have the right stimulus
Non-associative learning	Learning that is not based on associating a stimulus with a reward; through habituation or sensitization	Pollinators habituating to a fake predator and proceeding to foraging normally in the presence of such.

Though we have distinctly defined each important term, execution has not been as precise in the literature applying these terms to a natural pollination system. For example, "cues," "signals," and "stimulus" are often used interchangeably, despite the differences outlined in Table 1. Moreover, most work on pollinator learning has focused on testing different stimuli against known rewards, mainly sugar. There is a general lack of studies testing non-nutritious rewards on pollinators, which is surprising given the number of other potential rewards flowers can provide, such as scent or resin.

Additionally, the literature currently lacks a proper identification of key reinforcers in pollination. In theory, any floral trait could be acting as a reinforcer, and any reward could also play this double role. Another overlooked aspect of pollination is the role of nonfloral elements, such as leaves. It is unlikely that leaves act as unconditioned stimuli, but leaves may act as reinforcers or potentially as conditioned stimuli for pollinators, such as leaf-cutting bees.

Even well-studied pollination systems can lack these basic definitions. For example, let us look at the well-known example of scent collection by male orchid bees. It has been long assumed that scent is a reward for the male bees due to its role in mating (Vogel 1966; Milet-Pinheiro et al. 2021; Milet-Pinheiro and Gerlach 2017). However, there remains a lack of research testing the role of scent as a reward. Is scent itself a reward, or is the act of brushing the legs rewarding? How is scent being used by plants to guarantee more successful pollination and thus increase their reproductive success and fitness? How do we interpret the reward in this system if the source of the scent is not floral? (as described by Whitten, Young, and Stern (1993))

More research is needed to explore and define the stimulus, reinforcer, response, and reward in pollination systems and the role of non-sugar floral products in learning. From a pollination perspective, the interaction between stimulus, reinforcer, response, and reward is interesting, but it remains understudied. These questions are likely understudied because, typically, pollination biologists are usually focused on ecological aspects of the interaction rather than the cognitive mechanisms underlying the choices of the pollinators. In contrast, neurobiologists and animal learning psychologists are rarely focused on learning in the context of ecologically-relevant interactions. By merging these two fields, we can gain a deeper understanding of pollination.

Pollination is a stepwise process

Pollination can be interpreted as a stepwise process [adapted from Armbruster (2017)]. Armbruster's framework is an excellent way to consider which steps can be influenced through evolved preparedness of the pollinator. It is essential to clarify that we focus on true pollinators and not general flower visitors, which do not contribute to successful pollen transfer. From a plant perspective, there are eight key steps:

1. Pollinator attention and attraction (male flower)
2. Pollinator landing on and handling flower
3. Stamen contact and pollen deposition
4. Pollinator leaves flowers with pollen adequately attached to their body
5. Pollinator attention and attraction to a conspecific (female flower)
6. Pollinator landing on and handling flower
7. Pollen release on stigma
8. Flower departure

From an animal perspective, there are four key steps:

1. Pollinator attention and attraction to flower
2. Pollinator landing on and handling flower
3. Pollinator accesses a resource, usually nectar (which may or may not act as a reward)
4. Pollinator departs flower

These steps are cyclical, as depicted in Figure 2.

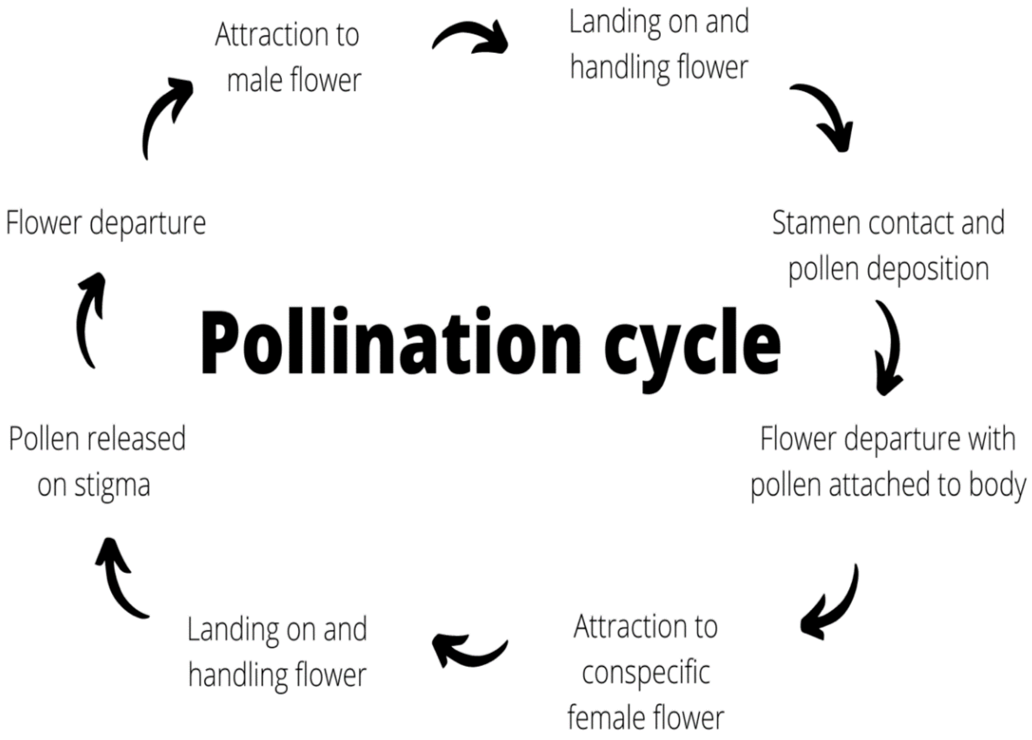

Figure 2 - Typical pollination cycle for plants and pollinators

For the pollinator, the pollination process, from attraction to pollen collection, is mediated by their sensory and cognitive systems and might be potential cases of the evolution of prepared learning. Interestingly, Armbruster defined the plant steps in the context of the pollinator, which suggests ways in which plants can modify the reliabilities and contingencies of cues for the rewards that they are offering (or not). For a plant, cues of attraction, flower anatomy and morphology, and pollen and nectar quantity can be manipulated, promoting the evolution of preparedness or contrapreparedness in pollinators.

Prepared learning as a framework for pollination studies

In addition to understanding specific behaviors, the theoretical framework of prepared learning also gives us a lexicon for describing and deepening our understanding of evolution's role in learning and how learning occurs in the natural world, filled with complex stimuli. For example, in a well-documented pollination system such as male orchid bee scent collection in the Neotropics, we can move beyond describing behavior and ask if scent itself is acting as a reinforcer and how the physical act of collecting scent might be triggering neural cascades of information processing in the bee's brain. By breaking pollination into a cycle of steps (Figure 2), one can ask more precise and informed questions based on better-informed hypotheses.

Pollination is a multimodal interaction where the same trait can be sending multiple signals due to its complex nature. In pollination, the stimulus is a complex combination of plant traits (mostly floral characteristics) such as petal color, petal texture, flower electrical potential, corolla size, shape, inflorescence architecture, flowering timing, etc. Those features may interact and can either hinder or enhance learning. Moreover, a floral trait may be multimodal. For example, petal color is a combination of hue, brightness, contrast, etc. A pollinator may respond to a combination of aspects of each trait or a combination of traits. There is also variation in learning due to temporal features of stimuli, and animals may learn an association of stimuli and response and the temporal relationship between conditioned and unconditioned stimuli.

Not all plant traits are cues, and not all cues are treated the same, and here is where preparedness can be acting on pollination. Flowers have cues to attract the pollinator, to guide them, and to convey information. Furthermore, pollinators can learn from each cue, have purely innate responses, or prepared learning can evolve in some but not all of these types of stimuli. We know that all flowers, or floral components, are not learned equally well, remembered equally as long, or extinguished with similar speed. There are constraints in a pollinator's sensory systems, which causes some cues to be perceived less frequently or not.

Additionally, there are constraints in the cognitive system, where perceived cues cannot be processed simultaneously. Pollinators might also not pay attention to cues in a given context even though they could perceive and process those same cues in a different context. A pollinator's natural history may help elucidate why an otherwise "perfectly fine" floral cue is not attractive to a species or group of pollinators, in which case a pollinator might be biologically prepared not to attend to some floral cues. We can apply the same logic to each of the steps listed by Armbruster in the previous section and use preparedness as a framework testing pollinator filters in natural systems, thus helping bridge ultimate and proximate explanations for pollination filters.

It is essential to keep in mind that although we are breaking pollination into steps, the process works as a cycle, and each step is affected by its previous step and affects its following step. For each step, we will explore the perspective of a naïve pollinator, who have never experienced that flower species, and the perspective of an experienced pollinator, who have interacted with that flower (or other flowers of the same species) before, since those previous interactions might play a role in their behavior. We are interested in what is happening cognitively and how preparedness plays a role in pollination for each step. In this breakdown, we present steps 2 and 3 as one single step. The predictions listed here are summarized on Table 2.

Table 2 – Predictions of prepared learning for plant-pollinator interactions based on the pollination steps of attraction and landing; handling the flower and accessing the resource; and departing the flower

Examples of predictions of prepared learning in pollination	
General	1. Prepared learning will evolve for floral cues that are reliably paired with rewards over ecological time in a given location.
Attraction	2. Preferences and biases for floral traits can evolve as a consequence of prepared learning for those flower traits 3. When presented with two or more floral traits, pollinators will pay more attention to, and be more motivated by, traits that coevolved in the context of prepared learning.
Handling	4. Prepared learning can prioritize the learning of persistent behavior for pollinators to access a resource

Departure	5. Pollinators will have enhanced memory for prepared learning

1. <u>Being attracted to a flower and landing on it</u>

The role of the type of resource being collected by the pollinator is an aspect of the pollination process that was highlighted by Armbruster (2017) as an overlooked topic. Not all pollinator foraging is equal. Depending on age, sex, role in a social group, or resource availability, a pollinator species might be foraging for different resources, which can significantly affect their cognitive processes and consequences for plant fitness. Thus, we propose that the pollinator motivation can act as a pollination filter. Therefore, the question emerges: how does motivation connect to the current state of the individual pollinator? When the same individual forages for different resources, that individual would be motivated by their current resource needs, which varies throughout life stages. It is common for pollinators to forage from different flowers in search of different resources. For example, male orchid bees are major pollinators in tropical forests (Roubik and Hanson 2004), collecting scents from flowers and mixing perfumes to attract females. However, males must also forage for nectar for food, and usually, nectar and scents are found in different flowers. So, a male searching for scent might not be motivated to visit a nectar flower and vice-versa.

If we assume that a pollinator is motivated and searching for a flower's resource (potentially), we can then explore their attraction to flowers. Armbruster (2017) discusses pollinators' attraction to flowers as the first step of pollination. He defines attraction as being mediated by floral advertisements, rewards in the form of the desired resource, and the timing of the interaction. Cognitively speaking, we can think of attraction to flowers as mediated by perception, attention, and bias. These three processes can act in conjunction, but it is important to make a distinction. For example, a pollinator might perceive a floral cue but not pay attention to it because of evolved preparedness towards a different cue (which would be equivalent to Armbruster's attraction filter).

Perception has to do with the ability to acquire information from the signal. Perception is mediated by the sensory systems and integrated into the brain of the animal. For example, for flowers pollinated by various animals (generalists), not all floral traits will signal the same information for every pollinator. Attention is the filtering of signals and focusing on one (or a few) of desired signals. Attention is an essential aspect of cognitive ecology, and failures in attention can often result in sensory overload and detrimental behavior.

Biases are tendencies toward or against a stimulus. Biases can be innate or acquired and can evolve through prepared learning. For example, preexisting innate biases will affect a naïve pollinator's attraction to a floral advertisement. What about an experienced pollinator? How can bias and learning interact in a prepared way? Although, to our knowledge, this has only been directly tested once (Maharaj et al. 2019), there are several cases in the pollinator social learning literature that can be explored as examples of prepared learning. For example, Dawson et al. (2013) and Jones, Ryan, and Chittka (2015) tested bumblebees on artificial floral arrays of two colors, and both studies found that experienced bees engaged in social learning. Dawson et al. (2013) showed that experienced bees trained on social cues changed their initial color bias to match the color presented with the conspecific. However, Jones, Ryan, and Chittka (2015) found that when naïve bees were presented with social cues on the alternative color, their initial bias did not change. Experienced bees did use social information when the social cue was placed on flowers that matched their initial bias. From these two studies and others, it is evident that bees learn and respond to social cues. However, this learning is mediated and influenced by initial bias and experience. Let us assume that bees learn a specific color cue better than an alternative color cue because they have been evolutionarily prepared to do so. In that case, we can see how social cues do not easily overcome prepared learning without prior experience and how the quality of that experience matters.

Another interesting, and slightly different example, is the cases of color biases that were not observed on an initial test but emerged when bees were tested under a social learning framework. Worden and Papaj (2005) were the first to describe social learning in bees, testing bumblebees on a two-colored floral array. Although the tested bees showed no initial color preference, the bees exhibited a preference for green over orange after being trained on a social information framework. The author's discussion is focused on the use of social information. However, we want to call attention to the emergence of a color preference for one color over another, despite bees trained on both colors receiving the same treatment. Once the bees were trained on learning, green emerged as a preferred color, despite being treated equally to the other colors before learning. We argue that this is an example of prepared learning for green, where the bees were quicker to learn and exhibited stronger responses when presented with green as a color cue in the context of social learning.

2. Handling the flower and accessing the resource

After attraction, the pollinator lands and proceeds to handle the flower, searching for the resource of interest. The handling of the flower and accessing of the resource are what Armbruster (2017) named the flower-visitor fit filter: pollinators are only effective if they can physically access the reward, make contact with the stamen for long enough to allow successful pollen deposition, and in a subsequent visit make contact with a conspecific stigma long enough for successful pollen deposit. In some cases, resources are easily accessible, requiring minimum handling from the pollinator. However, the resource of interest is concealed and not easily accessible in other cases, requiring complex handling. The complexity of handling skills, and the innovation and persistence that may be required to learn these skills, is a focus of recent work.

Many aspects of a flower can increase complexity, but Krishna and Keasar (2019) focus on flower morphology. In their experiment, bumblebees were tested on a gradient of morphological complexity that correlates with resource accessibility (nectar). The authors found that on their first choice, naïve bees landed on, and attempted to handle, complex flowers more often than expected by random choice and more often than experienced bees. When comparing the effects of experience on handling more complex flowers, and floral preference, their results confirmed that experience with handling flowers matters for success in accessing the resource (Krishna and Keasar 2019). This ability to handle complex and novel flowers is the basis for experiments on bees performing complex handling behavior in nonfloral contexts. For example, Loukola et al. (2017) presented bumblebees with a novel situation where they were required to move a small ball to a defined location in the arena in order to access the resource (nectar). There is no equivalent in nature to "bees playing soccer." However, since these behaviors are part of a bee's foraging repertoire, the bees could access the reward in that context the same way they would handle a novel flower searching for its nectar.

Bees in the previously described experiment learned to perform a task in a novel context in order to receive a reward that they were evolutionarily adapted to respond to. The authors of that paper discuss how a pollinator's cognitive flexibility is important in handling flowers and beyond, as demonstrated in their experiment under a novel context. One question that emerges is: what are the limits for what a bee would do for a nectar reward? We have seen bees move balls (Loukola et al. 2017) and pull on strings (Alem et al. 2016) to access a resource. But what behaviors do we observe on pollinator foraging (rubbing, scraping, tapping, pushing, etc.) that we can test on novel scenarios to shed light on how generalized these behaviors are? Preparedness can provide a framework for research on bee handling behavior.

The handling of a flower has best studied, perhaps, in the context of persistence behavior. Some pollinators must endure extreme situations to access a reward. That is the case of several Stanhopeinae orchids and male orchid bees (Euglossini). Adachi, Machado, and Guimarães (2015) describe in detail the pollination system on *Gongora bufonia*, a species of Stanhopeinae orchid. This pollination system is common across other orchids. Male Euglossini bees use their middle and back legs to hold on to the orchid's petals and use their front legs to brush droplets of scent (the resource they are searching for) from the flower's floral lip. While collecting scent, the bee "slips and falls" into the column of the flower multiple times. As the bee crawls back up to continue collecting the floral scent, it passes through the stigma and anther of the flower. Because of the position of the petals, the bee's wings are spread out, which facilitates the deposition of the pollinaria on the bee's scutellum; if a bee already had a pollinarium attached to its back, it can be deposited on the stigma as the bee moves up the flower and continues collecting scent. This interaction is called "slip-and-fall" pollination (Dressler 1982, 1968; Adachi, Machado, and Guimarães 2015).

