COMPARATIVE AND EXPERIMENTAL STUDIES ON THE FORAGING AND EXPLORATORY BEHAVIOR OF FOUR HONEY BEE SPECIES

By

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ABSTRACT

COMPARATIVE AND EXPERIMENTAL STUDIES ON THE FORAGING AND EXPLORATORY BEHAVIOR OF FOUR HONEY BEE SPECIES

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One of the primary challenges foraging animals face is deciding how to divide their time between exploitation of known resources and exploration for new resources. As foraging is costly, investment in exploration should be mediated by natural selection to balance its costs and benefits in ways are tuned to species life history (e.g., lifespan, reproduction rate, activity level), individual state (e.g., experience, hunger, cognitive abilities), and environmental conditions (e.g., reward predictability, distribution, abundance). Efforts to understand the role each factor plays in the exploitation-exploration tradeoff are complicated by the complex scenario-specific ways in which they interact. In addition, the lack of comparative information on exploratory behavior limits our ability to draw generalizations. In this dissertation, I use a combination of experiments and comparative studies in four honey bee species to examine how interactions between life history, individual experience, and environmental conditions shape investment in foraging and exploration. Each chapter addresses how the interaction between two factors (e.g., life history and environment, individual experience and environment) shapes honey bee exploration, or provides an in-depth look at a previously understudied aspect of Asian honey bee life history and foraging behavior that could play a role in shaping their exploratory behavior.

In Chapter 1, I investigate how evolved differences in life history interact with environmental reward context to shape worker investment in exploration in four honey bee species. Species that face higher mortality costs from exploration were generally less exploratory when confronted by a decrease in a familiar reward, but all species increased their investment in exploration as they experienced larger decreases in resource quality. These findings suggest that exploratory behavior has been tuned by natural selection to species life history but is also sensitive to the current environmental conditions. In Chapter 2, I use *Apis mellifera* to investigate how different past experiences with environmental predictability in the location or timing of rewards influence how honey bees search when those rewards are no longer available. My results show that honey bees that have had experience with unpredictable rewards are less precise but equally persistent in their search for vanished rewards, as compared with bees that have had experience with predictable rewards. This result suggests that a bee's experience with resource predictability shapes the way she searches but not her overall investment in exploration. In Chapter 3, I investigate the lifespan and foraging behavior of three honey bee species. For all three species, the age at which a bee first becomes active outside the nest was the primary predictor of her lifespan. Dwarf honey bee (*A. florea*) workers seem to have the longest lifespan, likely due to their much-delayed onset of flight outside the hive, supporting the idea that species that face higher costs from worker mortality should have longer-lived workers. Finally, in Chapter 4, I examine how investment in diurnal and nocturnal foraging activity by the giant honey bee (*A. dorsata*) changes across seasons and lunar cycles. I found that this species could be considered both diurnal and crepuscular, as well as facultatively nocturnal. The amount of nocturnal activity performed depended greatly on environmental illumination and the season, whereas crepuscular activity was extremely high regardless of season or illumination. This study paves the way for further investigations into the mortality costs associated with nocturnal foraging, and how those costs might shape their exploratory behavior during the day.

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INTRODUCTION

Foraging for resources is a necessary part of any animal's life, and the ability to successfully find and use those resources is paramount to the animal's survival and fitness. One of the primary challenges animals face when foraging for resources is deciding how to divide their time between exploitation of known resources and exploration for new ones. Each of these options provides unique benefits, but also has associated costs. Exploitation of known resources provides a guaranteed reward but might result in missing out on an even better reward in the environment, whereas exploration can lead to the discover of new, potentially higher-quality resources but is energetically costly (Stephens 1987; Lima and Dill 1990; Mehlhorn et al. 2015). Because these options are often mutually exclusive, how a foraging animal balances this tradeoff can have a significant effect on its fitness (Stephens and Krebs 1986; Kotler et al. 2004; Berger-Tal and Avgar 2012).

Given its importance, an animal's investment in exploration should be tuned by natural selection to maximize its benefits and minimize its costs. Previous studies on animal exploratory behavior highlight that many factors can influence when and how often an animal explores its environment. Certain environmental conditions can increase exploratory behavior, including environments where rewards fluctuate in quality or vanish altogether. Exploration is beneficial in such environments as it allows animals to find and exploit the best resources in a shifting landscape (Dunlap and Stephens 2012; Keasar et al. 2013; Dunlap et al. 2017). Similarly, a forager's motivational state and past experiences can change its willingness to pay the costs of exploration. In some species hunger reduces risky exploratory behavior, and individuals are often more willing to search or persistent in looking for a reward that has vanished if they have had more experience with it (Katz and Naug 2015; Townsend-Mehler et al. 2012; Al Toufailia et al.

2013). After all, when the animal is starving or uncertain that a better reward exists, the costs of search with no guarantee of a reward are expected to be very high. An animal's general foraging strategy also seems to influence the benefits it could gain from exploration: honey bee workers, which can use social information to find new resources, are much less exploratory than bumble bee workers, which must rely on individual search to find resources. The decreased exploratory tendency in honey bee workers is likely due to the high costs of individual exploration compared to the low costs and high benefits of using social information to quickly identify and exploit resources (Townsend-Mehler et al. 2011; Townsend-Mehler and Dyer 2012).

This previous research suggests that a forager's internal state (e.g., motivation, past experiences, cognitive abilities, etc.), environmental context (e.g., environmental variability, resource distribution and abundance, etc.), and species life history (e.g., lifespan, foraging strategy, etc.) can each play a role in determining her investment in exploration. Understanding how each of these factors shapes an animal's exploratory behavior is made more complicated, though, by the fact that none of them exist in isolation but instead interact in complex, scenario-specific ways to shape behavior. Most investigations into an animal's exploratory behavior have focused on the role of individual selection pressures within highly controlled environments (Eliassen et al. 2007; Katz and Naug 2015; but see Al Toufailia et al. 2013) and have studied only a single species at a time (Kramer and Weary 1991; Cook et al. 2013; Patrick et al. 2017; but see Townsend-Mehler et al. 2011 and Townsend-Mehler and Dyer 2012). How multiple factors interact to determine exploration in naturalistic foraging contexts remains an open question.

Answering this question requires an integrative approach. Experimental studies provide the opportunity to manipulate an animal's internal state and environmental context and examine the consequences for their exploratory behavior, while comparative studies allow selection pressures related to evolved differences in species' life history to be investigated. Observational studies might similarly be needed to identify and characterize differences in life history, and how foraging and exploratory behavior changes depending on the current state of the environment.

In this dissertation, I use a combination of comparative and single species experiments and observational studies to provide insight into how life history, individual experience, and environmental conditions, as well as interactions between the three, shape investment in foraging and exploration in honey bees (*Apis*). Honey bee species are ideal for comparative studies as they are closely related but vary in life history traits that are expected to shape the costs and benefits of exploration, including how they build their nests (commonly referred to as nest architecture), the environments they live in (e.g., tropical versus temperate climates), their body sizes, and their colony sizes (Seeley et al. 1982; Dyer and Seeley 1987; Dyer and Seeley 1991; Oldroyd and Wongsiri 2009). Similarly, their reliance on floral resources that are highly variable in availability, distribution, quality, and predictability within the environment (Edge et al. 2012; Delaney et al. 2015; Wood et al. 2018; Peat and Goulson 2005; Antoń et al. 2017) means that honey bees have likely evolved to be highly responsive to changes in environmental context. The first two chapters of this dissertation address how interactions between life history, individual experience, and environment shape honey bee exploration. The last two chapters of this dissertation provide an in-depth look at previously understudied aspects of Asian honey bee life history and foraging behavior that likely play a role in shaping worker exploratory behavior. This combination of comparative, experimental, and observational approaches provide a unique understanding of how exploration is tuned according to interacting selection pressures within individuals and species, and provides information on Asian honey bee biology and foraging behavior that will be helpful for further comparative investigations into species differences in exploration.

In Chapter 1, I use a comparative experimental approach to investigate how differences in life history between four honey bee species (*A. mellifera*, *A. cerana*, *A. dorsata*, and *A. florea*) shape worker investment in exploration, and how this investment is modulated by changes in environmental reward context. These four honey bee species differ in many factors that might shape the costs and benefits of exploration, including nest architecture, body size, and colony size (Dyer and Seeley 1987; Dyer and Seeley 1991), providing a natural system for testing the hypothesis that the exploitation-exploration tradeoff has been adaptively tuned by selection pressures related to life history differences among species. I found that species that pay higher costs for worker mortality, which is associated with open-nesting architecture (i.e., *A. dorsata* and *A. florea*), were generally less exploratory than species paying lower costs, namely those that nest in cavities (i.e., *A. mellifera* and *A. cerana*). However, all species tended to proportionally increase their investment in exploration as they experienced larger decreases in resource quality. These findings suggest that exploratory behavior has been tuned by natural selection according to species life history but is also sensitive to the current environmental conditions.

In chapter 2, I used *A. mellifera* to examine how a foraging honey bee’s past experience with environmental predictability in the location or timing of rewards influences her subsequent exploration when the world changes state and those rewards are no longer available. In two experiments, I compare the precision and persistence of search between honey bees that have experience in spatially or temporally predictable environments to the search exhibited by honey bees with experience in spatially or temporally unpredictable environments. My results show that honey bees that have experience with unpredictably located or timed rewards search more broadly in space or time, respectively, after those rewards vanish than do bees with experience with predictable rewards. However, a bee's past experience did not affect how persistently she searched. These findings suggest that a bee's experience with environmental predictability in reward location within a patch or reward timing from day to day shapes the precision with which she searches but not her overall investment in exploration.

In chapter 3, I use a comparative observational study to investigate how evolved differences in life history (particularly nest architecture) have shaped the lifespan and foraging behavior of three honey bee species (*A. mellifera*, *A. cerana*, and *A. florea*). These species evolved in different environments (i.e., tropical versus temperate climates), and vary in their nest architecture (Dyer and Seeley 1991; Oldroyd and Wongsiri 2009). For all three species, the age at which a bee first became active outside the nest was the primary predictor of her lifespan, but her activity level after she began flying generally did not influence her lifespan unless she was unusually active, in which case it reduced her lifespan. Open-nesting dwarf honey bee (*A. florea*) workers have the longest lifespans, due in large part to their much-delayed onset of flight outside the hive. Surprisingly, the tropical Eastern hive bee (*A. cerana*) has a longer lifespan than the temperate Western honey bee (*A. mellifera*) even though both species became active outside the hive at the same age. These species differences partially support the prediction that higher costs of worker mortality associated with open-nesting architecture should select for longer worker lifespans and reduced foraging activity. This study also paves the way for future investigations into how life history traits like lifespan and nest architecture interact with environmental habitat (i.e., tropical versus temperate) to shape foraging behavior.

In chapter 4, I examine how investment in diurnal and nocturnal foraging activity by the giant honey bee (*A. dorsata*) changes across seasons and lunar cycles. The giant honey bee has the unusual ability to forage during both the day and night during certain times of the month, namely when the moon is at least half full (Dyer 1985; Somanathan et al. 2009). However, lack of information on the frequency of nocturnal foraging and the factors that determine investment in nocturnal foraging have to date limited our understanding of *A. dorsata's* foraging behavior. Based on systematic observations of foraging activity throughout the diel cycle at all phases of the moon and across all seasons, I found that this species could be considered both diurnal and crepuscular, as well as facultatively nocturnal. The amount of nocturnal activity performed depended greatly on environmental illumination and the season, while crepuscular activity remained extremely high regardless of season or illumination. At times, nocturnal activity was even higher than diurnal activity, though this was seen only during the full moon and during certain seasons. This study paves the way for further investigations into the benefits and costs associated with nocturnal foraging, and how those benefits and costs might shape the foraging and exploratory behavior of *A. dorsata* during the day.