In the example above, we can see that the pollinator engages in physical contact with the resource (fragrance) and spends time handling the resource (collecting the scent). At the same time, the flower morphology allows the pollen to be attached and secured on the pollinator's body. The pollinator must repeatedly engage in handling the flower, despite being interrupted multiple times. The pollinator then visits another flower of the same species where it engages in the same behavior to deposit the pollen on the stigma. This continuing cycle of handling the flower, collecting the reward, and being interrupted, can be interpreted as a cycle of persistent behavior. In the context of the bees and orchids, we find an initial reward that is interrupted by a delayed reward, where the bee must persist on the collection behavior until it is satiated on the resource. This persistent behavior is also formally defined as the tendency for animals to continue engaging in a behavior despite frustrations such as lack of rewards, punishments, or obstacles (Amsel 1994). Persistent behavior develops when there is a high chance of a reward being present at that moment and absent after a response (Amsel 1994). Persistent behavior gives us a framework for understanding why a pollinator would go to such lengths to access a reward they need. The initial reward indicates a likelihood of a continued reward after the interruption is overcome. In this context, prepared learning can be acting in prioritizing the learning of persistent behavior to access a vital resource for the male orchid bee – access to scents that are key for mating success and increased fitness. When a seemingly extreme behavior is placed in the context of prepared learning theory, it is possible to examine it and proposed and test hypotheses and predictions on how this came to evolve and why this behavior is still present today.

3. Departing the flower

Departing the flower consists of when the pollinator stops collecting the resource up to when it physically leaves the flower. When a pollinator decides to leave a flower is a critical decision that is usually assumed to be made based on depletion of a resource or satiation. However, pollinators can also decide to depart a flower before satiation due to perceived or realized danger, competition, or frustration. In addition, foraging bees collecting resources for their colonies might return to the colony without being full when the value of the information they carry exceeds the value of the resource (pollen or nectar) they are collecting (Dechaume-Moncharmont et al. 2005). Unfortunately, flower departure is one of the least studied steps of the pollination process.

Although the decision to leave a flower, and the act of leaving itself, can take only a few seconds, this step can be crucial. From the plant's perspective, a successful departure can increase the chances of successful pollination, giving it time for pollen to be properly attached to the animal's body (Maad and Nilsson 2004). For the pollinator, one way flower departure can affect cognition is in the context of memory formation and interruption of interference. Interference happens when disruption is introduced during learning, which might impact memory formation and learning itself (Mendl, Laughlin, and Hitchcock 1997). As associations are consolidated in a pollinator's memory, the flower might benefit from having a pollinator lingering instead of moving on quickly and risking interference by other factors before memory is consolidated.

Learning theory predicts that more important associations will be remembered for a more extended period of time (McNamara and Houston 1987; Dunlap and Stephens 2009). More specifically, prepared learning predicts enhanced memory, with associations being remembered longer and forgotten less (Dunlap & Dexheimer, *in press*). Based on this theory, we predict that departure time is one way flowers can "manipulate" pollinators by increasing the departure time to allow for memory formation. Going back to the orchid bee example described above, male orchid bees hover over the *Gongora* flowers for 2-5 seconds while transferring the scent collected with the front legs to their back legs (Adachi, Machado, and Guimarães 2015), which might be necessary for memory consolidation time. In the same example, we can also consider the time a male spends trying to climb the column back to the top of the flower before the last collection bout as an example of how departure time can be used to secure the pollen on the body of the pollinator. It is important to note that memory consolidation can happen *en route* to the next flower in many cases.

Another way the pollinator's departure might play a role in prepared learning is tying it back to motivation, which we explored on the first step of the cycle. It is in the plant's best interest that the pollinator stays long enough to allow for pollen attachment from the stamen or pollen deposition on the stigma. One way flowers can take advantage of that is by offering limited resources (quantity and/or quality) to ensure an individual will depart with pollen adequately attached to their bodies and go and search for other (conspecific) flowers. For example, plants can distribute rewards in multiple inflorescences (Harder and Cruzan 1990) to promote several visits. Flowers can also ensure that the reward (pollen) is delivered directly to the bee through sonication

(Cardinal, Buchmann, and Russell 2018). These and other strategies can keep pollinators motivated to visit flowers long enough and promote successful pollen transfer.

Conclusion

Pollination is often a multimodal interaction. Animal pollinators are exposed to visual, chemical, taste, and other sensory inputs. Prepared learning can be a useful evolutionary strategy for an animal because they are not taking in all the cues and hindering learning. From the pollinator's perspective, plant traits can be prepared stimuli, and a pollinator's behavior can be a prepared response. From the perspective of the plants, there should be stronger selection for floral cues that their pollinators are prepared to learn from or contraprepared to learn from to reduce visits from unwanted floral visitors. It would be interesting to investigate if prepared learning plays a role in pollinator shifts, for example, where a plant would face stronger selection to fine-tune learning from one pollinator to the detriment of another.

The question that motivated this review was *why animals learn some cues better than others?* By studying prepared learning in plant-pollinator interactions, we gain a better understanding of learning in an ecologically and evolutionarily relevant context. The framework proposed in this review provides us with the information needed to predict patterns of prepared learning in pollinators. For example, we can use the pollination steps (attraction, handling, departure) to draw predictions of prepared learning on pollinator-plant interactions (Table 2). Furthermore, those predictions can be tested directly on experiments of pollinator learning in the wild. By testing predictions for prepared learning in plant-pollinator systems, we can move from *post hoc* explanations to the novel investigation of the age-old question of why pollinators learn some flower cues better than others.

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Chapter II: Spectral sensitivity of the orchid bee *Euglossa dilemma* - Color is in the eye of the beeholder

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Abstract

Diurnal pollinators often rely on color cues to make decisions when visiting flowers. Orchid bees are major tropical pollinators, with most studies focusing on scent collection and chemical ecology. The objective of this study was to measure spectral sensitivity and characterize color vision on the orchid bee *Euglossa dilemma* and compare it to the known spectral sensitivity of other closely related bees. We compared *E. dilemma's* spectral sensitivity and opsin protein structure to four closely related corbiculate bees. *E. dilemma* is a trichromat, with peaks on Green, Blue, and Ultraviolet in similar regions to other measured bees. Ultraviolet photoreceptors seem to be the most conserved among the compared bees, while blue photoreceptors and opsin proteins were the least conserved. We have also developed a color hexagon for orchid bees based on measurements of color spectral sensitivity for *E. dilemma*, which can be used in future works on color vision behavior in orchid bees. We found no differences between male and female *E. dilemma* visual systems despite neuroanatomy and behavioral differences reported in the literature. In this study, we lay the foundation for color vision studies in orchid bees.

Keywords: *visual system, pollination, corbiculate bees, opsin, sensory ecology*

Introduction

Color vision is an essential aspect of most pollinators' lives. For several groups of diurnal pollinators (bees, butterflies, hummingbirds, etc.), color has proved to be a critical floral signal, conveying information about nectar quantity (Melendez-Ackerman, Campbell, and Waser 1997) and affecting pollinator decision making (flower choice) (Chittka and Menzel 1992). However, color itself is not a physical property of a flower but a combination of the light reflected from a surface that is being perceived by an animal's sensory system. In other words, color is a cognitive construct. Therefore, color can be understood as a cognitive construct (Skorupski and Chittka 2009), and it can be studied as a perception trait (Garcia et al. 2020).

Perception is the first step in investigating visual ecology because it answers the question: what can this animal see? If flower color signals are conveying information, pollinators need to see that color for the signal to be used for making decisions. The cells on the eyes of an animal have neural terminations that transmit the signal perceived by the eyes through the nervous system, and this information is then processed in the brain. Once a signal reaches the brain, it can initiate the process of decision-making in response to that signal. Thus, color perception is studied by investigating the physiology and anatomy of pollinators by measuring the spectral sensitivity of an organism's eyes.

Bees have long been studied for their color vision, and flower color is important for foraging and decision-making for multiple species of bees (Menzel et al. 1988; Chittka 1992; Dyer and Spaethe 2008). There are thousands of species of bees currently described. However, the field of sensory ecology in bees is vastly dominated by studies on three species, namely the European honeybee (*Apis mellifera*), the buff-tailed bumblebee in Europe (*Bombus terrestris*), and the common eastern bumblebee in North America (*Bombus impatiens*). Other bee species were tested for spectral sensitivity (Peitsch et al. 1992; Van Der Kooi et al. 2021), but there is a significant emphasis on the three above-cited species. Bees have retinal cells that express three types of photopigments with maximum sensitivities around the color spectrum's Ultraviolet, Green, and Blue regions. While all species of bees share the presence of the three types of photopigments (Peitsch et al. 1992; Van Der Kooi et al. 2021), the wavelength where each bee's photopigments peaks might differ.

Although all bees have a similar pattern of spectral sensitivity, namely all bees are trichromats with peaks on Green, Blue, and Ultraviolet (Peitsch et al. 1992; Van Der Kooi et al. 2021), different species, and even related species have a different color perception, which is shown by differences in their spectral sensitivity. There is a high similarity between *B. impatiens* and *B. terrestris* in spectral sensitivity, with the Blue peak shifted towards short wavelengths by 12-13 nm *in B. impatien*s. Menzel et al. (1988) measured spectral sensitivity in *Osmia rufa*, a European solitary bee with the same three peaks on Green, Blue, and Ultraviolet. However, their green photoreceptors are shifted to longer wavelengths when compared to other species (572 nm in *O. rufa*, compared to 532 nm in *A. mellifera*). The social stingless bee *Melipona quadrifasciata* has an ultraviolet peak shifted to longer wavelengths while their blue receptor is shifted to shorter wavelengths when compared to honeybees.

Hypotheses on how evolutionary history and ecology shaped bee vision were tested by Peitsch et al. (1992) on comparing 26 species of bees and 17 other flying

Hymenoptera. Body size and proboscis length (two parameters that affect which flowers a bee will visit) do not seem to correlate with differences in spectral sensitivity. The same is true for polylectic (forage from several plants) and oligolectic (forage from few, usually related plant) bees – oligolectic bees seem to have a narrower distribution of spectral receptor types but are still within the distribution for polylectic bees. However, there was a difference between bees typically found in tropical forests (therefore exposed to dense vegetation and low-light intensity) versus bees that evolved in open space areas (typically exposed to higher light intensity) regarding their UV-receptors. It is important to note that dense vegetation also involves a shift in environmental light towards green. Bees from tropical forests have a peak on longer wavelengths than bees the fly on open areas, which have their peak shifted towards shorter wavelengths. There was no observable difference on the Green or Blue receptors. These differences suggest that a bee's evolutionary history plays a role in its current spectral sensitivity, opening the possibility for researchers to make predictions about a species spectral sensitivity based on its historical habitat distribution. Peitsch et al. (1992) focused exclusively on spectral sensitivity, and since their results were published, there has not been a comparable follow-up of studies testing these species for color discrimination.

A recent review on insect color vision (Van Der Kooi et al., 2021) offers an updated list of bees with spectral sensitivities measured. To date, only one species of bee has been found to have more than three types of photoreceptors: *Callonychium petuniae*, with the fourth photoreceptor at 593 nm (~yellow). There is still a gap in measuring male bees, with only one species of bee reporting data for males (*Apis mellifera*), despite an increase in the number of species measured. Apidae is the most sampled family of bees and the second most variable on the range of peak sensitivities, behind Megachilidae. Out of the 35 species of bees with known spectral sensitivity, only four species have been tested on behavioral trials for foraging, navigation, or nest recognition.

One aspect of color vision that is often overlooked in bee visual ecology is how male and female bees of the same species perceive color. Very few studies take into account male bees. For example, in Peitsch et al. (1992)'s massive work to measure spectral sensitivity in 43 Hymenoptera species, in only four species, males were tested, and out of these four, just one bee (*A. mellifera*). For decades, male bees were not considered relevant on color experiments or other behavioral assays (Lichtenstein, Sommerlandt, and Spaethe 2015). However, recent studies that tested male bumblebees on color learning showed that male bees perform as well as female worker bees in the laboratory (Lichtenstein, Sommerlandt, and Spaethe 2015; Wolf and Chittka 2016) and field conditions (Muth et al. 2021). There is still much research needed comparing male and female bee color vision.

Orchid bees are major pollinators in tropical forests with singular differences in foraging behaviors between males and females. Despite their immense value as pollinators, their visual system has not been investigated until now. Orchid bees are also an ideal group to test for ecological and sex differences in color perception. A recent study of a species of orchid bee brain (Brand et al. 2018) suggests sexual dimorphism in brain anatomy related to color vision. From these results, it is reasonable to predict that these differences in the brain affect color perception in male and female orchid bees. Although these brain differences could be related to several aspects of vision other than color perception because of the similar foraging demands for nectar from flowers for males and females, it is important to test if the observed brain morphology differences correlate with color vision and color use in orchid bees.

Color perception plays a role in pollination biology. By considering the pollinator's sensory ecology, one can better understand how pollination systems evolved and how pollinators sense and interact with their environment. The objective of this study was to measure spectral sensitivity and characterize color vision on the orchid bee *Euglossa dilemma* and compare it to the known spectral sensitivity of other closely related bees. Because of the neuroanatomy and behavioral differences, we hypothesized that there would be differences between male and female orchid bee visual systems. We have also developed a color hexagon for orchid bees based on measurements of color spectral sensitivity for *E. dilemma*, which can be used in future works on color vision behavior in orchid bees. Finally, we compared *Euglossa dilemma*'s spectral sensitivity and opsin protein structure to four closely related bees. We hypothesized that there would be low variation between the tested bees due to shared evolutionary history, and we predicted that the two orchid bee opsins would be the most similar.

Methods

Bee collection: Bees were collected from the non-profit botanical garden Flamingo Gardens in Davie, Florida. We used scent baits to attract males (Roubik and Hanson 2004; Eltz et al. 2011; Dressler 1982) and collected them from the scent baits using an insect net. We captured females when leaving or returning to nest boxes that have been placed in the park since 2018.

Bee shipping: We placed individual bees in 20mL scintillation vials containing a cotton ball soaked in honey-water (50% honey solution) and another cotton ball closing the vial. The use of cotton allows for airflow inside the vial. Each vial was wrapped in a paper towel and placed in a shipping cardboard box filled with Styrofoam padding to keep the vials from moving inside the box and ice packs to keep the box's interior cool and avoid overheating. Bees were shipped overnight and housed at the Morehouse Lab at the University of Cincinnati. Bees were kept in a 16.5x30x48-in plastic and mesh cage in a temperature and humidity-controlled room at a 12:12 light cycle to mimic their natural conditions. Bees were fed 20% sugar water ad libitum through feeders mounted to the side of the cage.

Spectral sensitivity measurements: We measured the spectral sensitivity of five males and five female bees. We measured bees for spectral sensitivity using microspectrophotometry (MSP). In MSP, we measured the light absorption by retinal photoreceptors in cryo-sectioned retinal tissue (Zurek et al., 2015). Before cryosectioning, bees were dark-adapted by placing them in a dark chamber overnight. Specimen preparation, cryosectioning, and MSP measurements were done under dim red light to avoid retinal tissue bleaching. We removed the head of the bee from the body using a sharp blade, and the head was embedded and flash-frozen in Tissue Plus OCT Compound (Fisher Healthcare, Houston, Texas). We cryosectioned the embedded heads in a Leica CM1860 cryostat at -20°C. Sections were 13 µm thick. Only the sections that contained retinal tissue were inspected in the MSP. Prior to measuring in the MSP, we placed the sections between two glass coverslips (22x22-1 Fisherfinest, Fisher Scientific, Pittsburgh, Pennsylvania) and immersed them in mineral oil (Fisher Scientific, Fair Lawn, New Jersey) surrounded by a ring of silicone grease (Dow Corning Corporation, Midland, Michigan).

We measured the absorbance of the retinal cells between 300 and 700 nm using a custom-built single beam, scanning MSP with a 32X Ultrafluar objective and a 32X Ultrafluar condenser (Carl Zeiss, Germany). The light source used was a xenon arc lamp, and using a monochromator, the light from 300 to 700 nm in steps of 1 nm. First, we measured a reference scan in an area with mineral oil but no tissue. Second, the measurements of the retinal tissue followed this procedure: the focal cells were measured, then photobleached for 60 sec using white light, and then re-measured. To confirm the presence of photopigments in the retinal tissue, we inspected the difference between the pre-bleach spectrum and the photobleached spectrum. We used the pre-bleach spectra of cells confirmed to have photopigments to model photopigment sensitivity using a visual pigment template (Govardovskii et al., 2000). We also measured the lens transmission of the crystalline cones from cryosectioned retinal tissue using a CRAIC microspectrophotometer. We estimated the sensitivities of the individual photoreceptors from the measured photopigments, and we modeled the visual system by incorporating the photopigment sensitivities with the lens transmission. Based on the orchid bee color vision model, we plotted the color measurements from known flowers (or other targets) on a color hexagon (Chittka 1992), which depicts the color space of an animal.