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CHAPTER ONE:

Adaptive tuning of the exploitation-exploration trade-off in four honey bee species[1]

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ABSTRACT

Foraging animals continually face the decision of whether to exploit known resources or explore for new ones, a decision with large implications for their fitness. Though animal foraging decisions have been extensively studied, we currently lack a deep understanding of how the exploitation-exploration tradeoff has evolved, including how it is shaped by divergent selection pressures between species. As a first step toward examining how the exploitation-exploration tradeoff has been adaptively tuned by natural selection, we compared the exploratory behavior of four honey bee species that differ in traits such as nest architecture, body size, and colony size. In a common behavioral context—exploratory behavior triggered by a decrease in quality of a known food resource—we found species differences in exploratory behavior that are consistent with selection arising from evolved differences in nest architecture, though the behavioral differences were also strongly influenced by the magnitude of the reward decrease. We had expected that species that build their nests in the open, and hence face a higher fitness cost of worker attrition compared with species that inhabit protective cavities, would be less likely to prolong unrewarded search when food declines in quality. The behavioral data were partially consistent with this expectation. However, at times environmental context strongly modulated species differences in behavior that would be expected based on nest architecture. Overall, our results suggest that the resolution of the exploitation-exploration tradeoff has been adaptively tuned between species by a number of interacting selection pressures.

INTRODUCTION

Animals face a fundamental choice when foraging for resources: whether to continue collecting food at a known resource or to explore to find a new one. Often, these options are mutually exclusive, and entail a tradeoff between investment in exploitation or exploration. Exploiting a known resource provides a guaranteed reward but might result in missing out on an even better reward in the environment. Exploring, on the other hand, can lead to the discovery of new, potentially higher-quality resources, but is energetically costly, especially if no reward is discovered (Stephens 1987; Lima and Dill 1990; Mehlhorn et al. 2015). Whether to exploit or explore is a choice that foraging animals must make throughout their lives, and thus is expected to have a cumulative effect on an animal’s fitness (Stephens and Krebs 1986; Kotler et al. 2004; Berger-Tal and Avgar 2012). We would therefore hypothesize that fitness costs associated with species-specific life history patterns will define how natural selection has shaped the resolution of this tradeoff.

Much prior theoretical and empirical research has investigated the circumstances under which animals should switch between foraging options (see Pyke 1984; Lima and Dill 1990; Mehlhorn et al. 2015; Pyke 2019 for selected reviews on this topic). The exploitation-exploration tradeoff, however, has not received as much attention. Previous studies on this topic have generally tested foragers who undergo extensive training before being tested in highly controlled environments (Eliassen et al. 2007; Yogeswaran and Ponnambalam 2012; Katz and Naug 2015), limiting the real-world applicability of the insight we gain from these experiments since foraging animals generally do not have detailed experience of the options and their payoffs when foraging in natural environments (McNamara 1982; Wajnberg et al. 2000). In addition, most prior studies of this tradeoff have employed experiments with only a single species (Kramer and Weary 1991; Cook et al. 2013; Patrick et al. 2017); hence they are not designed to reveal if or how the exploitation-exploration strategies of different species have diverged under the influence of species-specific selection pressures. Comparative studies can provide insight into this question by enabling the study of additional selection pressures that otherwise could not be investigated in studies of a single species. Prior studies of bumble bees and honey bees investigated the effect of foraging strategy (individual exploration vs social recruitment) on exploratory tendency (Townsend-Mehler et al. 2011, Townsend-Mehler and Dyer 2012), but these species are not especially closely related, making it difficult to isolate the most important selection pressures associated with evolutionary divergence.

To examine the hypothesis that the exploitation-exploration tradeoff may have been adaptively tuned by selection pressures related to biological differences among species, we compared four species of *Apis* to look for differences in how easily search is triggered and how long it persists before foragers accept a known food option. Though our ability to isolate the roles of specific selection pressures is limited by our examination of only four species, we provide the first comparative information on differences in the exploitation-exploration tradeoff in honey bees, and discuss how these differences are consistent or not consistent with hypotheses regarding the tuning of the tradeoff.

To examine differences in exploration between honey bee species, we used an assay designed to provoke a well-established psychological phenomenon known as a “negative incentive contrast effect” (Flaherty 1982). This phenomenon occurs when a forager experiences an unexpected decline in the quality of a food resource. The effect is a marked depression in the rate of consumption of the resource – below that of control bees that have experienced only the lower-quality reward – coupled by a tendency to explore the surrounding environment (Flaherty 1982). Studies of rats (Pecoraro et al. 1999) and bees (Waldron et al. 2005; Weigmann and Smith 2009; Townsend-Mehler et al. 2011; Townsend-Mehler and Dyer 2012), as well as mathematical models (McNamara et al. 2013), have suggested that the adaptive function of the negative incentive contrast effect is to ensure exploration when there is evidence from recent past experience that better foraging options would be available in the environment. Thus, we use this effect to compare how easily exploration is triggered by a reward downgrade, and how long it persists before bees decide to exploit the reduced reward.

Honey bees (genus *Apis*) offer an excellent model for exploring evolutionary divergence in foraging strategies. The age-based division of labor in honey bees means that foraging bees are focused solely on food collection, without distraction from other tasks such as cleaning the hive or rearing brood, let alone personal reproduction. Also, reliance on floral nectar simplifies experimental studies of how exploratory foraging behavior changes in response to varying costs and benefits (Robinson 1992; Seeley 1995). In addition, the diversity of *Apis* species offers a powerful tool for studying evolved species differences in exploratory behavior. Honey bee species are closely related, but differ markedly in traits such as colony size, body size, lifespan, and nest architecture that we expect could influence the fitness correlates of food gathering and search behavior (Seeley et al. 1982; Dyer and Seeley 1987; Dyer and Seeley 1991; Oldroyd and Wongsiri 2009; Ruttner 1988; Bhagavan et al. 2016). In this study, we examined foragers of four honey bee species representing large variation in these traits to harness the power of the comparative approach for gaining insights into differences in the tuning of the tradeoff between exploration and exploitation.

Nest architecture, in particular, is expected to shape the costs and benefits of exploration as it has large consequences for colony demography, and hence for the evolution of worker traits that could be correlated with foraging costs and benefits. Two strikingly different patterns of nest architecture are seen among *Apis* species. In Asia, giant honey bees (e.g., *A. dorsata*) and dwarf honey bees (e.g., *A. florea*) nest in the open on a single comb protected by a curtain of bees. In contrast, Eastern hive bees (e.g., *A. cerana*) and the Western hive bees, *A. mellifera* (originally distributed in Europe, Africa, and West Asia), build multiple parallel combs in an enclosed cavity. Open-nesting species have significantly fewer brood cells per worker as compared to cavity-nesting species (Table 1.1) but require a large worker population to maintain the protective curtain that covers the nest (Seeley et al. 1982). Because all four of the honey bee species studied here exhibit similar developmental times (18-22 days; Kapil 1959; Sandhu & Singh 1960; Seeley 1985; Tan 2007), but open-nesting species have lower brood per worker ratios, the colony's capacity to replace workers lost to mortality is expected to be lower in open-nesting species than in cavity-nesting species (see Table 1.1; though there is no data available on *A. dorsata*, it is expected that they exhibit a similarly low brood per worker ratio as *A. florea* given the similarities in their cell per worker ratios; Dyer & Seeley 1991). This decreased rate of worker replacement, in combination with the need to maintain a protective curtain, would make the cost of worker mortality higher for open-nesting colonies as compared to cavity-nesting colonies (Dyer & Seeley 1991). The need to maintain this excess of workers seems to have selected for physiological and behavioral traits in open-nesting species that reduce foraging costs and hence prolong worker lifespan (Dyer and Seeley 1987; Dyer and Seeley 1991; Underwood 1991). Similarly, we hypothesized that the higher cost of worker mortality for open-nesting species (as compared with cavity-nesting species) should also have influenced the costs and benefits of exploitation vs exploration. Specifically, given the high costs of unrewarded search (e.g., energy and time expended without the compensation of a reward, opportunity costs of not visiting rewarding resource, risk of predation, wing wear; Lichtenberg et al. 2020), our hypothesis predicts that, all else being equal, open-nesting species would be quicker to call off search and accept a downgraded reward.

Table 1.1. Species differences in colony demographic traits in honey bee species.

Property	*A. florea*	*A. dorsata*	*A. cerana*	*A. mellifera*
Cells/Worker Ratio	0.80[2]	0.74[2]	3.56 [2]	3.99 [3]
Brood/Worker Ratio	0.59 [2]	No data	1.88 [2]	1.77 [3]
Average Lifespan (days)	61.2 [4]	No data	28.6 [5]	30-40[6]

Two other important traits that vary dramatically among *Apis* species, and that could produce divergent fitness costs associated with search behavior, are colony size and body size. Average colony size varies among species from 6,000 workers in *A. florea* to upwards of 50,000 workers in *A. dorsata* (Table 1.2; Seeley et al. 1982). These large differences in colony size, and presumably the size of the foraging workforce (Lindauer and Watkin 1953; Seeley 1983), may influence how the costs and benefits of individual decisions have an impact on colony fitness. In particular, we might expect that unrewarded exploration by foragers should result in lower total and per-capita costs in species with larger colonies, with their larger foraging workforce ensuring a larger number of successful foragers to compensate for those who persist in searching without reward (Lindauer and Watkin 1953; Seeley 1983). If so, then species with larger average colony sizes would be more likely to prolong search after a reward is downgraded than species with smaller average colony sizes (e.g., *A. dorsata* search the most and *A. florea* search the least). As for worker body size, among the species studied here the mass of unloaded foragers varies over a five-fold range from the smallest (*A. florea*) to the largest (*A. dorsata*) species (Dyer and Seeley

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1987; Dyer and Seeley 1991; Underwood 1991). Larger body size is expected to produce higher costs of exploration, as the metabolic cost of flight increases with body size (Harrison and Fewell 2002; Townsend-Mehler and Dyer 2012). Based on body size considerations, larger bees would be expected to show less exploration than smaller bees (e.g., *A. dorsata* to search the least and *A. florea* to search the most for a better reward). In summary, increasing colony size would be expected to select for increasing exploration; in contrast, increasing body size would be expected to select for decreasing exploration. These traits give rise to contrasting predictions about exploration tendency that are not mutually exclusive from those based on the hypothesized selection pressures associated with nest architecture and colony demography, which instead predict high exploration in cavity-nesting species and low exploration in open-nesting species (Table 1.2).

Table 1.2. Predicted trait effects on exploration tendencies. Increasing body size and increasing colony size lead to opposing predictions for exploration tendencies but should both result in relationships between size and amount of exploration if they play a large role in determining exploration.

Species	Nest Architecture		Colony Size		Body Size	
	Nest type	**Predicted Effect on Exploration**	**Number of workers[2,7]**	**Predicted Effect on Exploration**	**Worker mass (mg)[7,8]**	**Predicted Effect on Exploration**
A. florea	Open	Decreased	6,000	Decreased	23	Increased
A. dorsata	Open	Decreased	>36,000	Increased	115	Decreased
A. cerana	Cavity	Increased	8,000	Mildly Decreased	50	Mildly Increased
A. mellifera	Cavity	Increased	15,000	Increased	77	Mildly Decreased

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In this study, we describe for the first-time differences in the exploratory behavior of four honey bee species in a semi-natural foraging context. We studied exploration by free-flying bees using an assay where a high-quality food source was downgraded in quality (i.e., the sucrose concentration was reduced) to varying degrees. This downgrade in reward triggered behavior typical of a negative incentive contrast effect (Flaherty 1982): bees repeatedly probed the feeder and flew around as if searching for the better food they expected to find there. We then measured each bee's propensity to engage in unrewarded exploration by recording the latency to resume feeding and probing behavior at the test feeder before resuming feeding. These indicate resistance to feeding, which we assume correlates with exploration of the environment (Flaherty 1982; Townsend-Mehler et al. 2011; Townsend-Mehler and Dyer 2012).

To take advantage of the naturalistic environmental context of our study, we broadened our study of exploration by including ecological variables such as season and the magnitude of the decline in resource quality. The behavior of honey bee foragers is strongly influenced by seasonal availability of resources (Al Toufailia et al. 2013; Danner et al. 2016), as are colony food stores (Szabo & Heikel 1987; Seeley 1995). Foraging bees tend to explore more when at a higher nutritional state (Katz & Naug 2015) or when colony food stores are larger (Katz & Naug 2016; Dyer et al. in preparation), so it is likely that seasonal differences in resource availability will influence a forager's investment in exploration. As our study was conducted over multiple seasons, we could examine how species varied in their exploration between seasons, providing a more naturalistic perspective on species differences in exploration. Outside of seasonal resource availability, resources can also decline in reward on a shorter time scale, such as when they become depleted by competitors or change in quality with humidity (Corbet et al. 1979). The impact of such a decline can depend on environmental context, where declines of equivalent magnitude can yield differing responses based on their relationship to the global average (McNamara et al. 2013). Species can also differ in their response to the same decline in resource quality (Townsend-Mehler et al. 2011; Townsend-Mehler & Dyer 2012), suggesting that the interaction between reward quality and exploration may be complex. By examining declines in resource quality to levels above, equivalent to, and below the average recently experienced by foragers, we provide insight into how current environmental resource context influences exploration. The inclusion of these ecological variables complicates our power to test hypotheses about the evolution of species differences, on top of sample-size constraints from the small number colonies and species being compared. However, it provides a fuller and more ecologically relevant picture of how exploration is shaped in honey bee species, as it could be tuned by these environmental factors as well as the interacting selection pressures of nest architecture, colony size, and body size.