Data analysis: We used the R package “Pavo” (Perceptual Analysis, Visualization, and Organization of Spectral Colour Data) (Maia et al. 2019) to estimate the sensitivities of the photoreceptors and create a visual system for *Euglossa dilemma*. More specifically, we used the function “sensmodel” to estimate the sensitivity curves, the function “vismodel” to create the color vision model, and the function “colspace” to design the orchid bee color hexagon based on the parameters of the visual model.

Gene and protein comparison: To confirm that our measured photoreceptors corresponded to the three expected opsins, we used the published genome of *E. dilemma* (Brand et al. 2017) and conducted a tBLAST search on GenBank to confirm that each photoreceptor corresponded to an opsin in their genome. We then conducted a tBLASTn search on GenBank to compare *E. dilemma*’s opsins with the opsins of four closely related bee species. We focused on three parameters, namely Query cover, E-Value, and Identity. Query cover is the percentage of the query length that is included in the aligned segments; E-value, or expected value, is the number of alignments expected by change, which should be a value close to 0 for good alignments; Identity is the highest percentage identity for a set of aligned segments to the same subject sequence. We used the parameter Identity to measure the similarity between the opsin proteins.

Results

Male x Female comparison

We collected 14 pre-bleach spectra from the 10 measured bees, which fell into three categories: four measurements for short, four for medium, and six for long wavelengths. There were no differences between male and female photoreceptor measurements, so we combined the data for the following analysis. We also took 11 unique measurements of lens transmission on two male bees, and those measurements were averaged and included in our color vision model.

Building the color vision model

We built a color vision model by using the pre-bleach spectra to estimate the peaks of maximum sensitivity of the pigments (lambda max) using the "visual template" function in "Pavo" (Maia et al. 2019). The 14 raw photoreceptor absorbance measurements were spread into three categories, which fall on the light spectrum's ultraviolet, blue, and green regions. The three peaks of the graph correspond to 347 nm (ultraviolet), 429 nm (blue), and 537 nm (green). We then processed the lambda max values and used the lambda max values and the lens transmission spectrum to build the orchid bee color vision model.

It is important to note that the Blue photoreceptor measurements were the most variable even though all three photoreceptors showed high variation. Blue photoreceptors were the most measured, but upon a closer look, we identified several Blue reads that also had peaks on for UV that were most noticeable when comparing the pre-bleached and post-bleached curves. These Blue-UV curves were identified as UV metarhodopsin measurements, as described by Cronin et al. (2000). Those measurements were removed from our analysis.

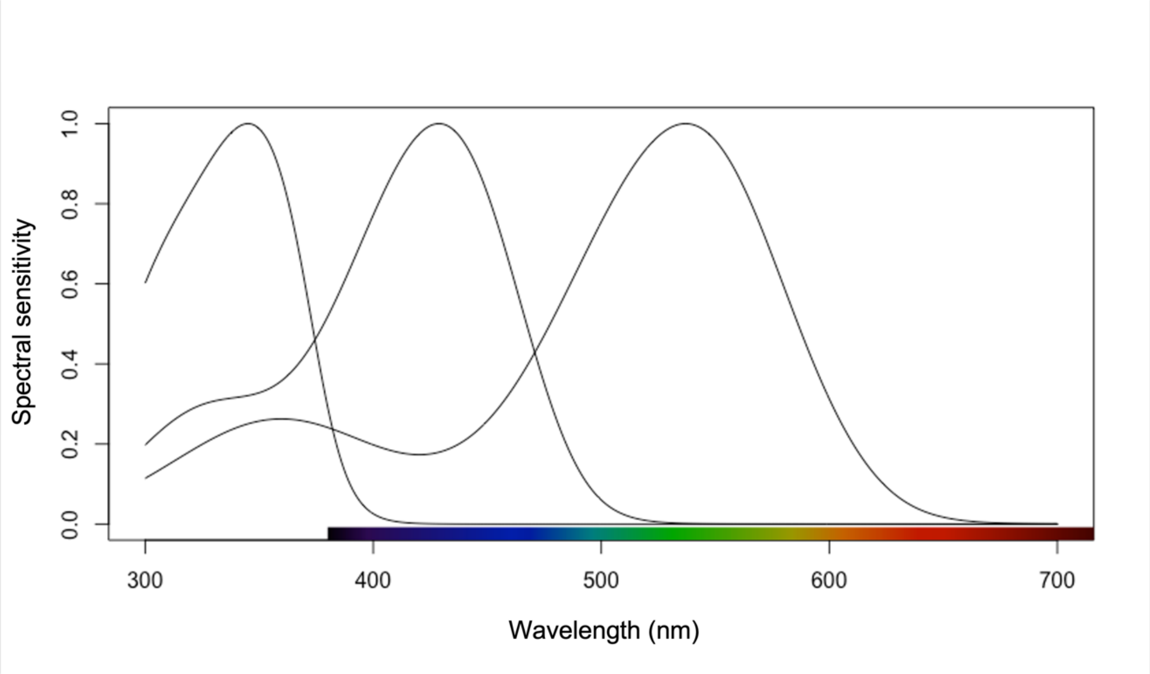

Figure 1 – Spectral sensitivity curves of the orchid bee *Euglossa dilemma*.

Orchid Bee color hexagon

Using the orchid bee color visual model, we created a color hexagon (Chittka 1992), which depicts the color space an animal can perceive. In Figure 2, we plotted several target colors from the "flower" database on the R package "Pavo" on the orchid bee color hexagon.

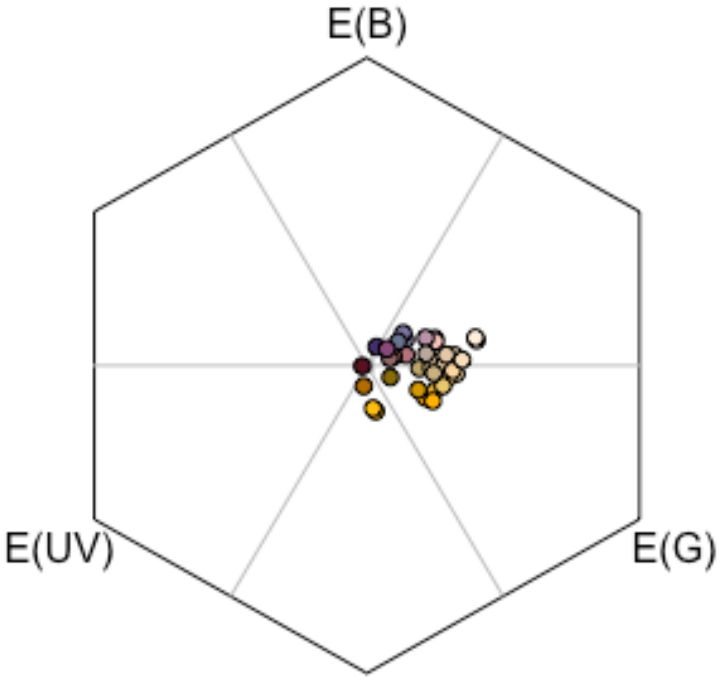

Figure 2 – Color hexagon depicting several color points on the color space of *Euglossa dilemma*.

Gene and protein comparison

Using the annotated *E. dilemma* genome (Brand et al. 2017) we identified the four opsin proteins and listed them alongside the described honeybee opsin genes (Table 1).

Table 1: Opsin genes in honeybees (*Apis mellifera*) and orchid bees (*Euglossa dilemma*). Gene names and annotation based on the Hymenoptera Genome Database (Elsik et al.

2016)

Opsin gene	**Expression**	**Honeybee gene**	**Orchid bee gene**
Blue sensitive opsin (Blop)	Compound eye and brain	GB41643	Edil_04295
UV sensitive opsin (Uvop)	Compound eye and brain	GB51369	Edil_09953
Long-wavelength sensitive opsin 1 (Lop1)	Compound eye and brain	GB50196	Edil_07671
Long-wavelength sensitive opsin 2 (Lop2)	Ocelli and brain	GB50034	Edil_07551

We compared the amino acid sequences, and we summarized the differences in Tables 2 – 5. The amino acid sequence was only available in a partial format for some of the closely related species, denoted in the tables below. This opsin does not affect color vision because Lop2 is found only in the ocelli and not principal eyes. Therefore, we removed it from further analysis. We also removed the partial elements. Then, we compared the three opsins across corbiculate bees (Figure 3).

Table 2 - Summary for Ultra-violet sensitive opsin:

Bee species	Query Cover	E-value	Identity
Honeybee (*Apis mellifera*)	90%	9e-76	96.90%

Bumblebee (*Bombus impatiens)*	99%	2e-75	96.12%
Orchid bee (*Eufriesea Mexicana)*	100%	1e-78	99.2%
Stingless bee (*Melipona quadrifasciata*)	100%	5e-74	93.80%

Table 3 - Summary for Blue sensitive opsin:

Bee species	Query Cover	E-value	Identity
Honeybee (*Apis mellifera*)	94%	9e-39	50.59%
Bumblebee (*Bombus impatiens)*	90%	5e-44	55.56%
Orchid bee (*Eufriesea Mexicana)*	100%	5e-77	40.29%
Stingless bee (*Melipona quadrifasciata*) **partial**	30%	1e-30	80.77%

Table 4 - Summary for Lop1 Long-wavelength sensitive opsin:

Bee species	Query Cover	E-value	Identity
Honeybee (*Apis mellifera*)	100%	1e-163	72.04%
Bumblebee (*Bombus impatiens)* **partial 1**	100%	1e-135	74.91
Bumblebee (*Bombus impatiens)* **partial 2**	100%	7e-88	57.47%
Orchid bee (*Eufriesea Mexicana)*	NA	NA	NA
Stingless bee (*Melipona quadrifasciata*)	100%	9e-47	78.50%

Table 5 - Summary for Lop2 Long-wavelength sensitive opsin:

Bee species	Query	E-value	Identity

	Cover		
Honeybee (*Apis mellifera*)	99%	2e-114	59.50%
Bumblebee (*Bombus impatiens)*	NA	NA	NA
Orchid bee (*Eufriesea Mexicana)*	NA	NA	NA
Stingless bee (*Melipona quadrifasciata*)	NA	NA	NA

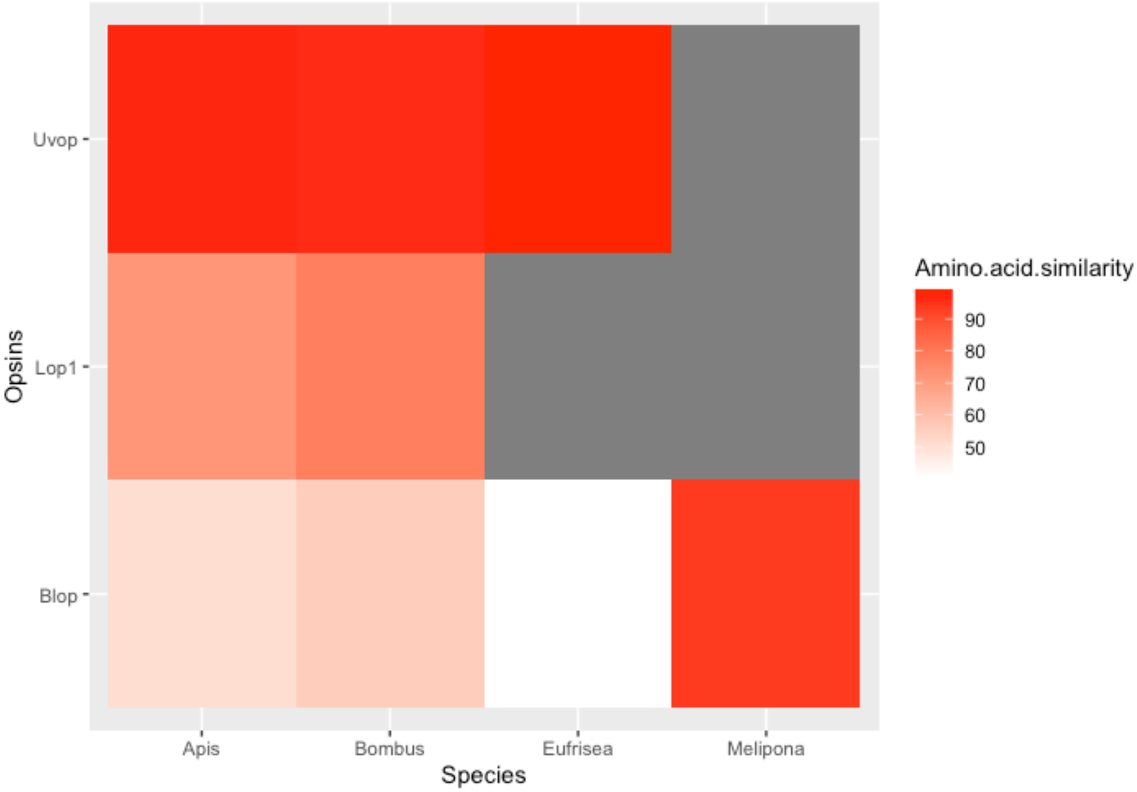

Figure 3 – Heatmap with opsin protein amino acid comparison between *Euglossa dilemma* (orchid bee) and the corbiculate bees *Apis mellifera* (honeybee), *Bombus impatiens* (bumblebee), *Melipona quadrifasciata* (stingless bee), and *Eufrisea mexicana* (orchid bee). Gray areas in the graph represent pairs of opsins that were not compared due to a lack of data.

Discussion

This study presented the first measurements of spectral sensitivity in orchid bees. We found evidence for three photoreceptors (Ultraviolet, Blue, and Green), which is typical for Hymenoptera. Our reported peak sensitivities are also within the range for other corbiculate bees. Our spectral sensitivity measurements show no differences between male and female orchid bees, despite brain anatomy differences (Brand et al. 2018) and distinct foraging behaviors (Dressler 1968; Roubik and Hanson 2004; Armbruster and Webster 1979). This is interesting and sheds a light on how brain anatomy and behavior might not be mediated by physiological differences on the photoreceptor level.

The spectral sensitivity measurements were clearly defined in three categories, matching the expectation that orchid bees are trichromat for UV, Blue, and Green. However, the measurements were highly variable, which added challenges for cleaning and processing data. We also had a small sample size for the lens transmission measurements, which were taken from male bees only. Future studies should focus on sampling a larger number of bees to investigate the extent of individual variability. Moreover, it would be interesting to compare the Florida population with other populations of *E. dilemma* in Mexico and Central America, although we do not expect differences in the peaks we reported here.

Our comparison of the orchid bee *E. dilemma* visual system with other known closely related corbiculate bees did not show many differences. Corbiculate bees are a monophyletic group comprising some of the better-studied bee species: honey bees, bumble bees, stingless bees, and orchid bees (Michener 2007; Bossert et al. 2019) (Figure

4a). Figure 4b shows a comparison of spectral sensitivity of four representatives of the corbiculate bee clade; in Table 6, we can see that our measurements for orchid bees are similar to its closely related species. It is important to notice that the peak for the UV photoreceptors seems to be the most conserved among corbiculate bees. Although this seems to agree with the conclusions of Peitsch et al. (1992) since the bees from tropical systems (orchid bees and stingless bees) have longer UV peaks, the differences we found are not likely biologically relevant (1 nm for *Euglossa dilemma* and 3 nm for *Melipona quadrifasciata*). It is also important to notice that Peitsch et al. (1992) found most differences among species for the UV photoreceptors and not the Blue or Green photoreceptors, and Table 6 shows the opposite trend.

Opsin genes are generally conserved among Hymenoptera (Van Der Kooi et al. 2021), and our results seem to confirm that: UV sensitive opsins (Uvop) and Long Wavelength sensitive opsin 1 are highly conserved in corbiculate bees. However, the Blue sensitive opsin (Blop) has considerable variability between corbiculate bees and within orchid bees, with the variation within orchid bees being higher than the variation between *Euglossa dilemma* and the other corbiculate bees (Figure 3). The opsin protein comparison, combined with the spectral sensitivity comparison, seem to indicate that the majority of diversity in visual systems in corbiculate bee is in the Blue sensitive opsin and photoreceptors. More studies would benefit from investigating whether these genetic and physiological differences are translated into functional and behavioral differences between corbiculate bees.