Given the higher mortality costs faced by open-nesting species with their need to maintain a protective curtain, the hypothesis that nest architecture primarily shapes exploratory behavior predicts that the open-nesting species, *A. dorsata* and *A. florea*, would invest less time in exploration following a reward downgrade than would the cavity-nesting species, *A. cerana* and *A. mellifera* (Table 1.2). This prediction would be supported by a tendency to resume feeding at the downgraded reward more quickly in open-nesting species, particularly if the downgrade resulted in food concentrations higher than the environmental average that foragers had recently experienced. If instead body and colony size, which are positively correlated in how they vary across species, have primarily shaped the exploratory tendencies of these species, our hypotheses would predict a correlation between exploratory tendencies (e.g., latency to resume feeding) and colony or body size. In particular, the hypotheses would give contrasting predictions, with an increase in exploration predicted with increasing colony size (e.g., *A. dorsata* and *A. mellifera* searching more than *A. cerana* and *A. florea*), but a decrease in exploration predicted with increasing body size (e.g., *A. dorsata* and *A. mellifera* searching less than *A. cerana* and *A. florea*). These would lead to dramatically different patterns than what would be expected if nest architecture primarily shapes species' relationships with exploration, since the open-nesting species are the largest (*A. dorsata*) and smallest (*A. florea*) in both body size and colony size (Table 1.2). If seasonal resource availability determines the exploratory behavior of these species, species would be expected to exhibit similar correlations between season and investment in exploration (e.g., showing reduced exploration in seasons of lower resource availability), though the magnitude of investment might differ between species. Finally, if the magnitude of resource quality decline determines exploration, all species should exhibit the most exploration after the largest decline, though they might differ in amount of exploration or in the amount of change between downgrade levels.

MATERIALS AND METHODS

General Setup

We conducted the study from July 2018 to April 2019. Experiments on *Apis mellifera* were conducted on the Michigan State University campus in East Lansing, Michigan from July-September 2018. Experiments on the three Asian honey bee species were conducted on the campus of the National Centre for Biological Sciences - TIFR in Bangalore, Karnataka, India from October 2018-April 2019. We studied one colony each of *A. mellifera* and *A. dorsata,* two colonies of *A. cerana*, and three colonies of *A. florea*. Multiple colonies were used for *A. cerana* and *A. florea* because previous colonies absconded (i.e., abandoned their nest) during the experiment, which is typical behavior for these species. The use of a single colony for *A. mellifera* and *A. dorsata*, a common practice in studies of learning, memory, and the regulation of foraging in bees (Seeley 1995), makes the underlying assumption that any between-colony variation in these psychological processes is small relative to the effects of the experimental manipulation. Here, we had to make the additional assumption that variation between species would also be much greater than within- or between-colony variation, though we acknowledge that this assumption is not necessarily true. However, we found no significant differences in latency to resume feeding between the two *A. cerana* colonies, or in latency or probing behavior between the three *A. florea* colonies studied here, lending some support to our assumption (Table S1.1), though we did find a significant difference in probing behavior in *A. cerana* (P = 0.04107). *Apis mellifera* was housed in a five-frame nucleus hive that was regularly supplied with sugar water as needed. We collected *Apis florea* colonies from local beekeepers in Bangalore and maintained them in sheltered boxes on the NCBS-TIFR campus. *Apis cerana* colonies were similarly collected from local beekeepers and housed in six frame hive boxes on the NCBS-TIFR campus. We used a natural *A. dorsata* colony whose nest was hanging from a third-story ledge of a campus building (Figure 1.1). All colonies were studied in open, free-flying conditions. We monitored colony state for all four species, either through manually weighing the colony or tracking recruitment, to ensure all colonies were healthy, actively foraging, and did not experience large fluctuations in food stores. All colonies of Asian honey bees were located within half a kilometer of each other on the NCBS-TIFR campus.

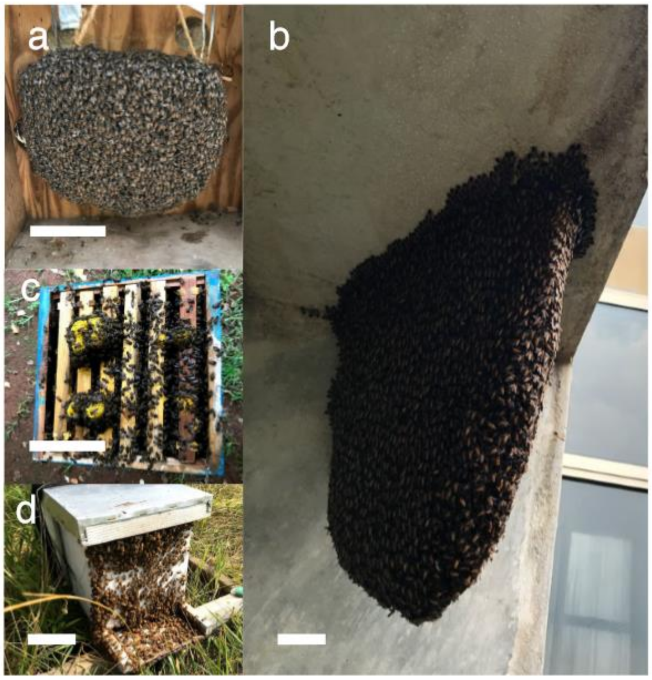

Figure 1.1. Example nests of four species of honey bees. Example nests of (a) *A. florea* in a sheltered wooden box, (b) *A. dorsata* hanging from a building ledge, and hives of (c) *A. cerana* in a closed hive box (opened solely for this picture), and (d) *A. mellifera* in a nuc box are shown. All Asian honey bee nests were maintained and tested on the NCBS-TIFR campus in Bangalore, India, while the *A. mellifera* hive was maintained and tested on the Michigan State University Campus in Michigan, USA. Scale bars in each picture represent approximately 10 cm.

Training Protocol

We followed an assay developed by Townsend-Mehler (Townsend-Mehler et al. 2011; Townsend-Mehler and Dyer 2012) for honey bees and bumble bees. First, we trained foragers from each colony to its own low-quality "constant" feeder containing unscented sucrose solution at a low concentration (0.25M – 0.5M) sufficient to maintain a constant population of foragers at the feeder without eliciting a large amount of recruitment. The low-quality feeder was placed 12-15m from the colony, and bees were given access to it for a few hours every day.

After they were trained to the constant feeder, bees were individually marked using either dots of enamel paint or numbered tags. We then trained small groups of bees (3-4 at a time) from the constant feeder to a high-quality "test" feeder containing a 2M sucrose solution. The test feeder was located 5m away from the constant feeder; it consisted of a plastic bottle cap (2.5cm diameter, 1cm deep) inverted and filled with sugar solution. We fitted a metal screen across the opening, which enabled bees to land and feed without standing in the sugar water.

To recruit test bees, we brought a test feeder cap to the constant feeder and introduced individually marked bees to it using a pipette that also contained a 2M sucrose solution. Once test bees were feeding from the test feeder, we carried the feeder to the testing location and left it there until bees finished feeding. Most bees, after encountering better food, did learning flights (Wei and Dyer 2009), then flew back to the hive. The learning flights enable bees to learn landmarks around a feeding location so that they can find their way there on a future trip. A second bottle cap containing 2M sugar water was placed at the testing location so that test bees would be rewarded when they visited the test feeder without assistance. Test bees typically required two to three assisted trips to the test feeding location before they were able to locate the test feeder independently. All assisted and unassisted visits to the test feeder were recorded during the training period through visual monitoring of the test feeder. Bees that failed to find the test feeder on their own after four assisted trips were not used in the experiment. After test bees arrived at the test feeder on their own, they received an average of four additional unassisted foraging trips. As we trained the test bees in small groups, some bees found the feeder faster than others leading to slight variations in the number of unassisted training trips received by the time the test started.

Testing Protocol

After the test bees completed four unassisted visits to the test feeder, we elicited a negative incentive contrast effect by lowering the concentration of the sugar solution in the test feeder from 2.0M to 1.0M, 0.5M, or water. This downgrade in resource quality marked the start of the testing period, which lasted for 30 minutes after the test bees experienced the downgraded test feeder. To measure the disruption in feeding, we recorded the number of probes (short contacts) each test bee made at the downgraded food at the test feeder and the latency to resume feeding at either the test feeder or the constant feeder. Both feeders were recorded using a video camera for the duration of the test, so all contacts with either feeder were recorded. We considered a bee to be 'feeding' if she remained on the feeder with her head bent towards the sucrose solution for at least 20 seconds; contacts lasting less than 20 seconds were recorded as probes. As bees rarely feed through a series of extended probes (Townsend-Mehler et al. 2011), we felt confident counting contacts less than 20 seconds as probing, not feeding, events. However, because we tested bees in small groups, if a bee appeared to be feeding (prolonged stationary contact with the feeder) but was interrupted by another bee, we considered her to be feeding if she quickly landed again and the total duration of her contacts was over 20 seconds. If a bee failed to resume feeding within the 30-minute testing period, then her latency was assigned as 30 minutes. We randomized the downgrade concentration among tests within each species. Sample sizes varied based on species and downgrade concentration, with an average of 20 bees tested in each treatment group in each species.

Statistical Analyses

We analyzed the results of this experiment using R v3.3.2 (R Foundation for Statistical Computing, Vienna, Austria). We separately analyzed the 0.5M and 1M downgrade conditions from the water condition because the water condition required bees searching for a reward to make multiple decisions: whether to abandon the test feeder to return to the constant feeder and whether to resume feeding. In contrast, the 0.5M and 1M conditions required only the latter decision.

To analyze the 0.5M and 1M downgrade conditions, we fit separate models for our response variables of latency to resume feeding and number of probes at the test feeder before feeding (called 'number of probes'); both were considered metrics of exploration as they signaled a non-acceptance of the downgraded reward. Latency to resume feeding was analyzed using a general linear mixed-effect model ('lmer' function in R v3.3.2). As the residuals of the final model were not normally distributed, the response variable latency to resume feeding was square-root transformed leading to normally distributed residuals. Number of probes was analyzed using a generalized linear mixed effects model with a negative binomial distribution ('glmer' function in R v3.3.32), as it consisted of over dispersed count data. In both models, the fixed effects of nest architecture (open or closed), species (*A. cerana*, *A. mellifera*, *A. dorsata*, or *A. florea*), concentration downgrade (2M to 1M, 0.5M, or water), season (autumn, winter, summer, early, or late), the interaction between species and concentration downgrade, the interaction between concentration downgrade and season, and the interaction between species and season were considered in candidate models. Colony ID was included as a random effect in all candidate models to account for between-colony variation. Model selection was performed, with the final model chosen based on AICc. Season was determined based on temperature and floral resource availability. For the work conducted in India, where we followed local practice for naming the seasons, the months of October – December were considered 'autumn', January – February were considered 'winter', and March – April were considered 'summer'. For the research conducted in Michigan, experiments conducted in July were considered early summer, while experiments conducted in August and September were considered late summer. (Across the Northeast of the US, nectar resources are far more abundant in early summer than in late summer; Delaney et al. 2015.) The final model for latency and final model for number of probes were both analyzed using ANOVA. Post-hoc tests were conducted where appropriate using a Tukey adjustment to reduce the likelihood of Type 1 errors.