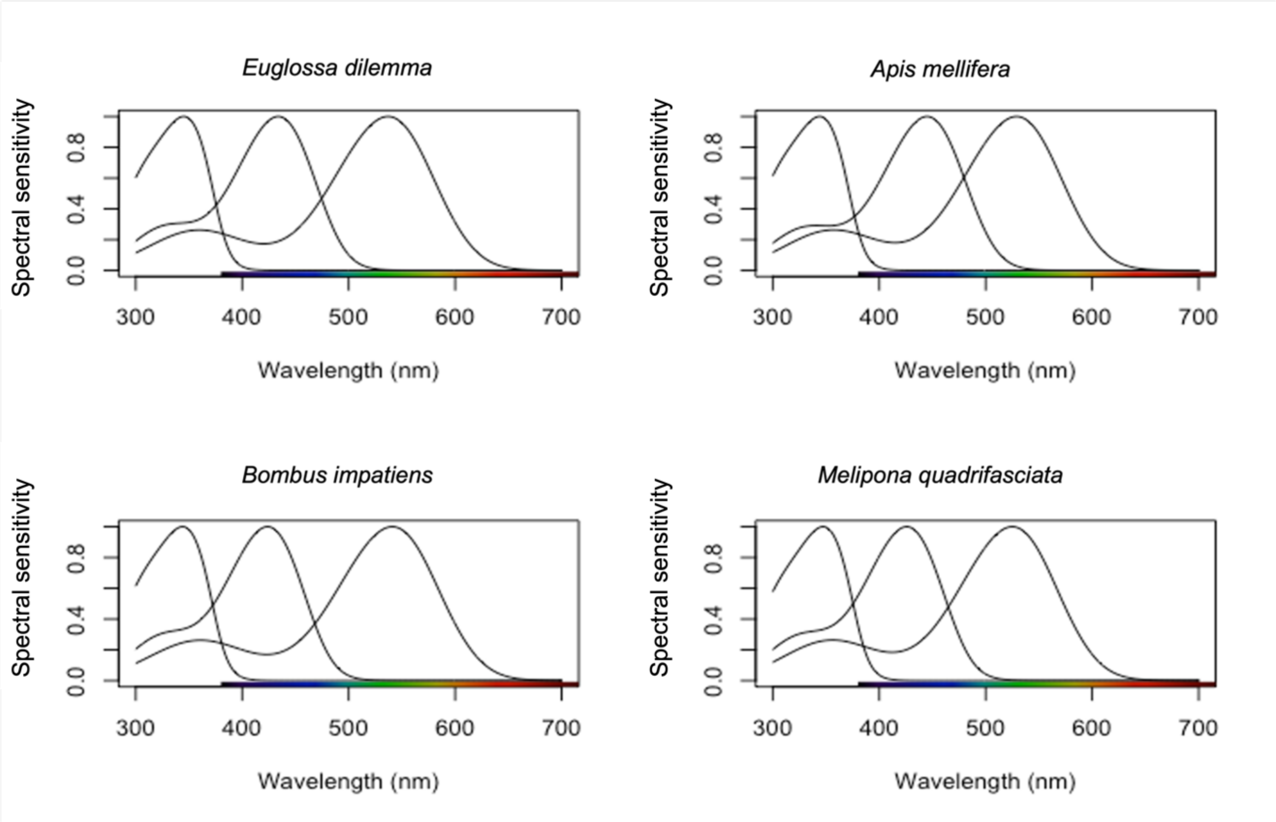

Figure 4 – Plotted spectral sensitivity of corbiculate bees (data for *Apis mellifera, Bombus impatiens,* and *Melipona quadrifasciata* was extracted from Van Der Kooi et al. 2021).

Table 6 – Peak sensitivity for the UV, Blue, and Green photoreceptors of four representative species of corbiculate bees

Species name	UV sensitive peak	Blue sensitive peak	Green sensitive peak
Bombus impatiens	346	424	541
Melipona quadrifasciata	349	426	525
Apis mellifera (female)	346	445	529
Apis mellifera (male)	346	445	540
Euglossa dilemma	347	429	537

Orchid bees provide significant ecosystem services, comprising about one-quarter of the diversity of pollinators in tropical forests (Roubik and Hanson 2004). By better understanding *E. dilemma*'s visual system, we can add to the growing literature on pollinator sensory ecology. This study also expands the possibilities for orchid bee studies. Most studies on orchid bee pollination and behavior are focused on scent collection and olfaction. Our results lay the foundation for the field to branch from olfactory studies only and expand into color vision studies. Pollination is a multimodal sensory experience and adding color vision to our knowledge will help us understand orchid bee foraging and pollination in a more holistic way.

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Chapter III: Color choice and preference in the tropical orchid bee *Euglossa dilemma*

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Abstract

Pollinators assess their environment and make decisions on which flowers to visit. Several factors can affect flower choices, such as floral availability throughout a season, time of day, and temperature. Biological factors particular to a pollinator, such as pollinator's sex, their previous experiences during a foraging trip, and learning, can also play a role in decision-making mediated by color. Orchid bee pollination is intimately linked to foraging behavior. This paper presents the first study of orchid bee color vision use in *Euglossa dilemma* and we focused on two aspects of color vision: color choice and preference. Blue and yellow emerged as easiest pair of colors to test, with 26.3% of males and 32.3% of females tested on this color responded to the protocol. Our results show that humidity and time of day played a role in color choice. Individual male and female orchid bees show variability in their color preferences in our tested colors. However, we found no constant preference for these bees overall. We also find that the preferences of bees are not significantly affected by the abiotic or biotic factors measured. Further, a bee's preference was not predicted by its initial color choice. Decision-making and preference are complex aspects of pollinator behavior, with fitness consequences for the pollinator and the plants they visit. By testing orchid bees in the field with non-invasive tests, we can better understand how tropical pollinators interact with their environment and make decisions based on their color vision.

Keywords: *behavior, pollination, tropical ecology, color vision, decision making.*

Introduction

Pollinators assess the environment in which they are located and make decisions on which flowers to visit. Several factors can affect flower choices, such as floral availability throughout a season (Hegland and Totland 2005), time of day (Fowler, Rotheray, and Goulson 2016), and temperature (Whitney et al. 2008; Norgate et al. 2010). Biological factors particular to a pollinator, such as pollinator's sex (Church, Plowright, and Loyer 2001), their previous experiences during a foraging trip, and learning (Forrest and Thomson 2009; Gumbert 2000), can also play a role in decision-making mediated by color.

A key aspect of decision-making for pollinators is preference. Preference is a bias towards or against a stimulus. Preferences can be innate, a byproduct of the animal's evolutionary history (Lunau and Maier 1995; Schiestl and Johnson 2013) or learned based on their life experiences (Schiestl and Johnson 2013; Maharaj et al. 2019), or both. Animals have preexisting biases and preferences that affect which cues they attend to and how they will respond to stimuli (Shettleworth 2010; Stevens 2013). There are many ways in which context can affect preference. The visual environment, such as brightness and contrast to background, can alter how colors are perceived and preferred (Finnell and Koski 2021). Preferences can also be affected by the time of day and circadian rhythms (Lazopulo et al. 2019). However, it is not known how abiotic factors that fluctuate with time of day, such as temperature and humidity, affect preference.

Color preference plays a role in pollination—the first studies on bee color choice date back over 100 years (Menzel 2012). Since then, honey bees and bumble bees have become models for bee color vision studies (Menzel 2012; Leadbeater and Chittka 2007).

Results from these two groups are usually generalized to other bee species, despite differences in their natural history. When comparing bumblebee and honeybee color vision, Dyer, Spaethe, and Prack (2008) suggested that the evolutionary history of the species plays a role in the differences in visual acuity and color discrimination – bumble bees evolved in temperate patchy environments, thus requiring better vision acuity, while honey bees evolved in tropical forests and thus were exposed to environments with an abundance and diversity of flowers that benefits better color discrimination. These predictions follow a similar logic from Peitsch et al. (1992), who proposed that temperate and tropical bees would have evolved differences in their visual systems due to differences in light intensity in their habitats. Bumble bees have been shown to have an innate preference for blue (Muller 1881, cited in Gumbert 2000), and testing bumblebees in a context of equal, medium and high color preference showed that even high preference can be overcome if the quality and consistency of the resource are manipulated (Maharaj et al. 2019). In a different species, Menzel et al. (1988) showed that the solitary bee *Osmia rufa* discriminates colors better than honey bees and has a bias towards violet, which was not completely overcome by learning during their experiments. Moreover, the description of pollination syndromes usually include color as an indicator of potential pollinators (for example, Krakos and Austin 2020), highlighting how color preferences can shape our understanding of the relationship between pollinators and the flowers they chose to visit.

Pollinator preference can be measured in field or laboratory assays. Field assays can focus on an array of flowers (natural or artificial) and record visitation data or focus on an individual pollinator and expose it to an array of flowers and record choice. In laboratory studies, pollinators are presented with an array of flowers and allowed to make choices in a controlled environment. Some studies take a more reductionist approach and consider a pollinator's first choice as their preference.

Orchid bees (tribe Euglossini) are major pollinators in tropical ecosystems (Roubik and Hanson 2004) and are closely related to honeybees and bumblebees (Michener 2007; Bossert et al. 2019). Orchid bee pollination is intimately linked to foraging behavior. Male orchid bees can forage over a few kilometers daily, while females have much more local ranges. Both sexes forage for nectar (energy source), but males actively search for and collect scents (possibly to attract females) while females collect resin (used to build a nest) and pollen (used to feed larvae) (Dressler 1982; Roubik and Hanson 2004). Despite having well-described behavior for many aspects of their natural history (Roubik and Hanson 2004), to date, only a few studies with wild orchid bees describe their behavior, with the focus on mating (Eltz, Roubik, and Whitten 2003; Eltz, Roubik, and Lunau 2005) or nest behavior (Saleh and Ramirez 2019). Very little is known about how orchid bee use their color vision. This is surprising, given the fact that orchid bees comprise approximately one-fourth of the total bee abundance in Neotropical forests (Roubik and Hanson 2004), acting as pollinators for multiple species of plants (Armbruster 2017), across distant areas (Roubik and Hanson 2004; Gilbert 1980). A recent study of the brain of a species of orchid bee (Brand et al. 2018) suggests that dimorphism in brain anatomy may be related to color vision. Male orchid bees have larger eyes and medulla (the brain region associated with color vision), suggesting that they invest more in their visual systems than females. These differences might affect color vision and color choice in male and female orchid bees. It is likely that orchid bees share cognitive similarities with honey bees and bumble bees due to their shared evolutionary history. However, there is a remarkable difference between orchid bees compared to their close relatives, namely that orchid bees are not eusocial (Roubik and Hanson 2004), but some species can be primitively or facultatively social (Saleh and Ramirez 2019; Brand et al. 2017; Freiria, Garófalo, and Del Lama 2017). This suggests they may handle information and make decisions differently than other eusocial bee species, as division of labor and sociality are predicted to affect cognition (Lihoreau, Latty, and Chittka 2012). Orchid bees may face different challenges as non-eusocial bees. While a eusocial bee can be specialized on a specific task, a non-eusocial orchid bee might benefit from more plasticity in their behavior since they are required to perform different tasks throughout their lifetime and may not benefit from the work done by a nestmate.

A major reason why orchid bees and other tropical bees are understudied is the lack of affordable, accessible, and adequate methodology for testing wild bees in the tropics. Recent years have seen a greater focus on testing cognitive abilities of wild animals in the field, especially in the avian literature (Morand-Ferron, Cole and Quinn 2016) In bees, a new methodology (FMPER - Free Moving Proboscis Extension Response) for testing wild bumble bees and honey bees has been recently published (Muth et al. (2017), and has been successfully used in a series of studies to date (Muth et al. 2021; Manning et al. 2021). However, these studies are still primarily focused on temperate ecosystems, which do not always translate well to tropical forests' high temperature and high humidity.

This paper presents the first study of orchid bee color vision use in *Euglossa* *dilemma*. We focused on two aspects of color vision: color choice and preference; preference was measured as an individual's bias towards a color. In addition to field behavioral assays, we collected data on a series of abiotic (time of day, temperature, humidity), biological (sex, relative brain size), and procedural (length of trial, number of choices) factors to answer the question of which factors affect orchid bee color choice and preference. We hypothesized that males and females would have different color preferences for the colors tested, and that abiotic and biotic factors would affect both choice and preference. We predicted that sex (due to male and female distinct foraging behavior and neuroanatomy), time of day (because of a bee's prior experience with daily or seasonal flower turnover), and the number of choices (as a measure of a bee's experience) would play a role in color choice and preference.

Material and Methods

Bee collection and tagging: Orchid bees (*Euglossa dilemma*) were collected at Flamingo Gardens in Davie, Florida, in July and August of 2019. We used scent baits (1,4-Dimethoxybenzene) to attract males and nest boxes to attract females searching for cavities to build their nests. Bees were captured and released in the same location. Captured bees were tagged using scratch marks on their thorax (Pokorny et al., 2015) to avoid retesting the same individual. A subsample of tested bees was pinned for morphometric measurements (see below).

Bee testing: We used a recently developed methodology, FMPER (Free-Moving Proboscis Extension Response (Muth et al. 2017)), developed initially for testing wild bumblebees. FMPER consists of trapping an individual bee in a plastic vial large enough for the bee to move freely and presenting the bee with two strips of colored paper, which can contain a drop of sugar reward on the tip of the paper (Figure 01). While this method is successful in temperate bees (Muth et al. 2017; Collado et al. 2020) and laboratory studies (Amaya-Márquez et al. 2019), it has posed problems with field trials in tropical ecosystems where bees are found in high temperature and humidity (due to condensation inside the vial), and also for bees that are not primarily lapping feeding bees (due to a bee's difficulty in drinking from the strip of paper). Working over two years of the summer field season, we have successfully adapted this technique to be used with wild tropical orchid bees, and we believe the modifications we list below are valuable considerations for any species of tropical bee. First, we used a smaller plastic vial (8.5 cm height x 5 cm diameter) which facilitated the bee to see the colored strips and respond to the test. We also added as many holes as possible in the vial to increase airflow and reduce condensation inside the vial, since high moisture caused low visibility for the researcher and airflow helps control the temperature inside the vial. We monitored the temperature inside the vial with a thermohygrometer with a probe placed inside the vial to stop protocol before a bee overheated. We folded the strips of paper in half to create a crease so the drop of sugar water could be placed inside the crease and thus allow a suction feeding bee to drink from the strip of paper. Finally, we also reduced the sugar concentration from 50% to 20% sugar solution because high viscosity reduces orchid bee nectar intake (Borrell 2006).

Testing protocol: We tested four colors commonly used in bee color vision experiments: blue, green, orange, and yellow. Colors were presented in pairs for a total of six possible pairs of colors. Each bee was placed in an FMPER vial where they were presented with two strips of colored paper, both equally rewarding (20% sugar solution). Both strips of paper were introduced simultaneously, and we alternated the side in which the paper was introduced. If the bee extended its proboscis to a colored paper, we counted that as a choice and recorded the color and side. We allowed bees to drink from a paper for 3-5 seconds and waited a minimum of 60 seconds before the bee was presented with the next pair of colored papers. Pairs of paper were only presented when the bee was far enough from the end of the tube to ensure it could see both colors before making a decision. Bees were presented with the same pair of colors multiple times. Each bee was allowed to make as many choices as they were motivated to, and we ended a test once a bee stopped responding to the colored paper for longer than 30 minutes or if the temperature inside the vial was higher than 35^{o}C due to the risk of a bee overheating and dying. Bees that made five or more choices were considered to have completed the test, and we tested a minimum of 10 bees per pair of colors.

Color measurements and abiotic data: Spectral reflectance of each tested color was measured using an Ocean Optics USB-200+UV-VIS spectrometer with a PX-2 pulsed xenon light source (Maharaj et al. 2019). During field tests, we took note of the time of day in which a bee was tested and used a portable thermohygrometer with a probe to collect abiotic data on-site: the temperature outside the vial, temperature inside the vial, and humidity.

Bee morphometrics: bees that completed the protocol were frozen and later pinned. We collected morphometric measurements from the specimens (head width and intertegular distance) using a caliper. All bees were measured three times by the same person, and the final data points were averages of the three measurements.

Figure 01 – Picture of an orchid bee during testing.

Color preference: Color preference was assessed using Jacobs' Index (D) (Jacobs 1974; Gegear and Laverty 2005; Austin, Horack, and Dunlap 2019) for the equation $D = (r - p) / (r + p - 2rp)$; where r is the proportion of focal color selected and p is the proportion of focal colors available in the array. Originally, this index considered the array as all the colors simultaneously available to a forager. Here, because we employ an FMPER design, we consider as the array all the options a bee encountered throughout testing, even though we presented only two options at a time. Therefore, in our design, $p = 0.5$ for every bee since the two colors are equally represented; r is the number of times a specific color is chosen divided by the number of choices made. A value of $D = 1$ indicates a complete preference for the focal color, a value of $D = 0$ indicates no preference (random choice), and a value of $D = -1$ indicates a total preference for the non-focal color. For each pairing of colors, we arbitrary decide on a focal color, so that each of the four tested colors was represented at least once in our analysis.

Data analysis: To test if abiotic factors affect a bee's choice, we performed a Generalized Linear Model. Our model included temperature inside the testing vial, temperature outside, humidity, and time of day as predictors. We ran a variance inflation factor (VIF) analysis to test for multicollinearity, and we excluded any predictor with VIF>3 (Thompson et al. 2017). We tested bees for color preference using Jacob's Preference Index (D). We tested male and female color preference separately. It is worth noting that females were not tested on green, nor the pairing of blue and orange. For male preference, we ran a One-Way ANOVA with color pair as our treatment and preference (D) as the dependent variable. For female preference, we removed two bees from the analysis because they were outliers, and we ran a Welch One-Way test with color pair as our treatment and preference (D) as the dependent variable. To test for the effect of the first choice on preference, we performed a paired t-test comparing final preference with predicted preference based on the first choice. Due to the exploratory nature of our study, to test which factors affect orchid bee color preference, we used the automated selection model package *glmulti* (Calcagno and de Mazancourt 2010). Our dependent variable was the preference index D. Our preference model included bee sex, relative brain size, focal color and color pair, number of choices made by a bee, the temperature inside the testing vial, humidity, and time of day as predictors. All statistical analyses were conducted in RStudio (version 3.6.1).

Results

1. Color measurements and mapping on orchid bee color space

We used the R package "Pavo" to plot the tested colors on the orchid bee color hexagon. Details on how the orchid bee color hexagon was develop were explained in Chapter 2. Figure 2 depicts the four colors, with each dot representing the respective color.

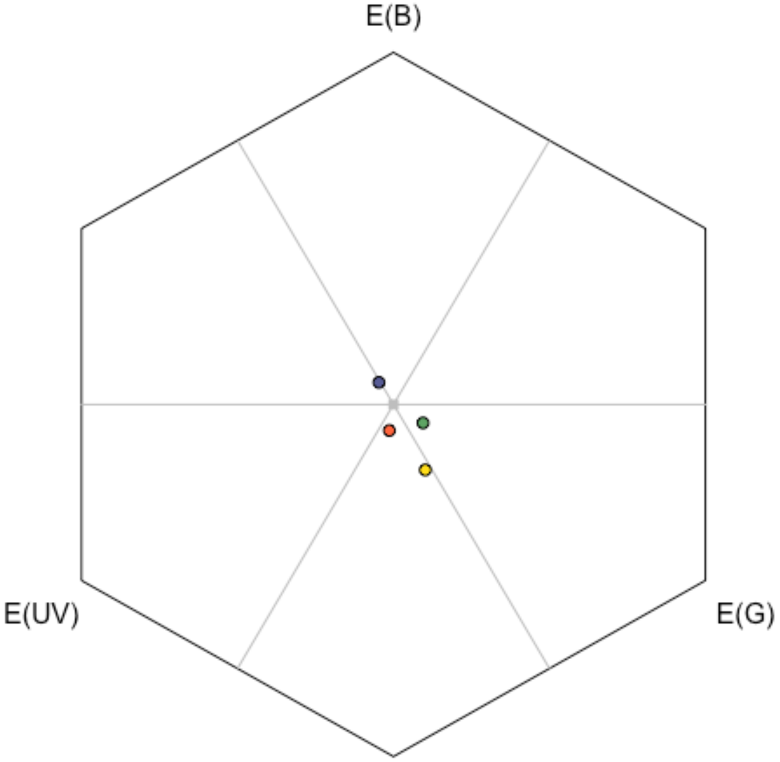

Figure 1 – The four colors tested in this experiment (blue, green, orange, and yellow) mapped on the orchid bee color space, represented by the color hexagon.

2. Sample size and testing

We tested 638 bees (562 males and 76 females) (Table 1). 178 (27.99%) made at least one choice, and 80 bees (12.54%) completed the minimum of 5 choices to be included in the statistical analysis (Figure 3 & Figure 4). The breakdown of bees that completed the protocol were 62 males (11.03% of male bees tested) and 18 females (23.68% of female bees tested). Blue and Yellow was the easier color pair to test, with 26.32% of males and 32.26% of females completing the protocol.

Table 1 – Sample size of field-tested bees.

Color	Total tested males	Males made 1+ choices	Males made 5+ choices	Total tested females	Females made 1+ choices	Females made 5+ choices
Blue and Yellow	38	18	10 (26.32%)	31	16	10 (32.26%)
Orange and Yellow	137	29	10 (7.29%)	45	16	8 (17.78 %)
Green and Yellow	115	24	10 (8.69%)	NA	NA	NA
Blue and Orange	88	29	11 (12.5%)	NA	NA	NA
Blue and Green	65	18	10 (15.38%)	NA	NA	NA
Orange and Green	119	29	11 (9.24%)	NA	NA	NA
Total	562	147	62 (11.03%)	NA	NA	NA

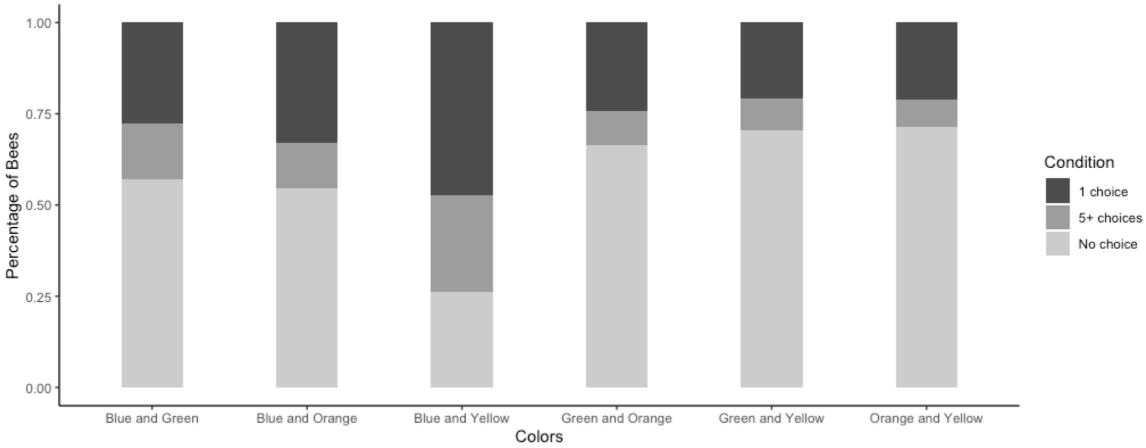

Figure 3 – Field-tested males. Proportions of bees that made zero choices, 1 choice, or 5 or more choices.

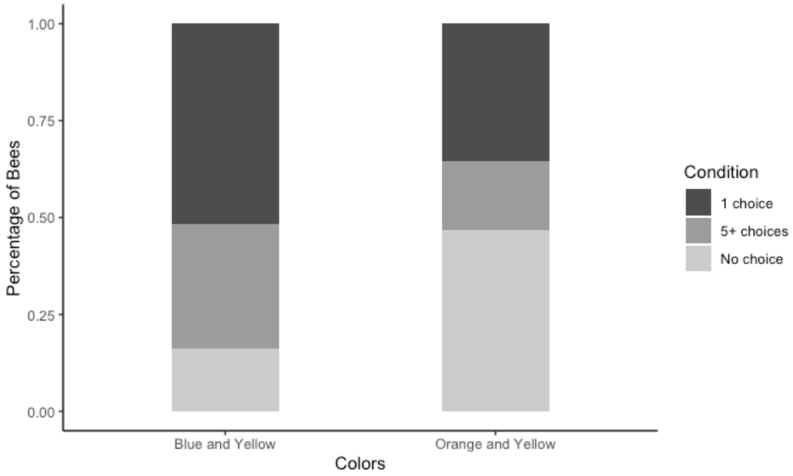

Figure 4 – Field-tested females. Proportions of bees that made zero choices, 1 choice, or 5 or more choices.

3. Do abiotic conditions play a role in choice?

We tested 625 bees (13 were eliminated due to missing abiotic data). We performed two analyses to answer the following questions:

A. How do abiotic factors influence a bee's ability to make a choice?
B. Out of the bees that made at least one choice, what abiotic factors influence how many choices a bee makes?

We scored bees as a 0 (no choice) or 1 (one or more choices) for A. For B, we used the number of choices as our dependent variable. A variance inflation factor analysis resulted in the exclusion of the two temperature measurements. Our final model included time of day and humidity. Our results, summarized in Table 2, show that both humidity (Figure 5A and Figure 6A), time of day (Figure 5B and Figure 6B), and the interaction between the predictors played a significant role in a bee's ability to make a choice and in how many choices a bee makes. Our results show that most bees chose between 9 AM – 11 AM (mean = 10 AM) and between 75 – 85% (mean = 80%) humidity (Figure 5). From the bees that made a choice, most choices were made on the same window of time and humidity (Figure 6).

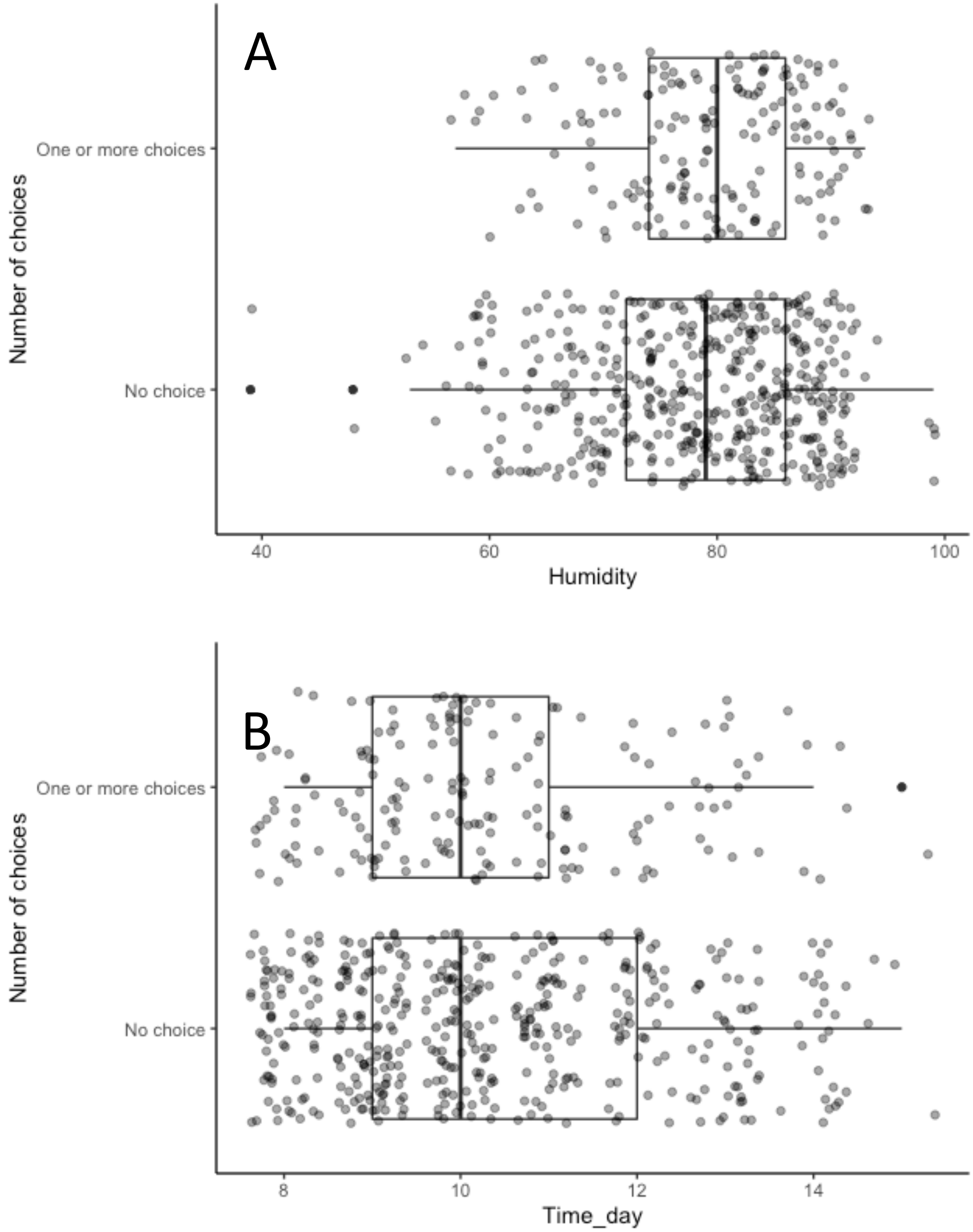

Figure 5 - The effect of humidity (A) and time of day (B) on a bee’s ability to make a choice. Each dot represents a tested bee for 625 bees, and a boxplot showing the median and quartile intervals is under-posed to the data.

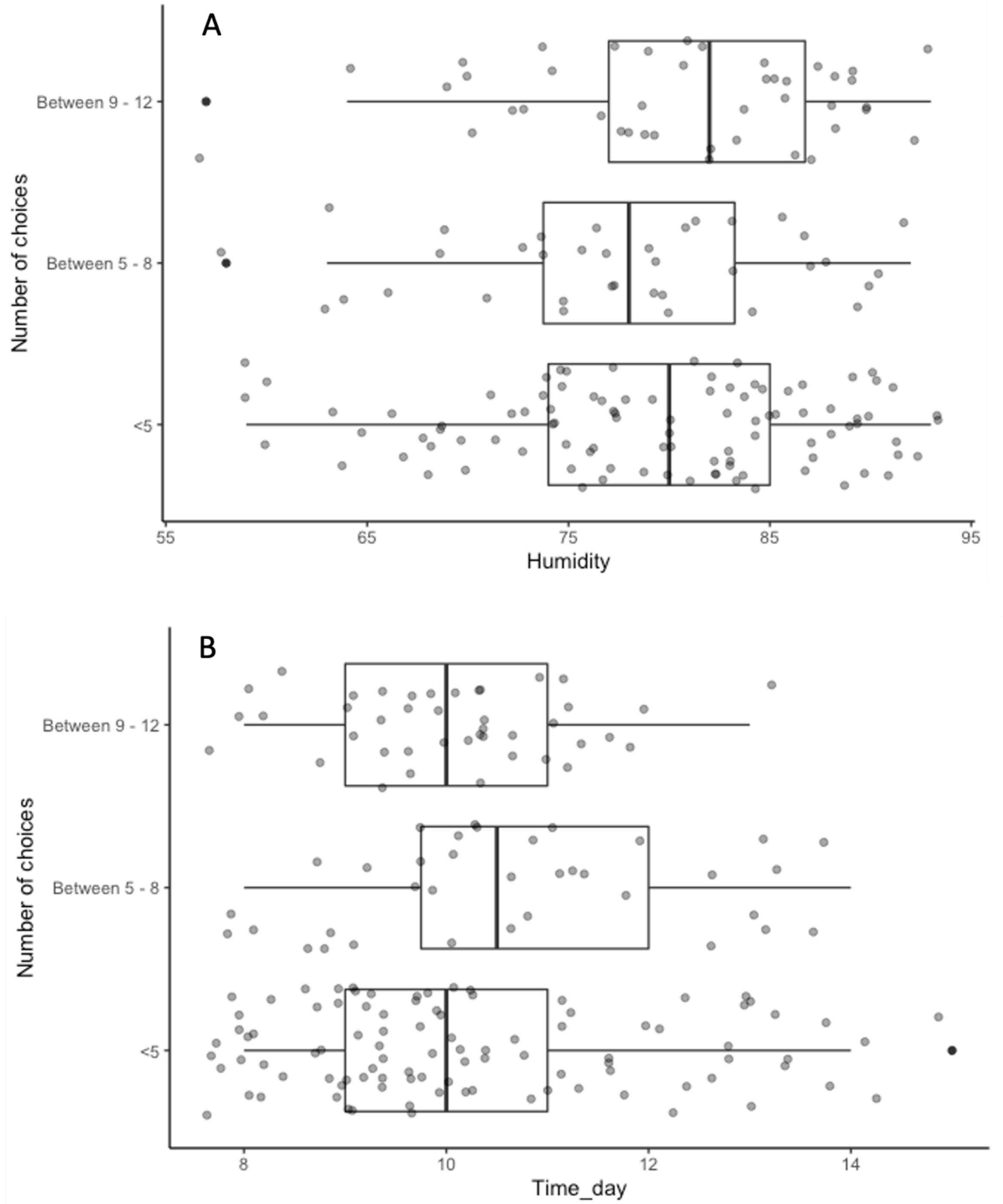

Figure 6 - The effect of humidity (A) and time of day (B) on the number of choices a bee makes. Each dot represents a tested bee for 175 bees, and a boxplot showing the median and quartile intervals is under-posed to the data.

Table 2 – Summary of generalized linear model testing abiotic conditions and choice.