In the water condition, we observed a unique behavior where some *A. dorsata* foragers began collecting water instead of going to the constant feeder and resuming feeding on the 0.5M sucrose solution. Before running analyses on the water condition, therefore, we first investigated if the *A. dorsata* foragers' latency to resume feeding or probing behavior on water were significantly different from that of those who fed on the sugar solution at the constant feeder. Because we found no significant differences between foragers who collected water or fed on sugar solution (Table S1.2), we included all *A. dorsata* foragers in the general analysis of the water condition. Latency was analyzed using a general linear mixed-effect model, while number of probes was analyzed using a generalized linear mixed-effect model with a negative-binomial distribution. For both response variables, candidate models including the fixed effects of nest type, species, season, and the interaction between species and season were considered, with colony ID included as a random effect. We performed model selection based on AICc, choosing final models with the lowest AICc value. Final models were then analyzed using ANOVA and post-hoc tests with a Tukey correction.

As a further window on species differences in exploration and its interaction with concentration downgrade, we performed Kaplan-Meier survival analyses on the data for latency to resume feeding with bees having a latency over 30 minutes censored ('survival' package in R v3.3.2). We analyzed pairwise comparisons between survival curves ('survminer' package) using a Benjamini-Hochberg (BH) adjustment. We similarly performed a redundant analysis of probing behavior using pairwise t-tests with a Bonferroni correction.

RESULTS

Behavioral Response of Bees to Resource Quality Downgrade

As expected, bees that returned to the test feeder to find that it had been downgraded from 2M to a lower sucrose concentration or water showed a pronounced negative incentive contrast effect, probing the feeder repeatedly without feeding and flying around. We interpreted these behaviors as exploration for the higher quality food they had expected to find. Eventually some bees resumed feeding on the downgraded sucrose solution; we interpret this resumption of feeding as a decision to end exploration and resume exploitation of the food resource. We found considerable variation among species and across experimental conditions in how intensively the bees probed at the downgraded test feeder during this exploration period and in the latency before the resumption of feeding. In addition, a unique behavior was seen in some *A. dorsata* foragers tested in the water condition. Of the 22 *A. dorsata* foragers tested in the water downgrade condition, 5 (23%) actually stayed at the test feeder and ingested the water, a behavior not seen at all in the other species. Overall, our results were generally consistent with the hypothesis that colony demography has played a role in shaping the exploitation-exploration decisions of honey bee species – the open-nesting species (*A. florea* and *A. dorsata*) tended to be quicker to abandon search and resume feeding while the cavity-nesting species (*A. cerana* and *A. mellifera*) tended to prolong search – but the results are complicated by interactions with experimental conditions and season.

Final Models Selected

In the experimental conditions where the food in the test feeder was downgraded from a concentrated (2M) to a more dilute sugar solution (the 0.5M and 1.0M conditions), our final models for both latency to resume feeding and number of probes included the fixed factors of nest type, species, concentration downgrade, season, and the interaction between species and concentration downgrade, as well as the random effect of colony ID (Tables S1.3 – 1.4). In the experimental condition where the food in the test feeder was downgraded to water – analyzed separately due to the more complex behavioral response it required – our final model for latency included the fixed factors of nest architecture, species, season, the interaction between species and season, and the random effect of colony ID (Table S1.5). The final model for number of probes in the water condition included only the fixed effects of nest architecture and species, as well as the random effect of colony ID (Table S1.5).

Relationship Between Nest Architecture and Exploratory Behavior

In the 0.5M and 1M condition, we found that open-nesting was associated with a significantly shorter latency to resume feeding as compared to cavity-nesting (X^2 = 9.4118, *DF* = 1, *P* = 0.0021559); probing behavior, a related measure of exploration, showed the same trend but was not significant (X^2 = 1.1704, *DF* = 3, *P* = 0.279311; Figure 1.2). In the water condition, we found no relationship between nest architecture and either latency to resume feeding (X^2 = 0.8896, *DF* =1, *P* = 0.34558) or probing behavior (X^2 = 2.4041, *DF* =2, *P* = 0.1210).

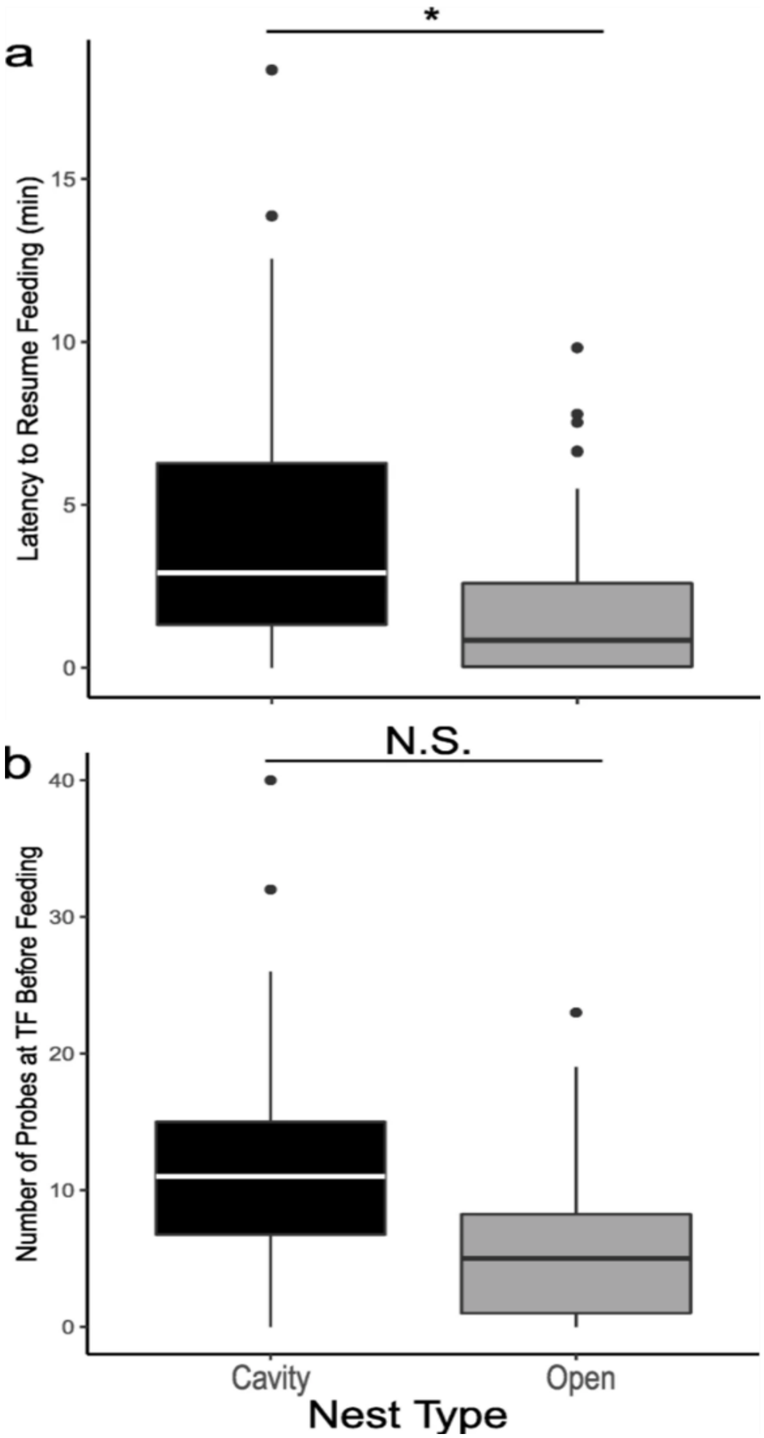

Figure 1.2. Latency to resume feeding and probing behavior of open- and cavity-nesting species. Boxplots of (a) latencies to resume feeding and (b) number of probes at the test feeder before feeding for open- and cavity-nesting species are shown. Nest architecture had a significant effect on latency to resume feeding (represented with *), with open-nesting species having significantly shorter latencies to resume feeding (X^2 = 9.41, *DF* = 1, *P* = 0.0022). Nest architecture did not have a significant effect on probing behavior (X^2 = 1.17, *DF* = 3, *P* = 0.28).

Interacting Effect of Species and Concentration Downgrade on Exploration

In the 0.5M and 1M conditions, we found that latency to resume feeding and probing behavior were significantly affected by species (latency: X^2 = 26.84, *DF* =2, P = 1.48e-6; probes: X^2 = 45.16, *DF* =2, *P* = 1.56e-10) and the concentration downgrade (latency: X^2 = 33.81, *DF* =1, *P* = 6.07e-9; probes: X^2 = 29.74, *DF* =1, *P* = 4.95e-8), as well as the interaction between these two factors (latency: X^2 = 11.27, *DF* =3, *P* = 0.010; probes: X^2 = 12.30, *DF* =3, *P* = 0.0064). When examining the interaction between species and concentration downgrade for latency to resume feeding, we found all species generally exhibited higher average latencies to resume feeding in the greater downgrade condition (from 2M to 0.5M) as compared to the smaller downgrade condition (from 2M to 1M) (Figure 1.3). The differences in latency to resume feeding from the 0.5M to the 1M conditions were larger and significant for both *A. cerana* (*P* = 0.0006; Tukey correction) and *A. mellifera* (*P* < 0.0001; Tukey correction), but was not significant for *A. florea*, which showed no significant decrease in latency to resume feeding between conditions (*P* = 0.9549; Tukey correction) or *A. dorsata* (*P* = 0.063; Tukey correction), though the difference for *A. dorsata* tended towards significance (Figure S1.1a). Similarly, when examining the interaction between species and concentration downgrade for probing behavior, we found that average number of probes was generally higher in the 0.5M condition as compared to the 1M condition (Figure 4), which again makes sense given that this involves a larger decrease in concentration from what bees expected. However, the decrease in number of probes between 0.5M and 1M conditions were only significant for *A. dorsata* (*P* < 0.0001; Tukey correction) and *A. mellifera* (*P* = 0.0002; Figure S1.1b), suggesting that the decreased probing of these two species in the 1M condition is driving the interaction seen between species and concentration downgrade for probing behavior.

An analysis of latency survivorship curves provides a redundant but nevertheless illustrative picture of the interaction patterns seen between concentration downgrade and species. In the 0.5M condition, both *A. dorsata* and *A. florea* searched significantly less than did *A. cerana* (*A. dorsata*: P = 0.00013; *A. florea*: P = 0. 00258; BH adjustment) or *A. mellifera* species (*A. dorsata*: P = 0. 00067; *A. florea*: P = 0.030; BH adjustment; Figure 1.3a). However, in the 1M condition species differences in latency were driven primarily by *A. dorsata*, who searched significantly less than any other species (*A. florea*: P = 2.7e-10; *A. cerana*: P =7.7e-11; *A. mellifera*: P =7.3e-7; BH adjustment), and by *A. mellifera*, who searched for a shorter period of time than did *A. cerana* (P = 0.028; BH adjustment; Figure 1.3b). Similarly, redundant analyses of probing behavior within the 0.5M concentration downgrade found that *A. dorsata* probed significantly less than *A. cerana* (P = 7.1e-5; Bonferroni adjustment) and *A. mellifera* (P = 4.2e-7; Bonferroni adjustment), and *A. florea* probed less than *A. mellifera* (P = 0.0095; Bonferroni adjustment; Figure 1.4a). As with latency, *A. dorsata* was the primary driver of species differences in the 1M condition, probing less than any other species (*A. florea*: P = 0.0011; *A. cerana*: P = 4.2e-6; *A. mellifera*: P = 0.00011; Bonferroni adjustment; Figure 1.4b).

In the water condition, we found no effect of species on either latency to resume feeding (X^2 = 1.55, DF =2, P = 0.46) or probing behavior (X^2 = 5.12, DF =2, P = 0.077). However, the effect of species on probing behavior in the water condition tended towards significance (P = 0.077); this trend was presumably driven mostly by the reduced probing of *A. florea* (Figure 1.5).