	Choice			
	Estimate	Std. error	Z score	p-value
Time of day	-1.069738	0.493722	-2.167	0.0303
Humidity	-0.137379	0.069412	-1.979	0.0478
Time of day * Humidity	0.01479	0.006434	0.0214	0.0214
	Number of choices			
	Estimate	Std. error	Z score	p-value
Time of day	-0.685869	0.213686	-3.210	0.001329
Humidity	-0.085489	0.029393	-2.909	0.003632
Time of day * Humidity	0.009004	0.002755	3.268	0.001083

4. Do orchid bees have a color preference?

We tested bees for color preference using Jacob's Preference Index (D) (Figures 7). Due to the different sample sizes, we opted for analyzing color preference on males and females separately. Due to pandemic constraints, females were not tested on all six color pairs. There were no differences for color preference between the tested colors for males (One-way ANOVA, F=2.042, p=0.0866) (Figure 7 A) or females (Welch One-way test, F=1.8262, p=0.2097) (Figure 7 B).

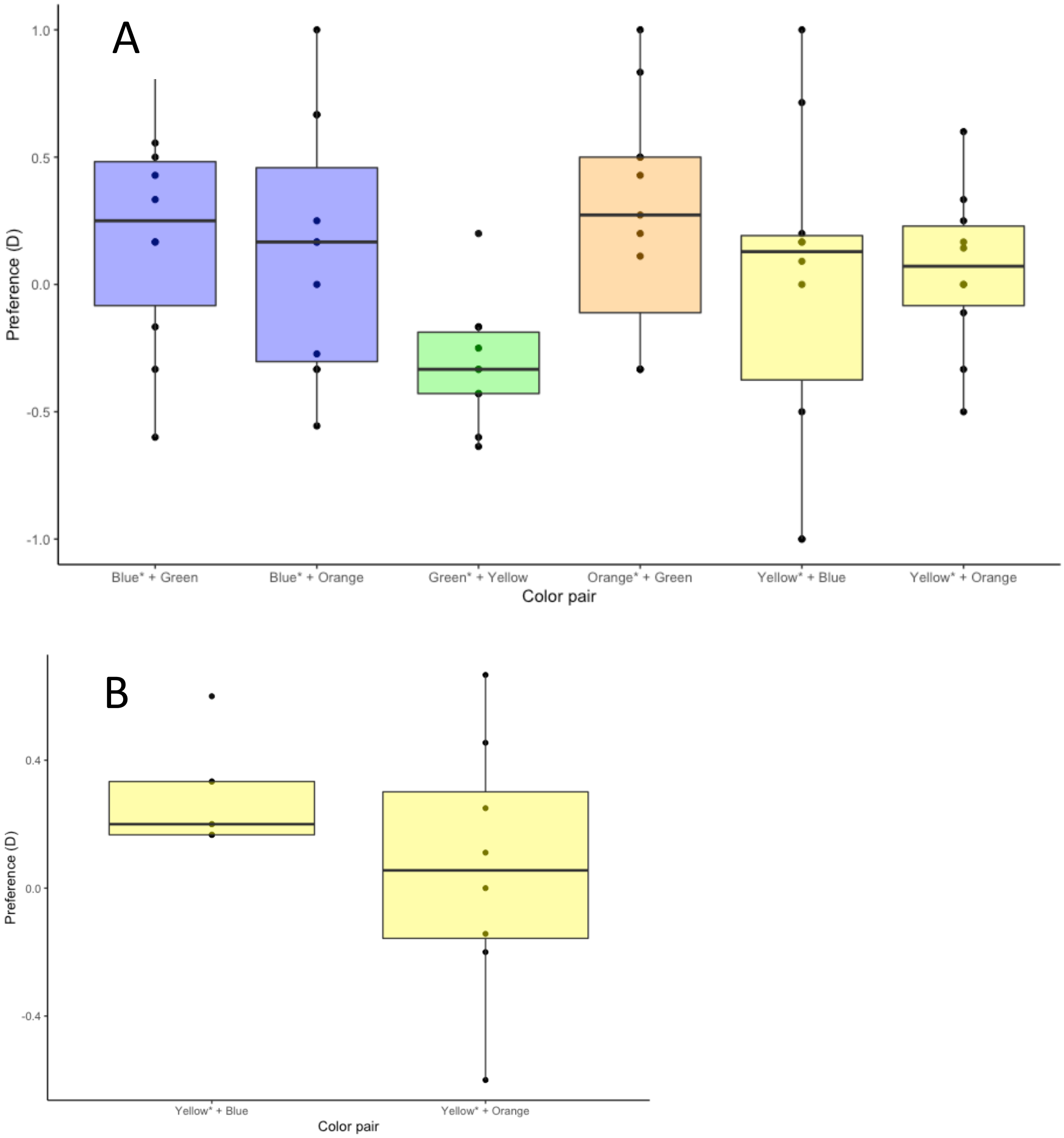

Figure 7 – Male (A) and female (B) orchid bee color preference per color pair. Color preference was calculated with Jacob's index D, where D>0 indicates preference for a color, D<0 indicates preference against a color, a D = 0 indicated random choice. For each pair of color, a focal color was assigned. Focal colors are represented with an asterisk on the color pair in the x axis. In this graph, each dot represents a tested bee.

5. Does the first choice predict overall preference?

To assess if the first choice predicts overall preference, we scored a bee's first choice as a 1 if it matched their preference or a 0 if it did not. 34 out of the 80 field-tested bees (42.5%) did not match their first choice with their final color preference. A paired t-test revealed that first choice is statistically significantly different from overall preference calculated from additional choices (t= 4.5135, p<0.001) (Figure 8).

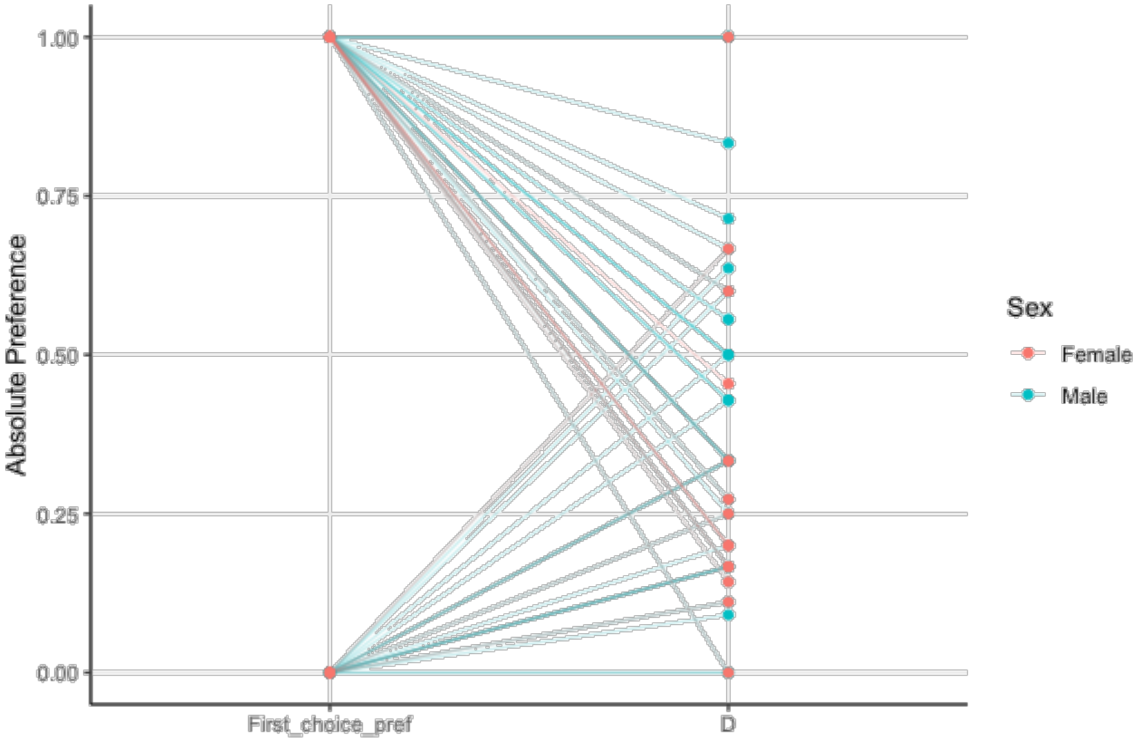

Figure 8 – Color preference does not match a bee's first choice. In this graph, each dot represents a bee. For the first column, first choice preference was assigned as either 1 if the bee's first choice was to the focal color, or 0 if the bee's first choice was to the alternative color. For the second column, D was calculated as the bee's absolute preference, which could vary from 0 (no preference for the focal color) to 1 (complete

preference for the focal color), with 0.5 being random choice. Red dots represent female bees, and blue dots represent male bees. Blue lines between columns connect bees from their first choice to their final preference. A straight line indicated a perfect match, and a sloped line indicates that first choice did not match color preference.

6. What factors affect color preference?

We then proceeded to ask which factors affect orchid bee color preference. We excluded five bees (three males and two females) from the analysis due to missing data for a total of 75 bees. The best fitted model included only number of choices (Figure 9 A). In order to test if number is choice is indeed biologically relevant and not just an artifact of our methodology, we truncated the data in 5 choices, so that every bee had the same number of choices. We ran the same analysis with this truncated data, except number of choices was not a predictor (Figure 9 B). The best model was the null model, which confirms that the abiotic and biotic factors we tested did not affect color preference.

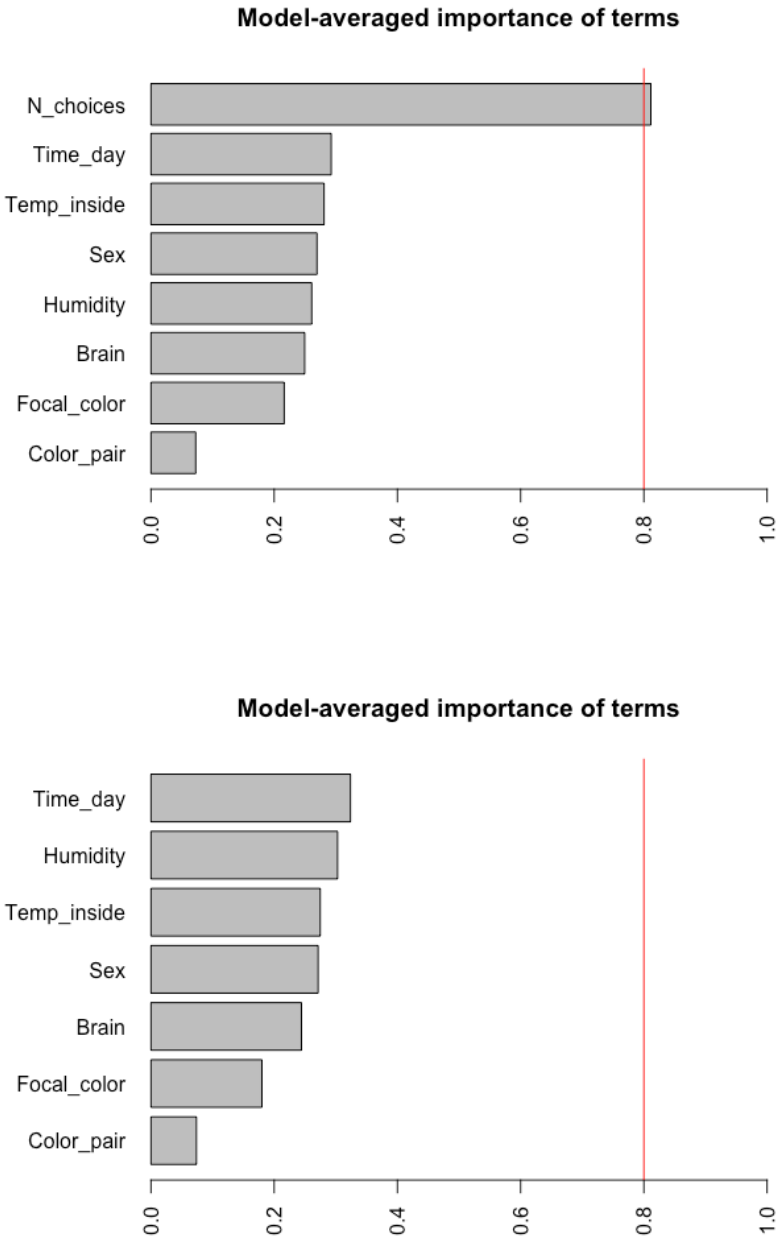

Figure 9- Importance of each tested factor for predicting color preference. Time of day, humidity, temperature inside the vial, sex of the bee, relative brain size, focal color or color pair did not predict preference, even when the number of choices was truncated (B).

Discussion

This study is the first experiment on color vision use in orchid bees. Our results show that environmental conditions, such as time of day and humidity, played a role on color choice. Individual bees showed color preference; however, we found no consistent preferences across bees for either sex. Color preference was not affected by the abiotic or biotic factors measured, and it was not predicted by a bee's initial color choice.

Color choice

One pair of colors emerged as the most effortless pair to test: blue and yellow. 26.3% of males and 32.3% of females tested on this color completed the protocol (Table 1). Moreover, males and females made more choices when presented with blue in combination with any other color. This result is interesting when considering that both human yellow and human blue are colors that honey bees (R Menzel 1985) and bumble bees (Muller 1881, cited in Gumbert, 2000) are known to be biased toward. However, we did not find an overall preference for blue or yellow in our color pairings. It is unclear from our results the reason why more bees completed the protocol with the Blue and Yellow pairing. Blue and Yellow were the most distant colors on the orchid bee color hexagon (Figure 1), which might indicate that these colors were perceived more distinctly by the tested bees. Another possible explanation is that these colors are more associated with nectar flowers. Future studies measuring color discrimination and comparing behavior testing with the colors of flowers available for these bees in their environment would be necessary to clarify this question.

Female bees were easier to test than male bees. One possible explanation for this can be related to motivation. We collected males from scent traps, so those were bees that were motivated to collect scent but maybe not motivated to collect nectar, which was the reward we offered them on our behavioral assays. It is possible that the male bees were satiated already when we started testing them. Alternatively, maybe scent collection is so vital that males were not attentive to the nectar reward. Future work on motivation and resource collection might elucidate some of these questions.

Motivation can also play a role in female decision-making. Saleh and Ramirez (2019) described that foraging females leave the nest searching for nectar and pollen, and different resources are prioritized depending on where a bee is in its natural social cycle. Foundress bees collect nectar (energy source) and resin (nest-building material), while subordinate bees collect nectar (energy source) and pollen (protein for the colony's young). For our study, females were captured near their nests. We noted if a female was leaving the nest, thus motivated to collect, or returning the nest, thus potentially already satiated. We also noted if females were carrying pollen or resin on their back legs. Unfortunately, our sample size was too small to test for predictions of how social behavior interacts with foraging and color preference. It would be interesting to track nests and test if females foraging for different resources and in various stages of their social cycle make different choices and have different color preferences.

Testing bee behavior in the field in tropical ecosystems is not trivial. There are few field-based assays for testing insect behavior, which is almost exclusively designed for temperate ecosystems. The best assays, like FMPER, are the ones that can be easily adapted for different climatic conditions. We tested if abiotic conditions (time of day, temperature, humidity) affect a bee's ability to choose and how many choices they make.

The temperature was excluded from the final model because it was autocorrelated with humidity. Time of day, humidity, and interaction play a role in a bee's ability to choose and how many choices a bee will make. This indicates an ideal window in which to test color vision on orchid bees in the field. Based on our results, we propose that between 9 AM and 11 AM and between 75 – 85% humidity is an ideal window for testing orchid bee behavior in the field. These results should help future studies, and they might translate to other bees and other types of behavioral assays.

Color preference

We did not detect consistent color preference across bees for any of the tested pairs of colors. However, as shown on Figure 7, individual bees did have a preference for specific colors. We did not test all pairings of colors with females, and more studies on female color choice are needed to investigate if they do show some preference for any of the colors we did not test in this experiment.

When looking at the individual preference of a bee, it is unclear if the mismatch between color preference and first choice tells us something about orchid bee biology or speaks to how color preference should be assessed in other bees. It does not look like a bee's first choice reflects their preference across a series of choices instead, bees sampled the colors available. Our results challenge behavioral studies that consider a pollinator's first choice as their preference (for example, on Muth et al. (2017)). When we test individuals in only one choice, we lose a considerable part of their behavior. We also believe that instead of imposing a minimum time or number of choices for testing a bee, studies should let bees choose if they are motivated and then analyze the whole data to define preference. For example, bee foraging arenas (for example, Austin, Horack, and Dunlap 2019) usually test only motivated foragers when they are out in a foraging trip. We do acknowledge that our threshold of a minimum of 5 choices was arbitrary; thus, it does not necessarily reflect a biologically relevant threshold.