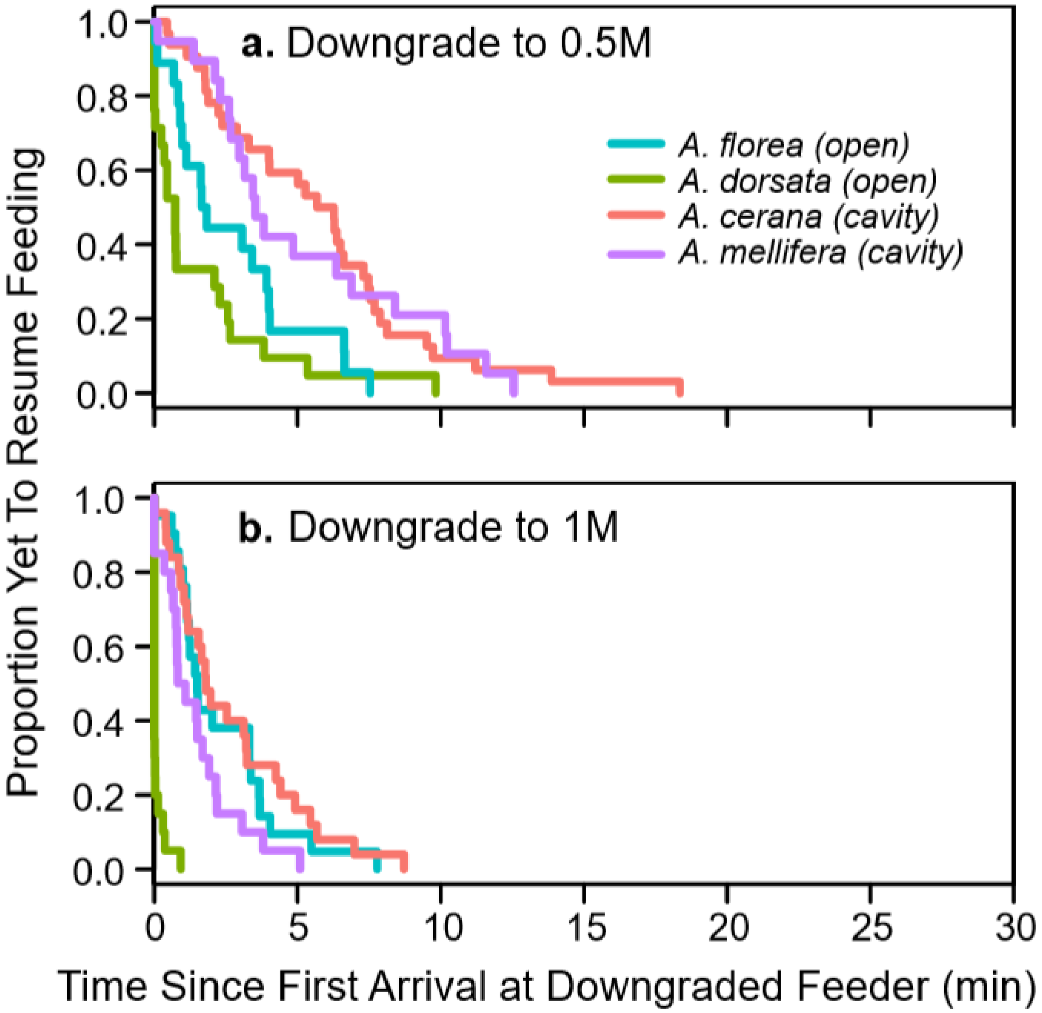

Figure 1.3. Survival curves for latency to resume feeding in each species across 0.5M and 1M downgrade conditions. Survival curves for each species are shown in downgrade conditions (a) 2M to 0.5M and (b) 2M to 1M. Open-nesting species were faster to resume feeding than cavity-nesting species in the 0.5M. *A. dorsata* consistently ceased searching and resumed feeding faster than any other species, and seems to be the primary driver of the species differences found in the 1M downgrade condition.

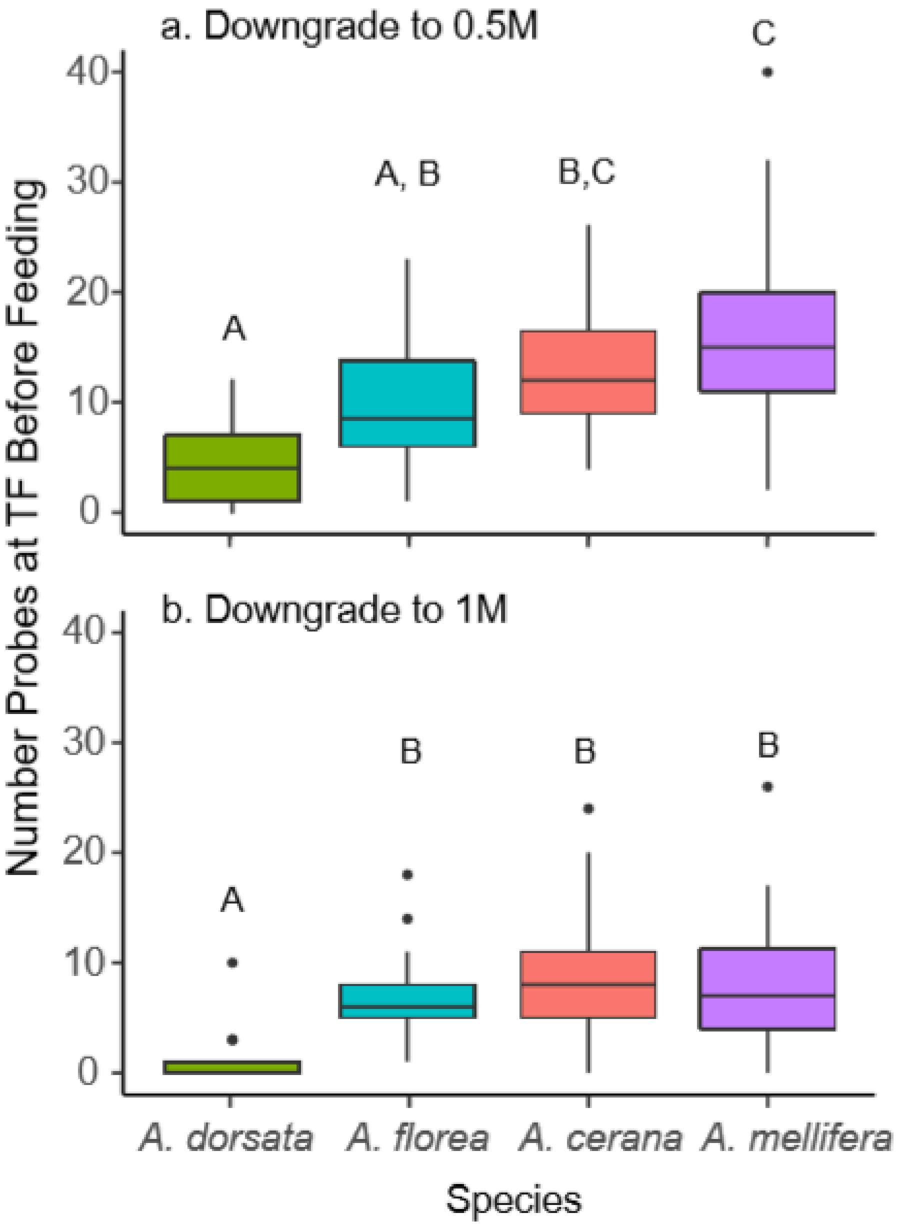

Figure 1.4. Probing behavior at the test feeder in each species across 0.5M and 1M downgrade conditions. Boxplots showing the number of probes made at the test feeder after the downgrade and before feeding for each species in downgrade conditions: (a) 2M to 0.5M and (b) 2M to 1.0M are shown. Significant species differences in probing behavior (X^2 = 45.1583, *DF* =2, *P* = 1.563e-10) were driven primarily by *A. dorsata*, who probed less than any other species in both conditions. Letters above each boxplot indicate if species behavior significantly differed (different letter) or not (same letter).

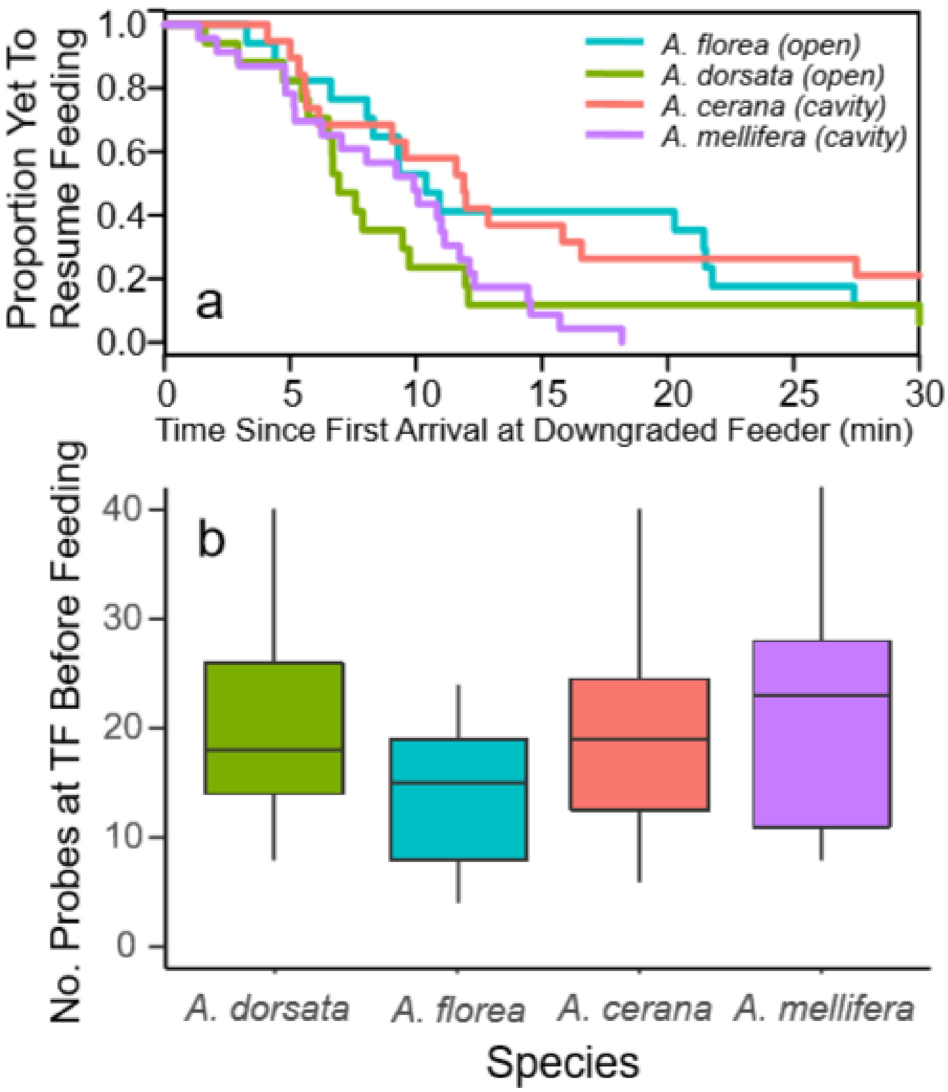

Figure 1.5. Latency to resume feeding and probing behavior in the water condition. (a) Log-rank survival analysis of the water downgrade condition found that species did not differ in their latency to resume feeding in the water condition (X^2 = 1.5516, *DF* =2, *P* = 0.46033). (b) Species did not differ in their probing behavior in the water condition (X^2 = 5.1175, *DF* =2, *P* = 0.0774).

Seasonal Patterns in Exploration

We found that the main effect of season was significant for both latency to resume feeding (X^2 = 19.52, *DF* =3, *P* = 0.00021) and number of probes (X^2 = 14.26, *DF* =3, *P* = 0.0026) in the 0.5M and 1M conditions, but did not have a significant effect on latency or probing in the water condition (X^2 = 5.11, *DF* =3, *P* = 0.164; season was not included in the AICc-selected model for probing behavior in the water condition). When examining bees tested in India, we found no pairwise differences in latency to resume feeding between bees tested in autumn, winter, and summer (autumn vs winter: *P* = 1.000; autumn vs summer: *P* = 0.28; Tukey corrections), though bees tested in summer tended towards a shorter latency than those tested in winter (winter vs summer: *P* = 0.068; Tukey correction; Figure 1.6a). Similarly, we found no differences in the probing behavior of bees tested in India between autumn and summer (*P* = 0.24; Tukey correction), nor between autumn and winter (*P* = 0.62; Tukey correction). However, we found that bees made significantly fewer probes in summer as compared to winter (*P* = 0.0088; Tukey correction; Figure1.6b). When examining *A. mellifera* bees tested in the United States, we found no differences in neither latency to resume feeding nor probing behavior between early and late summer (latency: *P* = 0.966; probes: *P* = 0.46; Tukey correction). We did not compare seasonal differences between Indian and U.S., as comparisons between seasons in tropical vs temperature climates would not be meaningful.