Moreover, abiotic (time of day, temperature, humidity), biological (relative brain size, sex), and methodological (focal color, color pair) factors did not play a role in preference. This indicates that although orchid bees are making decisions that are affected by time of day and humidity, their preference was not predicted by the external (environmental) or internal (biological) parameters we tested. The lack of differences in male and female preference is interesting, given that there are recorded differences in their foraging behavior (Roubik and Hanson 2004; Society and Journal 2018) and brain anatomy in regions related to insect vision (Brand et al. 2018). However, these results are congruent with Chapter 2 results, which did not find differences in orchid bee eye photoreceptors and color vision.

Decision-making and preference are complex aspects of pollinator behavior, with fitness consequences for the pollinator and the plants they visit. By testing orchid bees in the field with non-invasive tests, we can better understand how tropical pollinators interact with their environment and make decisions based on their color vision. This study describes an alternative methodology for behavioral field assays, determines the optimal time of day and environmental conditions for testing, and presents the first data on orchid bee color preference. By testing orchid bees in the field with non-invasive tests, we can better understand how tropical pollinators interact with their environment and make decisions based on their color vision.

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Chapter IV: Scent and color cues in nectar foraging in the orchid bee Euglossa dilemma

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Abstract

Pollinators visit flowers in search of different resources, such as food and nest materials. Male orchid bees are major tropical pollinators and are well known for their scent collection behavior; scents are used in the context of mating. However, little is known about how they find nectar flowers. Building upon prior work on color preferences in these bees, we tested if the presence of scent affected a bee's engagement with and motivation to complete a color choice assay and if the presence of scent affected male orchid bee color preference. We find that the presence of scent affected motivation but not participation in choice trials. Once bees made choices, we found that scent did not affect color preference. These results shed light on the context of choice, but we also tested learning of the scents themselves. We tested if male orchid bees learned to associate a nectar reward with two types of scent: common floral scents and mating specific scents. We were unable to detect learning with our protocol, but this does not indicate that these bees do not learn scent associations. Together, our results show that male orchid bees attend to scent cues even when tested on a choice paradigm of color cues. This might indicate that male orchid bees are evolutionarily prepared to attend to scent cues; however, more testing on the specific ways in which male orchid bees are prepared to attend to scent cues are warranted.

Keywords: *pollination, foraging, environmental cues, scent learning, FMPER*

Introduction

Animal pollination is intimately linked to foraging behavior. Pollinators visit flowers in search of different resources, such as food (usually in the form of nectar or pollen), materials for their nests (resin, oil, or leaves), and attractants for mating (scents). Pollinators use different environmental cues to locate and remember flowers and their resources. Some of the most studied cues are the color and odors used by bees on foraging trips. Color cues are important for bees when pollinating (Dyer, Paulk, and Reser 2011), and bees use color information to make decisions and learn about nectar-bearing flowers (Gumbert 2000; Garcia, Shrestha, and Dyer 2018). Scent cues are also important for bees when foraging for nectar (Raguso 2008; Wang et al. 2018) and collecting plant resins (Leonhardt et al. 2010; Leonhardt, Schmitt, and Blüthgen 2011).

Orchid bees are major pollinators in tropical forests (Roubik and Hanson 2004). Most of what we know about orchid bee pollination comes from studies on male orchid bees (but see Armbruster and Webster (1979 and Opedal et al. (2017) for examples on female orchid bee pollination). Male orchid bees forage for nectar, their primary energy source, but they are better known for collecting scents used in mating behavior (Dressler 1982; Eltz, Roubik, and Whitten 2003; Michener 2007; Pokorny et al. 2017). There has been extensive research on male orchid bee chemical ecology. Male orchid bees must collect a combination of different chemicals presented with floral scents, and it is the unique combination of those chemicals, called "perfumes," which are used in mate attraction and mating (Pokorny et al. 2013; Brand et al. 2020; Weber et al. 2016). This foraging pattern leads to individual bees visiting different species of flowers in search of different resources, which results in orchid bees acting as pollinators for multiple species of plants (Armbruster 2017) across a large area (Gilbert 1980; Roubik and Hanson 2004).

Scent collection behavior has been used for decades as a successful way to attract male orchid bees for research purposes. Male orchid bees visit baits and collect scents that were commercially purchased and placed on cotton balls or pieces of paper, with reports of bees being attracted to scents baits up to 1 kilometer away (Dressler 1982). Therefore, it has been widely assumed that these bees use odor cues to find the sources of the chemicals they are attracted to, which would explain why they collect scents from scent baits that do not resemble flowers. Scent perception in orchid bees was recently explored by looking at brain morphology (Brand et al., 2018). Whole-brain reconstructions revealed that male orchid bees have specialized macroglomeruli, a specialized structure of the olfactory system. There is one species of orchid bee, *Euglossa dilemma,* which is found in the United States, in South Florida (Skov and Wiley 2005; Eltz et al. 2011; Pascarella 2017). *Euglossa dilemma* is attracted to a mix of scents, including 1,4-Dimethoxybenzene (DMB), Eugenol, cineole, methyl salicylate, among others (Skov and Wiley 2005; Ramírez et al. 2010), with DMB being the most attractive scent (*personal observation*).

While much is known about scent collection in male orchid bees, fewer studies focus on male nectar foraging. Male orchid bees may use scent cues to locate nectar flowers, but like other bees, they likely also attend to other cues, such as color. Neuroanatomy studies by Brand et al. (2018) showed that male orchid bees invest more in their visual systems than females: males have larger eyes and medulla, a brain region associated with color vision in insects. In that study, the authors suggest that these differences may be related to sexual selection. However, foraging behavior may also play a role as a selective pressure driving sexual dimorphism in orchid bees' brains and the use of color information. Moreover, scent and color cues often interact with each other in the context of pollination, as reviewed by Leonard and Masek (2014). Work in other insects also uncovers these types of interactions. For instance, when explicitly testing for the effect of scent on color choice in the fly, *Drosophila suzukii*, Bolton, Piñero, and Barrett (2021) found differences in color preference in the presence of different scents.

In our previous work (Chapter 3), we tested male orchid bees in the field. We found no consistent color preferences across bees but rather an individual variability in preference and strength of preference. However, we found that males were harder to test than females, and we noted that males were captured from a scent bait. We hypothesized that females, collected when leaving their nests, were more motivated to collect nectar, while males found in scent baits were probably motivated to collect scent. We often think of the multimodal cues of flowers as acting to enhance the attraction to and learning of flowers (Leonard, Dornhaus, and Papaj 2011), but differences in task specialization due to sex differences in life-history might result in motivational differences in attending to all available perceived cues. Thus, males searching for scent could use the reliable cues of olfactory volatiles and ignore all other sources of information. Searching for scent may also simply be an incompatible behavior with learning about floral cues such as color. Or selective pressures on male orchid bee scent collection behavior might have resulted in evolutionary preparedness for attending to and learning scent cues over color cues. To answer the question of how scent functions in preference for color, we tested male orchid bee color choice and preference in the presence of scent.

We specifically tested if the presence of scent affected a bee's engagement with (participation) and motivation to complete a color choice assay and if the presence of scent affected male orchid bee color preference. We predict that the presence of scent will function as a distracting cue for males, in that male orchid bees are evolutionarily prepared to attend to scent cues over color cues. This prediction leads logically to a few specific predictions: we predict that scent can act as a distracting cue for males when they are foraging for scent and not nectar. We did not predict changes in preference but expected to find differences in participation (likelihood to make a choice) and motivation (number of choices a bee makes, time to make a choice, trial length). We also predict that the presence of a more preferred scent will result in bees taking a longer time to make decisions about color because a more preferred scent will be more distracting for the bee. This latency to make a decision will decrease in the presence of a less preferred scent and decrease further with no scent present. Finally, we test the specificity of scent cues as operating in sexual selection and not foraging and directly ask whether male orchid bees can learn food associations using scent cues. We specifically tested if orchid bees learn to associate a nectar reward with two types of scent: common floral scents and mating specific scents. Because the ability to learn is often quite generalized, we predict that male orchid bees would learn to associate a scent cue with a food reward. We expect learning to be faster when using a common floral scent than a mating-specific scent.

Materials and methods

Bee collection: We collected male orchid bees (*Euglossa dilemma*) at Flamingo Gardens in Davie, Florida, using scent baits (1,4-Dimethoxybenzene). For laboratory experiments, bees were shipped (described below). For experiments in the field, we captured and released bees in the exact location. Captured bees for field testing were tagged using scratch marks on their thorax (Pokorny et al., 2015) to avoid retesting of the same individual. For laboratory testing, these same scratch marks were used but were created in individually identifying patterns.

Bee shipping and husbandry: We placed individual bees in 20mL scintillation vials containing a cotton ball soaked in honey-water (50% honey solution) and another cotton ball closing the vial. The use of cotton allows for airflow inside the vial. Each vial was wrapped in a paper towel and placed in a shipping cardboard box filled with Styrofoam padding to keep the vials from moving inside the box and ice packs to keep the box's interior cool and avoid overheating. We shipped bees overnight and housed bees at the University of Missouri-St. Louis in a 16.5x30x48-in plastic and mesh cage in a temperature and humidity-controlled room at a 12:12 light cycle to mimic their natural conditions. We fed bees 20% sugar water ad libitum through feeders mounted to the side of the cage.

Experiment 1: Color preference in a laboratory setting: We tested 10 bees for their color preference using the Free-Moving Proboscis Extension Response (FMPER) protocol developed by Muth et al. (2017) and modified by me (as described in Chapter 3). FMPER consists of placing an individual bee in a plastic vial large enough for the bee to move freely and presenting the bee with two strips of colored paper, which can contain a drop of sugar reward on the tip of the paper. We tested four colors commonly used in bee color vision experiments: blue, green, orange, and yellow. Colors were presented in pairs for a total of six possible pairs of colors. Each bee was placed in an FMPER vial where they were presented with two strips of colored paper, both equally rewarding (20%

sugar solution). Both strips of paper were introduced simultaneously, and we alternated the side in which the paper was presented. If the bee extended its proboscis to a colored paper, we counted that as a choice and recorded the color and side. We allowed bees to drink from a paper for 3-5 seconds and waited a minimum of 60 seconds before the bee was presented with the next pair of colored papers. Pairs of paper were only presented when the bee was far enough from the end of the tube to ensure it could see both colors before making a decision.

Each bee was presented with all possible pairs of colors, for a minimum of 12 presentations per bee, controlling for the side in which each paper was presented and randomizing the order of presentation. Color preference was calculated using Jacob's Preference Index (D) for the equation $D = (r - p) / (r + p - 2rp)$; where r is the proportion of focal color selected and p is the proportion of focal colors available in the array. Originally, this index considered the array as all the colors simultaneously available to a forager. Here, because we employ an FMPER design, we consider as the array all the options a bee encountered throughout testing, even though we presented only two options at a time. $D=1$ signifies a complete preference for this index, $D=-1$ signifies complete aversion, and $D=0$ signifies random choice. Testing took place in a temperature- and humidity-controlled room at the University of Missouri-St. Louis.

Experiment 2 - color choice and preference in the presence of scent: We tested 72 male orchid bees for color preference using the FMPER procedure. We tested color preference in the laboratory in a room with a cotton ball soaked in a scent hanging from a top shelf, thus changing the context of the choices bees were making. For this scent context, we tested three conditions: Mineral Oil (control), Eugenol (less preferred scent), DMB (most preferred scent). We only tested two colors, blue and yellow. Bees were allowed to make up to 15 choices, and we calculated preference using blue as the focal color. Individual bees were tagged, and bees were tested on all treatments when possible. To test for participation, we scored each bee as a 0 if no choices were made or 1 if at least one choice was made. To test for motivation, we noted the number of choices a bee made, the time to make the first choice, the average time to make a choice, and the time to finish the trial. Color preference was calculated using Jacob's Preference Index (D), as explained above. All trials were timed.

Experiment 3 – Scent Learning in the field: We tested 100 male orchid bees on scent learning in the field at Flamingo Gardens. We used the scent-FMPER protocol developed by Amaya-Márquez et al. (2019), where bees are conditioned with scent instead of colored paper. The reward was a 20% sugar solution, presented at the tip of a sterile Q-tip. Bees were tested in one of 5 treatments: Mineral Oil (control), DMB (mating scent), Eugenol (mating scent), Geraniol (floral scent), Linalool (floral scent). Each bee was tested only once, and bees were tagged before releasing to avoid retesting bees. If a bee made at least one choice, we considered that it had responded to the protocol, and those bees were included in the data analysis. If a bee made more than 3 choices when presented with the scent cue, we considered that bee to have completed the protocol.

Data analysis: For Experiment 1, since each bee was tested on all possible color pairs, each bee had a score of preference for each color. We tested the preference in the presence of scent between the four tested colors with a repeated-measures ANOVA, where preference was the response variable, treatment (color) was a fixed effect, and bee identity was a random effect.

For Experiment 2, we tested participation by running an ANOVA where "participation" (scored as a 0 or 1) was the response variable and "type of scent" (Mineral Oil, Eugenol, DMB) were the treatments. We tested motivation by running a MANOVA. The number of choices, time to make the first choice, and trial length were the dependent variables, and scent treatment was the independent variable. We dropped the "the average time to make choices" variable because of issues with multicollinearity. For a post hoc analysis, we performed a Kruskal-Wallis test with a Bonferroni correction.

We also analyzed the effect of experience on testing the bees by comparing bees tested on two treatments. We performed a series of t-tests on the same parameters listed above. We tested color preference in the presence of scent for the first trial for each bee by running an ANOVA where "color preference" was the response variable and "type of scent (Mineral Oil, Eugenol, DMB) were the treatments. For the bees that were tested in all three treatments, we compared individual color preferences across treatments. Because the number of choices per bee per treatment did not always reach the minimum of 5 choices to calculate Jacob's preference index, we used a simpler preference index consisting of (#B - #Y) / (#B + #Y), where #B is the number of choices for blue, and #Y is the number of choices for yellow. We ran a repeated-measures ANOVA, where preference was the response variable, treatment (scent) was a fixed effect, and bee identity was a random effect.

For Experiment 3, when testing scent learning, we initially planned on building learning curves for the bees made at least three choices in the testing phase, and we were going to compare the speed and accuracy of learning between floral and mating scents.

However, no bees reached the threshold of three choices in the testing phase. We then conducted a Kruskal-Wallis test to test individual bees were making different choices under different treatments. All statistical analyses were performed in RStudio (version 3.6.1).

Results

Experiment 1 - Color preference in laboratory conditions

In Figure 1, we plotted the tested colors on the recently developed color hexagon for this species of orchid bee (Chapter 2). We compared the preference between the four tested colors and found no differences between the tested colors, but blue and yellow were the most often chosen colors (Figure 2) (One-Way ANOVA F=0.654, p=0.586). These results match our field-tested bee preference (Chapter 3).

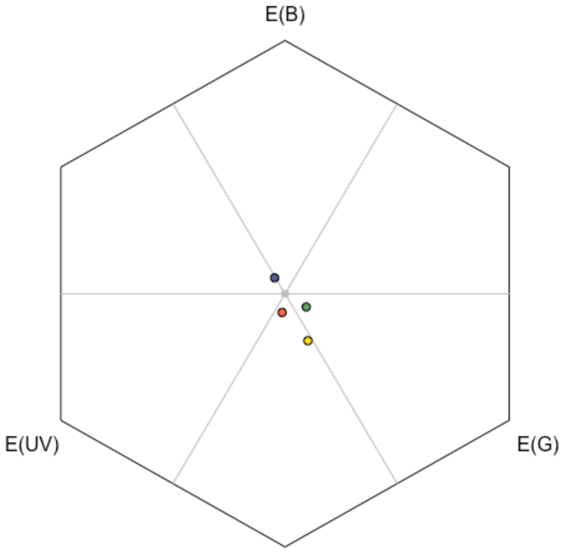


Figure 1 – *Euglossa dilemma* bee color hexagon depicting the four tested colors: blue, green, orange, and yellow

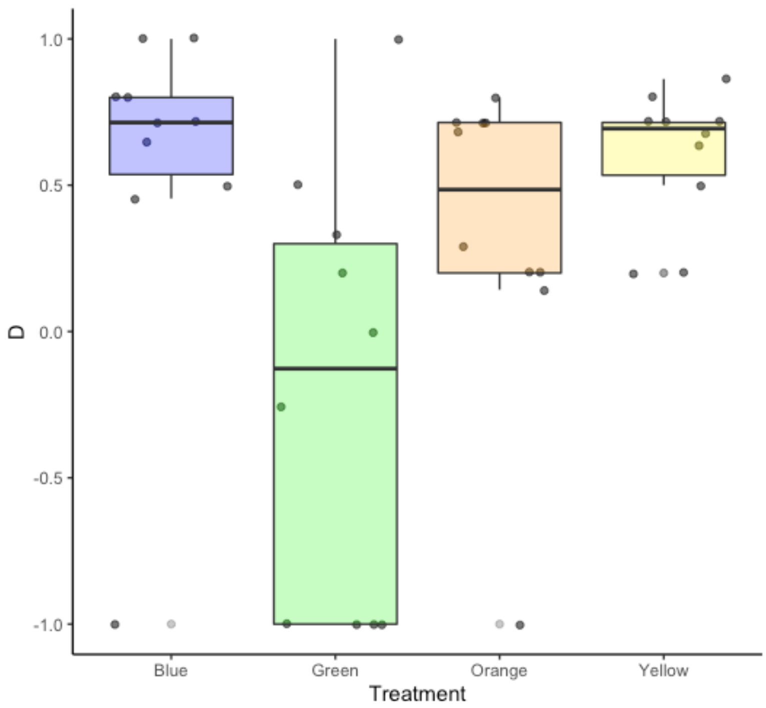


Figure 2 – Results of experiment 1 where we measured color preference for each of the 4 colors across 10 bees in a laboratory setting. Each dot in the graph represents a tested bee, and each color's data includes the choices of each other colors.