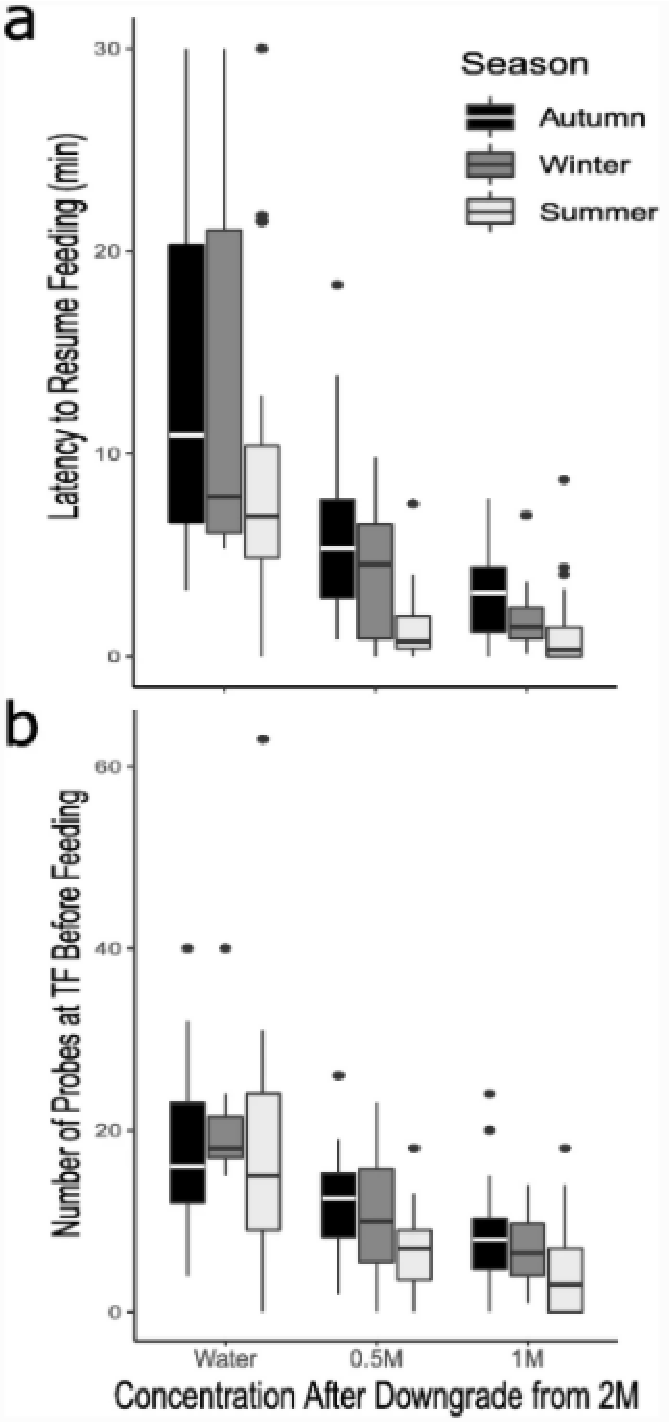

Figure 1.6. Seasonal differences in exploratory behavior. Boxplots of bees' (a) latencies to resume feeding and (b) number of probes at the test feeder after the downgrade are shown. Only data for Asian honey bees are shown, as no seasonal differences were found for *A. mellifera*. Season had a significant effect on (a) latency and (b) probing behavior, but few pairwise differences were seen between seasons when compared over all concentration downgrades. However, bees did make significantly fewer probes in summer than in winter (*P* = 0.0088). The names for seasons (following local usage in Bangalore) refer to the following time periods: autumn: Oct-Dec; winter: Jan-Feb; summer: March-April.

Relationship Between Exploration and Body or Colony Size

We found no correlation between body size or colony size and latency to resume feeding or probing behavior. As can be seen in Figure 1.7, the largest and smallest bees (the open-nesters *A. dorsata* and *A. florea*, respectively) instead are more similar to each other than they are to the species of intermediate size (the cavity-nesters *A. cerana* and *A. mellifera*). Though body size and colony size each significantly affect latency to resume feeding and probing behavior, differences in body size and colony size are effectively equivalent to species differences.

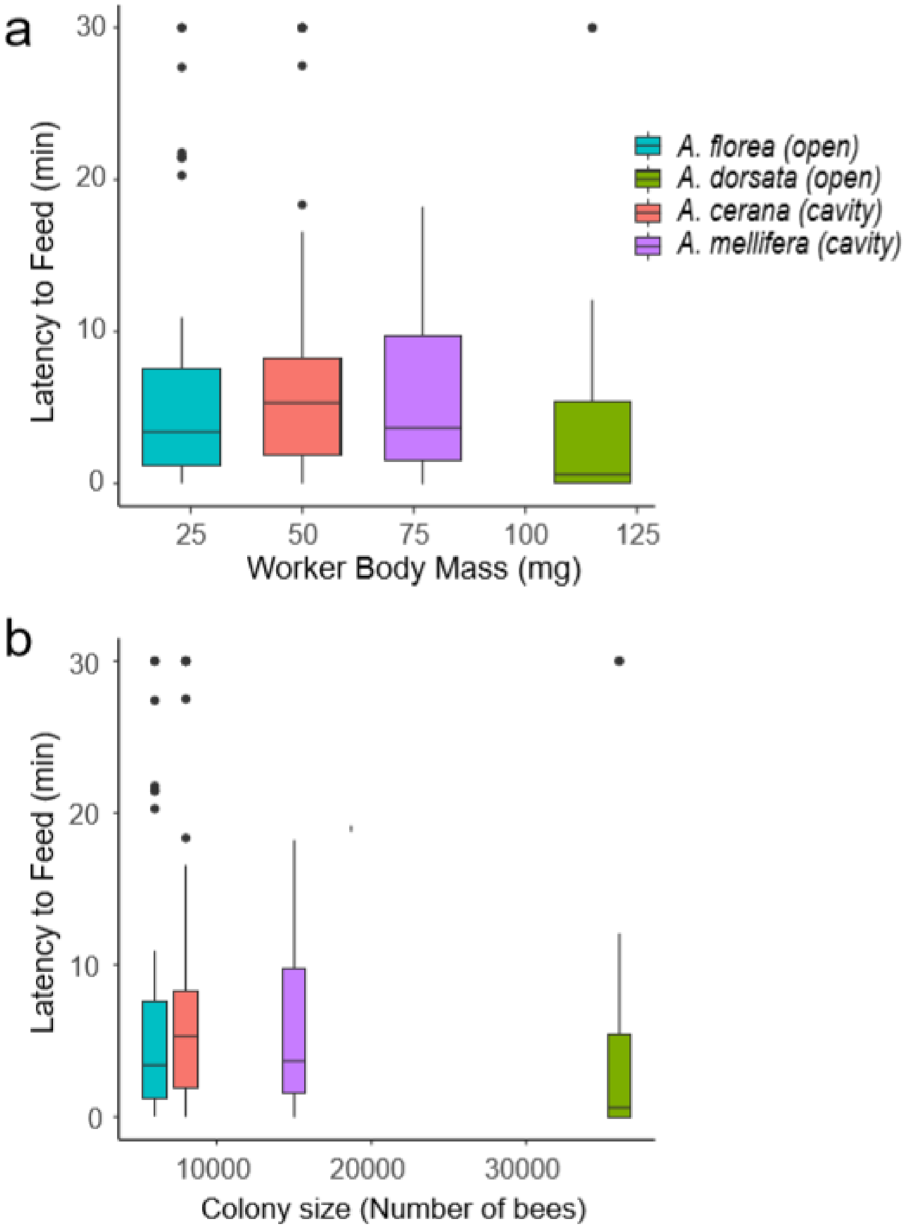

Figure 1.7. Relationship between body and colony size and latency to resume feeding. Boxplots showing the relationship between (a) body size and (b) colony size to latency to resume feeding are shown. There is no obvious relationship between either factor and latency to resume feeding, as would be expected if either played a primary role in shaping the exploratory behavior of honey bees.

DISCUSSION

In this study, we investigated the evolution of the exploitation-exploration tradeoff in four honey bee species using the negative incentive contrast effect, with particular attention paid to the role of nest architecture in shaping the tradeoff. Although the practical constraints (doing complicated experiments in field conditions with only four species) limit our power to test hypotheses about which selection pressures shape the exploratory behavior of these species, we do find results that are consistent with the hypothesis that open-nesting species should be less tolerant of the costs of search, and therefore less exploratory. We found that open-nesting was associated with a significantly shorter latency to resume feeding as predicted, but was only weakly associated with probing behavior in the 0.5M or 1M conditions (Figure 1.2). We found that *A. dorsata* was particularly and consistently intolerant of paying the costs of search. Understanding the significance of these results is complicated by the difficulty in disentangling the effect of nest architecture from that of other selection pressures. In particular, it is challenging to disentangle the effects of nest architecture and species on exploratory behavior considering they are correlated in this study, with the additional interaction between species and response to the concentration downgrade exacerbating that challenge.

When examining the exploratory tendencies of these honey bee species, we found, as seen in previous studies (Townsend-Mehler et al. 2011; Townsend-Mehler and Dyer 2012), that species differences in exploratory behavior were dependent in part on the magnitude of the downgrade in resource quality: a bigger downgrade leads to more protracted search (Figures 1.2, 1.5, S1.1). However, these differences in latency to resume feeding and probing behavior between larger (2M to 0.5M) and smaller (2M to 1M) downgrades in concentration were only significant for some species (Figure S1.1). The two open-nesting species, *A. dorsata* and *A.*

florea, were quicker to resume feeding than the two cavity-nesting species, *A. cerana* and *A. mellifera*, after a larger (2M to 0.5M) downgrade in resource concentration, but exhibited smaller, nonsignificant decreases in latency to resume feeding following a smaller (2M to 1M) downgrade. Similarly, all species probed more on average after experiencing a larger downgrade in resource concentration. But, only *A. dorsata* and *A. mellifera* made significantly fewer probes in the face of a smaller downgrade in concentration. These interactions suggest that after larger resource quality declines, open-nesting species do invest less in exploration than cavity-nesting species. However, at smaller resource quality declines, only *A. dorsata* differs in exploratory behavior from the two cavity-nesting species, which is not fully consistent with the hypothesis that open-nesting species will show less exploration.

To consider the interactions between selection pressures such as nest architecture and the environmental context in which exploration is studied, we first consider the condition with the largest downgrade, from 2M to 0.5M. This resulted in a reward that was identical to what bees had experienced for several days at the constant feeder. Here, although all four species were delayed in resuming their foraging, we found that *A. dorsata* and *A. florea*, the two open-nesting species (and the largest and smallest, respectively, in both colony size and worker body size), have significantly shorter latencies to resume feeding on the downgraded resource as compared to *A. cerana* and *A. mellifera*, the two cavity-nesting species (Figure 1.3a). Similarly, *A. dorsata* probed at the test feeder significantly less than *A. cerana* and *A. mellifera*, and *A. florea* probed less than *A. mellifera* (Figure 1.4a).

These patterns, stronger for the latency data than for the probing data, are consistent with the hypothesis that the open-nesting species, with their need to maintain an excess of workers to form a protective curtain (Dyer and Seeley 1987; Dyer and Seeley 1991; Underwood 1991) should have evolved to be averse to foraging costs that could increase worker mortality, and specifically to costs associated with unrewarded search. However, these findings are complicated by the difficulty in disentangling the effect of nest architecture from that of other selection pressures, and by the fact that it is *A. dorsata* in particular who drives much of the differences seen in probing behavior in the 0.5M condition.