Experiment 2 – Part A: Color choice in the presence of scent in laboratory conditions

We tested 72 bees in 128 behavioral assays. Out of the 128 assays, in 99 of them, a bee made at least one choice. Out of the 99 bees that made at least one choice, 34 bees were tested on Mineral Oil, 32 bees tested on Eugenol, and 33 bees tested on DMB. Participation was not affected by the presence of scent. Bees were not more likely to participate in the trial (make at least one choice) under different scent treatments (F=1.505, p=0.229). For the bees that did make a choice, we compared the first trial for each bee. 15 bees were tested on DMB first, 17 on Eugenol, and 21 on Mineral Oil. A one-way multivariate analysis of variance revealed a statistically significant difference between the scent treatments on the combined dependent variables (number of choices, time to make the first choice, and trial length) (F(3, 49) = 4.706, p=0.005774). Follow-up univariate Kruskal-Wallis, using a Bonferroni adjusted alpha level of 0.0167 showed that there was a statistically significant difference in trial length (F(2, 53) = 22.8, p < 0.00001) between scent treatments. All pairwise comparisons between groups were significant (Figure 3). Bees took longer to complete a trial in the presence of scent, with bees taking the longest latencies with Eugenol, followed by DMB, compared to the mineral oil control.

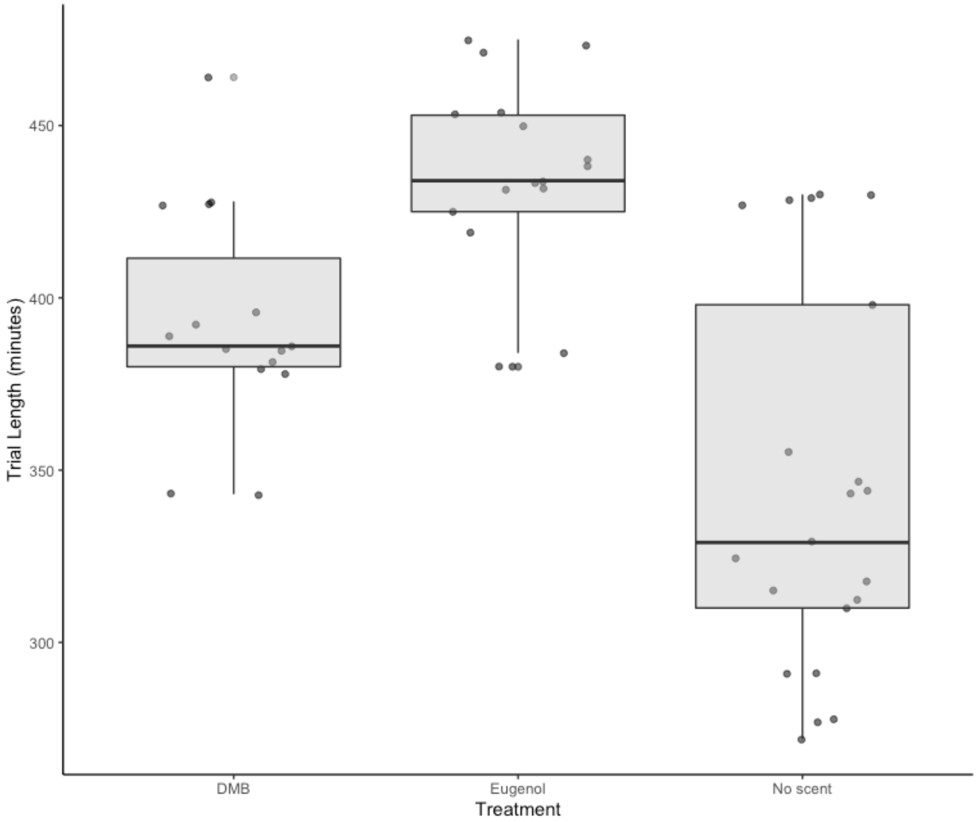
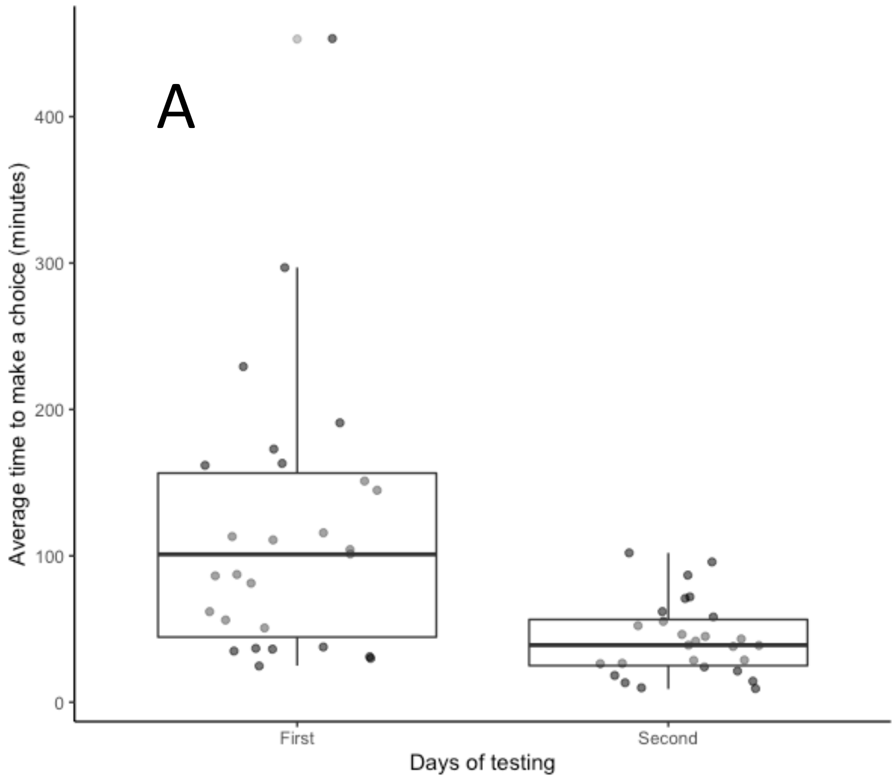


Figure 3 – Trial length in minutes per scent treatment for the three treatments: DMB, Eugenol, and Control (no scent). Each dot represents a tested bee. The two scent treatments were significantly longer than the control treatment, with bees tested in the presence of Eugenol taking significantly longer to complete a trial than bees tested in the

presence of DMB

Twenty-seven bees were tested in 2 treatments for 54 trials (13 tested first in color, 14 tested first in scent). For these bees, we found an effect of experience, with bees making more choices (t= -7.4705, p<0.001) and faster choices (t= -5.5458, p<0.001) on the second day of testing regardless of the presence of scent. There were no differences in time to make the first choice, time to finish the trial or preference between the two days (Figure 4 and Table 1).



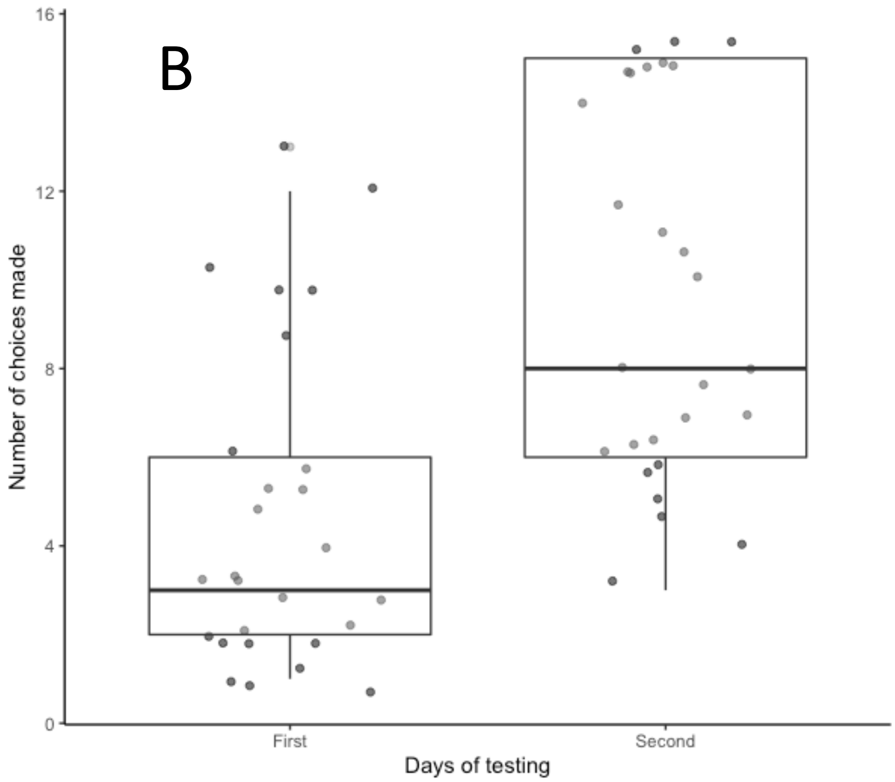


Figure 4 – Comparison of bee motivation between the first and second days of testing on average time to make a choice (A) and the number of choices (B).

Table 1 – Series of t-tests comparing bees on the first day of testing versus the second day of testing. Significant values are bold-faced.

Parameter	t value	p-value
Number of choices	**t=-7.4705**	**p<0.001**
Time to make the first choice	t=1.7280	p=0.09716
Average time to make a choice	**t=-5.5458**	**p<0.001**
Time to finish trial	t=0.50321	p=0.6192
Preference	t=1.138	p=0.2655

Experiment 2 – Part B: Color preference in the presence of scent

We took a subset of bees were tested on all treatments and calculated their preferences.

We tested if the presence of scent affects color preference. Color preference was not affected by the presence of scent (F=0.049, p=0.953).

Experiment 3 - Scent learning in the field

For this experiment, we tested scent learning on *E. dilemma* in the context of nectar foraging. We tested 100 bees on four scents and one control treatment, and 46 bees responded to the protocol (Table 2). Unfortunately, no bees reached the threshold of at least three choices on scent to account for learning. The number of choices a bee made was not affected by the treatment (Kruskal-Wallis chi-square = 8.0427, p=0.09003).

Table 2 – Number of bees tested on scent learning per treatment.

Treatment	**Type**	**Number of tested bees**	**Number of bees that made at least one choice**
Mineral Oil	Control	18	7 (38.89%)
Geraniol	Floral	22	9 (40.91%)
Linalool	Floral	19	10 (52.63%)
Eugenol	Mating	17	7 (41.18%)
DMB	Mating	24	11 (45.83%)
Total	---	100	46 (46%)

Discussion

Scent is an important environmental cue for orchid bees. This is the first study to investigate the role of scent on nectar foraging in male orchid bees. We found that the presence of scent did not affect participation, but it did affect motivation for color choice, with bees taking longer to complete their choices when the scent was present than when the scent was not present. This is different than what we expected since we expected that both participation and motivation would be affected by the presence of scent. The specific scent also matters in this result. We predicted that the presence of DMB would have a larger effect than Eugenol, based on the general difference in observed attractiveness of these odors for bees coming to baits in the field. However, we found the opposite of this prediction between these two scents, with bees taking longer to choose in the presence of Eugenol. This shows that the specific scent matters in the context of orchid bee color decision-making.

When testing for the effect of experience, we found that individual bee behavior was affected by the presence of scent by comparing bees that were tested twice. On the second day of testing, bees made more choices and made choices faster. This effect of experience might indicate that bees can overcome some of the effects of scent on color choice by being exposed to the same choice paradigm more than once. In a natural context, we expect that the effects of distraction posed by scent would be less pronounced if the scent in question were common enough for the bee to have encountered in previous nectar foraging trips.

The presence of scent did not affect color preference. We tested color preference for each bee's first trial across all three treatments. Our results differ from a recent paper in

Drosophila (Bolton, Piñero, and Barrett 2021), where color preference was scent specific in some cases, demonstrating how odors interact synergistically with color cues. However, as with any cognitive test, the specific choice of stimuli matters, and we may always find a similar result with a different combination of stimuli. We did observe high individual variability on preference in male orchid bees (Figures 2 and 5). This is consistent with our field results (Chapter 3), where we did not detect preference on a population level, but we did observe high individual variability on color choice and preference.

We tested male orchid bees for scent learning. We had hypothesized that male orchid bees would learn the floral scent association better than the mating scent and that there would be no learning on the control. Our results show that male orchid bees did not learn to associate a sugar reward with a scent cue regardless of the type of scent presented. And the presence of scent did not affect choice. But male orchid bees did respond to the scent FMPER protocol, much like the stingless bees tested by Amaya-Márquez et al. (2019). It is important to note that our results do not necessarily show that male orchid bees cannot learn from scent cues. As with any new test of learning, especially in a species that is not well studied for learning, many parameters of the learning test itself, such as the concentration of the scent cue, or the context of the learning test, such as how the bees were caught, can affect the behaviors we are measuring. For instance, if males on a scent trap are foraging for scent and not nectar, perhaps they are not motivated by the sugar reward we offered, which can explain why males made so few choices across trials. This agrees with our laboratory tests on color preference, where we found an effect of the presence of scent on how male orchid bees participated and made choices in our color choice experiment. Ideally, one should test scent learning associated with a sugar reward by testing bees foraging in flowers. However, it is much harder to collect male bees in flowers than it is on scent traps. Another caveat is that we only used unscented rewards. It is important to note that Amaya-Márquez et al. (2019) had higher success with conditioning bees when using scented rewards and on the second day of testing bees. However, we could not test individual bees across multiple days because our experiment was conducted in the field during a pandemic.

In this study, we laid the foundation for the study of scent as an environmental cue for male orchid bees. Together, our results show that male orchid bees attend to scent cues even when tested on a choice paradigm of color cues. This might indicate that male orchid bees are evolutionarily prepared to attend to scent cues; however, more testing on the specific ways in which male orchid bees are prepared to attend to scent cues are warranted. Out of the many questions that remain unanswered about the role of scent on orchid bee foraging and pollination, we suggest two as the most pressing and, potentially, most interesting. First, although it is widely assumed that male orchid bees are rewarded by scent collection, to this date, one question that has never been tested is: are scents acting as a reward for male orchid bees? This question can be answered from a physiological perspective by investigating if scent collection triggers a rewarding cascade in the brains of male orchid bees. It is important to test if scent is rewarding or if the mechanical act of scent collection is what is rewarding.

The vast literature on bee learning has taken advantage of using sugar solutions, a proxy for nectar, as a reward. We know from the bee learning literature that bees learn associations when using sugar as a reward. However, there are few studies addressing the question of non-nutritive rewards, such as scent. The second question we would like to propose is: do bees learn with non-nutritive rewards? If scents are shown to be rewarding for male orchid bees, they will be an ideal group to test non-nutritive rewards. This question can be answered from a behavioral perspective by conditioning bees using scent as a reward and testing if male orchid bees respond to scent the same way they would respond to another reward, such as nectar.

Motivation is an important element of decision making, and male orchid bees are less motivated to engage in nectar foraging type of experiments in the presence of scent. Male orchid bees take longer to complete decisions when in the presence of scent. However, this effect changes when individual bees are exposed to the same behavioral assay more than once. Bees make faster and more decisions on the second day of testing regardless of the presence of scent. And although we found differences in foraging time and experience, we did not observe changes in color preference. Future studies should focus on testing color preference on a larger sample size of bees across different scents to further test differences among scent treatments. We were not successful in conditioning male orchid bees in the field to learn to associate a scent cue with a sugar reward, regardless of being presented with a mating-specific scent or a common floral scent. Future studies on the role of scent as an environmental cue for male orchid bees will surely deepen our understanding of the foraging behavior of these important pollinators.

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