We next consider the response of bees to a smaller downgrade, from 2M to 1M. As mentioned previously, generally species showed a milder disruption in their feeding behavior as compared to the 0.5M downgrade condition though these differences were not always significant; this makes intuitive sense given that the downgrade experienced in the 1M condition was smaller. In this condition, the downgraded sucrose solution (1M) is still better than the longer-term value of 0.5M that the foragers had experienced prior to being introduced to the 2M test feeder. Theoretical work has shown that the time spent searching during a negative incentive contrast effect should vary depending on the long-term average quality of the environment one is in, where one should search longer when resource quality falls below the environmental average (McNamara et al. 2013). It makes sense, then, that foragers of all species should more quickly accept the 1M downgrade than they did the 0.5M downgrade. These results are supported by previous studies that also found *A. mellifera* to not react strongly to a sucrose concentration downgrade from 2M to 1M (Townsend-Mehler et al. 2011). The notable exception here was the behavior of *A. dorsata*, who reacted much more strongly than any other species and was the primary driver of species differences in exploratory behavior (Figure 1.3a, 1.4a). This result suggests that nest architecture alone is not sufficient to explain the species differences.

It is puzzling why *A. dorsata* stood out so dramatically in this condition, with 60% of the bees feeding almost immediately when they encountered the downgraded resource. One possible explanation is that *A. dorsata* workers face a much more intense set of mortality risks than the other species, leading to even stronger selection to reduce the costs of exploration than would be expected due to nest architecture alone. The nests of *A. dorsata* colonies are built in very exposed places, and the workers are large and targeted by a wide variety of bird (drongos, bee-eaters) and insect (*Vespa* wasps) predators (Seeley et al 1982; Fry 1983; Kastberger and Sharma 2000), all of which take workers one at a time. In the open-nesting *A. florea*, by contrast, predation commonly involves the destruction of the whole nest (Seeley et al. 1982). In addition, *A. dorsata* foragers routinely forage on moonlit nights (Dyer 1985, Young et al. in preparation), when they are presumably subject to mortality from nocturnal predators, navigational errors, and, more recently, anthropogenic light. The attrition rate from all of these factors could have favored the evolution of even more careful, and cost-reducing, foraging behavior in *A. dorsata* than in the other species. Isolating the effect of nest architecture on exploratory behavior is therefore made more challenging by the interactions with environmental context and requires additional research on a wider range of open- and cavity-nesting species.

While our results are partially consistent with the hypothesis that nest architecture has led to selection pressures that have shaped the exploitation-exploration tradeoff in honey bees (particularly when examining exploration in a context matching that of the environment's long-term average), we find less evidence consistent with the hypotheses that body size or colony size play a large role in shaping exploration in these species. If body size was more important than colony size in determining these species' willingness to engage in unrewarded search or vice versa, we would expect to find a relationship between size and latency to resume feeding. Specifically, we would expect larger bees to search less than smaller bees, and/or bees from species with larger colonies to search longer than those from species with smaller colonies (Dyer and Seeley 1987; Dyer and Seeley 1991; Harrison and Fewell 2002). Instead of a discernible relationship, though, we find clustering by nest architecture such that the largest and smallest bees, who also come from species with the largest and smallest worker body sizes and foraging work forces, are more similar to each other than to the intermediate-sized bees (Figure 1.7). However, it is important to point out that body size and colony size lead to opposite predictions in terms of their effects on exploratory behavior (Table 1.2). These opposite effects would have the strongest impact on the high and low extremes, which are the two open-nesting species in this study, and could potentially cancel each other out. Because body size and colony size are positively correlated and lead to opposing predictions in our study species, we cannot distinguish if the lack of relationship seen here is due to neither trait having an effect on exploration or instead both having similarly sized effects that lead to a net impact that is negligible since they work against each other. In addition, it is possible that particularly large or small body sizes might lead to risks or mortality costs that bee species with intermediate body sizes such as *A. cerana* and *A. mellifera* are buffered from (Müller et al. 1996; Chole et al. 2019; Kerr et at. 2019)). Further research on species that exhibit more variation in body and colony size in both open- and cavity-nesting species is therefore necessary to disentangle the potentially interacting influences of nest architecture, body size, and colony size on the exploitation-exploration tradeoff.

The downgrade condition from 2M to water was unique in that it required bees first to abandon the no-longer-rewarding test feeder, and then decide when or if to resume feeding on the lower concentration in the constant feeder. Perhaps it is unsurprising then that we did not find differences in the exploratory behavior between species or based on nest architecture when they experienced a downgrade from 2M to water. This more complex behavioral response, combined with the added complication of their past experience with the environmental context, likely overwhelmed any potential species differences in exploratory behavior that might have otherwise existed. In this condition, when the previously highly rewarding resource downgraded in quality to be completely unrewarding, it became lower than the long-term average for the environment. We would expect all bees to search longer in this case (McNamara et al. 2013), and that is what we found (Figure S1.2). It is possible, then, that the effect of reward history was stronger than that of nest architecture, making it impossible to identify species differences. This idea is strengthened by the fact that differences in the probing behavior between species tended towards significance (driven primarily by the decreased probing of the open-nesting species *A. florea*), raising the possibility that the added differences between this condition and the others decreased our power to identify the effect of nest architecture. The water condition highlights the difficulty in isolating the effects of any single selection pressure in a naturalistic foraging context, as the effect of the environmental context is ubiquitous and potentially overwhelming.

Throughout the experiment, it was clear that *A. dorsata* is the most intolerant of paying search costs of the four species studied (Figure 1.5). For example, the majority of *A. dorsata* foragers did not even seem to register the downgrade to 1M and fed immediately, a behavior not seen in any other species (Figure 1.3b, 1.4b). This intolerance of unrewarded search was expressed in a surprising way in the water downgrade condition, as some (23%) *A. dorsata* workers readily accepted water as a resource. In these instances, foragers would exhibit temporary disruption after experiencing the water in the test feeder, but instead of returning to feeding at the low-quality feeder (the only source of sucrose solution that they had been trained to), bees would begin to collect water during the test period. Interestingly, these bees that collected water did not cease searching any later than did those who ended search by feeding on the 0.5M sucrose solution (Table S1.2). This water collection behavior, like the strong intolerance to paying search costs, could be a result of *A. dorsata's* tendency to nest in very exposed locations, such as on tree branches, rock cliff faces, or building ledges. These nests are exposed not only to predation but to large amounts of direct sunlight (Dyer and Seeley 1991). Perhaps the heat from direct exposure to sunlight would lead to increased need for water collection to help regulate colony temperature (Mardan and Kevan 1989; Dyer and Seeley 1991). Indeed, most of our observations of bees accepting water occurred in the hottest and driest season of the year. It is important to note that water collection behavior is seen in other species, including *A. mellifera*, during periods of high temperature (Visscher et al. 1996). The water collection behavior seen in this experiment, while not unique to *A. dorsata*, could potentially be a result of high temperatures exacerbated by the fact that *A. dorsata* nests are often exposed to direct sun (as they were in this experiment).

We conducted this study over a range of seasons and did find that the season in which a bee was tested impacted its exploratory behavior in the 0.5M and 1M conditions (Figure 1.6). In particular, Asian honey bees seemed to be quicker to accept downgraded food, hence less tolerant of unrewarded exploration, during summer than in spring or winter (Figure 1.6), though this difference was only significant for their probing behavior. This apparent readiness to abandon search could be a result of decreased resource availability during the summer months. In India, where the three Asian honey bee species were tested, resources are available throughout the year. However, summer months tend to have fewer floral resources than other seasons (Singh and Kushwaha 2006; Santhosh and Basavarajappa 2016), possibly leading to increased colony need and therefore decreased willingness to pay the costs of unrewarded search. The lack of a seasonal effect seen in the behavior of *A. mellifera* is likely due to the fact that all bees were tested during summer. Though there often more resources available in early summer than late summer in the Midwest (Delaney et al. 2015), any change in resource availability seems to be too small to have impacted colony need in any significant way. Though seasonality seems to have an impact on behavior at least within tropical climates, it is difficult to compare these effects across the Asian honey bee species and *A. mellifera* as they were tested in tropical and temperate environments respectively. An interesting follow up to this study would therefore be comparing the behavior of *A. mellifera* in a temperature vs tropical environment.

Given the small number of species tested, it is impossible to conduct a rigorous phylogenetic analysis. Still, it is interesting to consider how the trait differences we see map onto the phylogenetic relatedness between species. If some kind of "phylogenetic constraint" were responsible for the exploratory behavior of these species, we would expect *A. dorsata* to be more similar to the cavity-nesting species than to *A. florea*, as giant honey bees share a more recent common ancestor with cavity-nesting bees than dwarf bees (Raffiudin and Crozier 2007). As the earliest-diverging species of those tested, we would expect *A. florea* to be the most divergent in behavior from the other species tested. However, we found consistent results that *A. dorsata* was more similar to *A. florea* than to *A. cerana* or *A. mellifera*, and that it was the most divergent from all other species (Figures 1.2, 1.3). The behavior observed in this study therefore aligns more closely with behavioral predictions based on selective forces related to nest architecture than with a simple pattern of divergence related to phylogeny.

As mentioned throughout this discussion, our study is limited by the difficulty in isolating the effect of any one of many potentially interacting selection pressures on exploration, particularly when examining only four species and only one to three colonies per species. Within-species variation in behavior has been studied extensively in *A. mellifera*, with behavior being influenced by factors such as season, climate, and colony genetics (Page & Fondrk 1995; Page et al. 1995; Pankiw 2003; Danner et al. 2016). When studying these species over multiple seasons, we again found differences in behavior related to seasonal differences, though by studying all three Asian honey bee species over the same seasons we hoped to minimize potentially confounding effects of seasonal differences in resource availability between species. However, it is still possible that colony state differences could exist between colonies even when studied in the same environment, and so future research comparing honey bee species should explicitly consider that. The decision to study *A. mellifera* in a different climate weakens our ability to draw comparisons between it and the Asian species but was motivated by the goal of studying each species in the climates in which they evolved. Future studies should investigate behavioral variation in these species, particularly that of *A. mellifera*, which has been distributed throughout the world for beekeeping. In addition, it would be particularly interesting to investigate behavioral differences in exploration between *A. mellifera* subspecies that evolved in different climates (e.g., *A. mellifera ligustica* vs *A. mellifera scuttelata*), as previous studies have shown that such races can differ in their sucrose responsiveness and foraging behaviors (Pankiw 2003). Though interpretations of our between-species comparisons must consider the limited sample size, the differences we found are strengthened by the lack of between-colony differences seen between *A. cerana* colonies and *A. florea* colonies in this study (excluding differences in probing in *A. cerana*; Table S1.1). However, another worthwhile line of future study would be investigating between colony and between experiment variability in behavior.

Overall, our study opens new paths for future studies investigating the evolution of the exploitation-exploration tradeoff in honey bees. We suggest hypotheses and predictions for how this tradeoff has been tuned according to multiple selection pressures and provide the first comparative study of exploratory behavior between temperate and tropical honey bee species in a naturalistic foraging setting. We found species differences in exploration partially consistent with the hypothesis that nest architecture plays some role in the tuning of the exploitation-exploration tradeoff, though these findings are at times driven primarily by *A. dorsata* or complicated by environmental context. Our results highlight that the tuning of exploration in these species is nuanced, and that it is important to take the context of the current environment into account when examining species differences in exploration. Future studies on exploration should investigate a wider variety of species and colonies in order to isolate the causative effects of particular selection pressures and to investigate the extent of inter- versus intra-specific behavioral variation in exploration. We suggest that particular attention be paid to the role of nest architecture in shaping these relationships. In addition, further examination of how environmental conditions and context shape the resolution of this tradeoff could provide important insight into the nuances of exploration.

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APPENDIX

Table S1.1. Within species colony differences in latency and probing behavior. Model output results examining between colony differences in latency to resume feeding and probing behavior following a downgrade in *A. florea* and *A. cerana*.

	Sum of Squares	DF	F value	P-value
A. florea				
Latency to Resume Feeding				
ColonyID	51.6	2	0.4252	0.6558
Residuals	3214.4	53		
Number of Probes				
ColonyID	94.38	2	1.2622	0.2914
Residuals	1981.55	53		
A. cerana				
Latency to Resume Feeding				
ColonyID	147.2	1	2.8503	0.09557
Residuals	3821.4	74		
Number of Probes				
ColonyID	239.9	1	4.3229	0.04107
Residuals	4106.8	74		

Table S1.2. Differences in latency and probing behavior between *A. dorsata* foragers feeding on water and sucrose solution. Model output results comparing latency to resume feeding and probing behavior in *A. dorsata* foragers tested in the water condition. We analyzed latency of *A. dorsata* foragers in the water condition using a general linear model with latency to resume feeding as the dependent variable and collected water status (yes or no) as a fixed factor. We similarly analyzed probing behavior using a generalized linear model with a negative binomial distribution. We then analyzed the models using 1-way ANOVA.

	Sum of Squares	DF	F value	P-value
Latency to Resume Feeding				
Fed on Water (yes or no)	94.52	1	1.6673	0.2121
Residuals	1077.19	19		
Number of Probes				
Fed on Water (yes or no)	125.4	1	0.6557	0.4281
Residuals	3633.7	19		

Table S1.3. Model selection for latency to resume feeding in 0.5M and 1M conditions. The final selected model is bolded.

Model	AICc	Delta AICc	Model Weight
Latency ~ NestType + Species + FinalConcentration + Season + Species*FinalConcentration + (1|ColonyID)	**405.137**	**0.000**	**0.509**
Latency ~ NestType + Species + FinalConcentration + Season + (1|ColonyID)	407.003	1.866	0.200
Latency ~ NestType + Species + FinalConcentration + Season + Species*FinalConcentration + FinalConcentration*Season + (1|ColonyID)	407.150	2.013	0.186
Latency ~ NestType + Species + FinalConcentration + (1|ColonyID)	408.380	3.243	0.101
Latency ~ NestType + Species + FinalConcentration + Season + Species*FinalConcentration + FinalConcentration*Season + Species*Season + (1|ColonyID)	414.640	9.503	0.004
Latency ~ NestType + Species + (1|ColonyID)	438.992	33.855	0.000
Latency ~ NestType + (1|ColonyID)	439.427	34.290	0.000

Table S1.4. Model selection for number of probes at the test feeder in 0.5M and 1M conditions. The final selected model is bolded.

Model	AICc	Delta AICc	Model Weight
NumProbes ~ NestType + Species + FinalConcentration + Season + Species*FinalConcentration + (1|ColonyID)	**1039.980**	**0.000**	**0.830**
NumProbes ~ NestType + Species + FinalConcentration + Season + Species*FinalConcentration + FinalConcentration*Season + (1|ColonyID)	1044.275	4.295	0.097
NumProbes ~ NestType + Species + FinalConcentration + Season + (1|ColonyID)	1045.509	5.528	0.052
NumProbes ~ NestType + Species + FinalConcentration + Season + Species*FinalConcentration + FinalConcentration*Season + Species*Season + (1|ColonyID)	1047.457	7.477	0.020
NumProbes ~ NestType + Species + FinalConcentration + (1|ColonyID)	1054.030	14.050	0.001
NumProbes ~ NestType + Species + (1|ColonyID)	1081.897	41.916	0.000
NumProbes ~ NestType + (1|ColonyID)	1093.005	53.025	0.000

Table S1.5. Model selection for latency to resume feeding and number of probes at the test feeder in the water condition. The final selected model is bolded.

Model	AICc	Delta AICc	Model Weight
Latency to Resume Feeding			
Latency ~ NestType + Species + Season + Species*Season + (1|ColonyID)	**252.267**	**0.000**	**0.735**
Latency ~ NestType + Species + Season + (1|ColonyID)	255.906	3.639	0.119
Latency ~ NestType + Species + (1|ColonyID)	256.507	4.240	0.088
Latency ~ NestType + (1|ColonyID)	257.344	5.077	0.058
Number of Probes			
NumProbes ~ NestType + Species + (1| ColonyID)	**599.987**	**0.000**	**0.514**
NumProbes ~ NestType + (1|ColonyID)	600.289	0.302	0.442
NumProbes ~ NestType + Species + Season + (1|ColonyID)	605.116	5.130	0.040
NumProbes ~ NestType + Species + Season + Species*Season + (1|ColonyID)	609.670	9.684	0.004

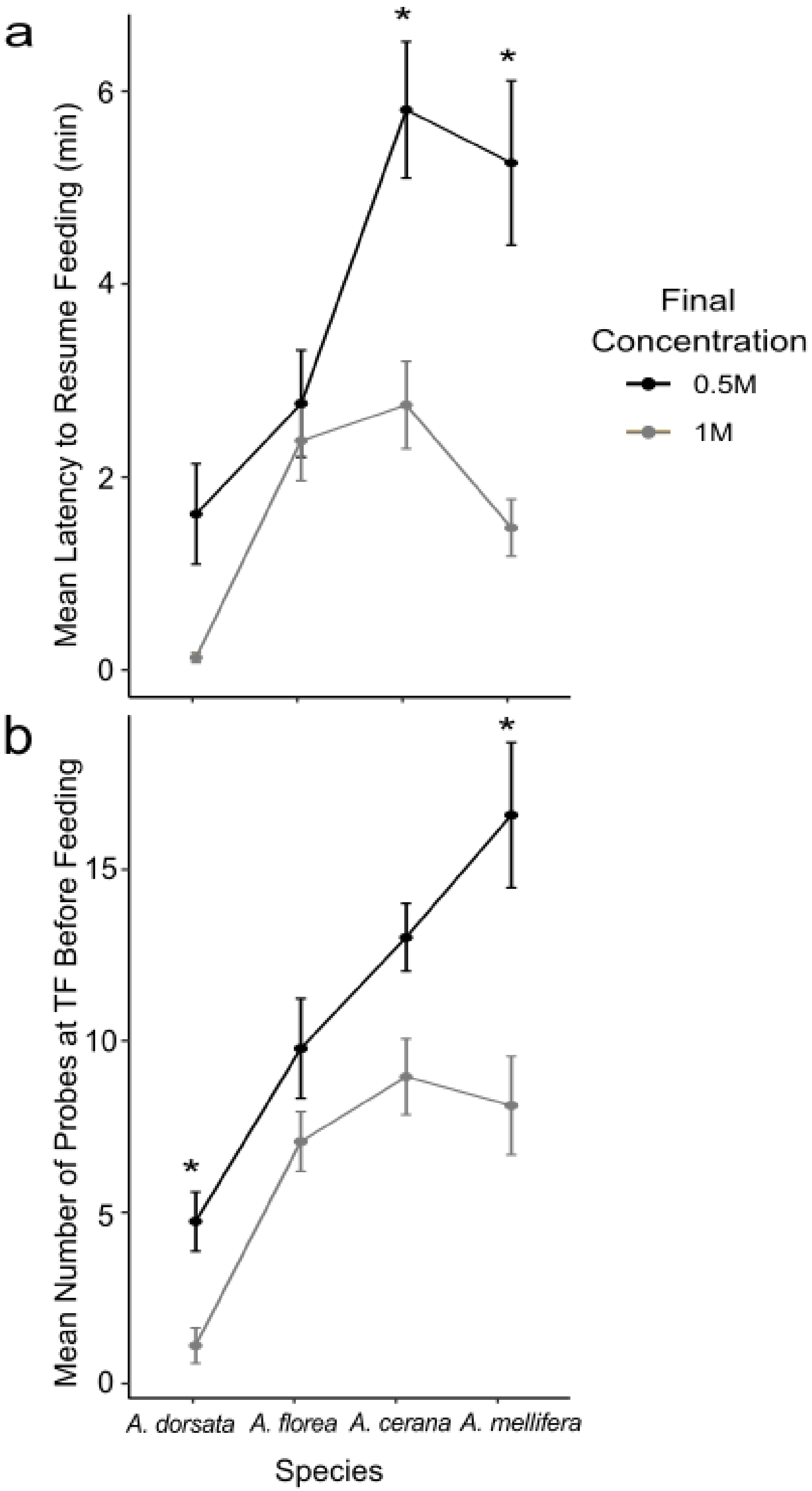

Figure S1.1. Mean latency to resume feeding and number of probes before feeding in the 0.5M and 1M downgrade conditions. Bees generally had (a) longer latencies to resume feeding and (b) probed more in the 0.5M condition than in the 1M condition. Significant differences between 0.5M and 1M conditions are represented by *. Error bars represent standard error.

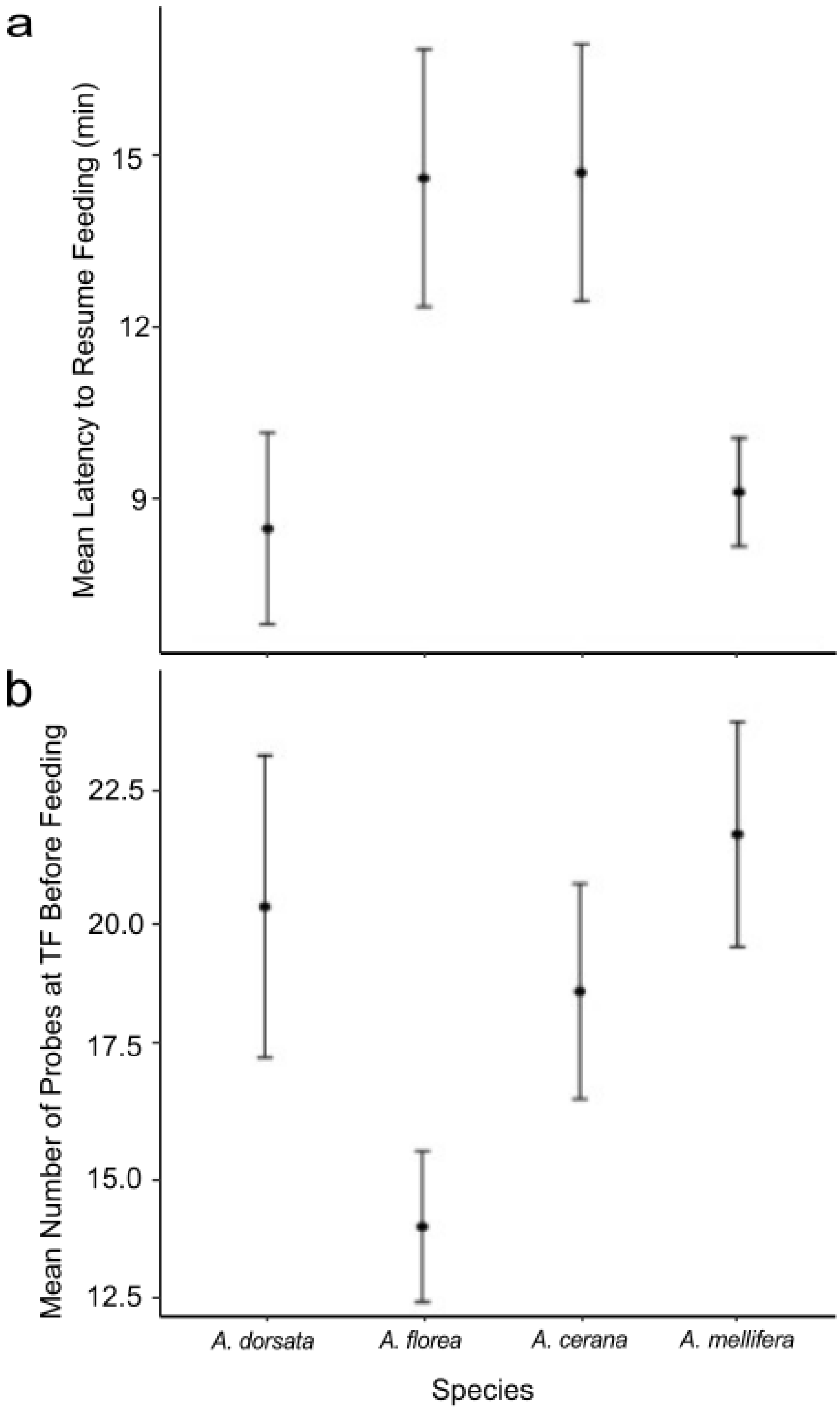

Figure S1.2. Mean latency to resume feeding and number of probes before feeding in the water condition. No species differences were seen in (a) latency to resume feeding (X^2 = 1.5516, *DF* =2, *P* = 0.46033) or (b) probing behavior (X^2 = 5.1175, *DF* =2, *P* = 0.0774) during the water condition. Error bars represent standard error.

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CHAPTER TWO:

Past experience with spatial or temporal resource unpredictability shapes exploration in honey bees

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ABSTRACT

Foraging animals must repeatedly decide whether to exploit currently available resources or explore to gain information and locate new resources. Balancing these two often mutually exclusive options is made more difficult by environmental changes that can occur predictably, as seen in daily patterns of nectar secretion in flowers, or unpredictably, as seen when resources are depleted by competing foragers. Recent experiences with predictable and unpredictable rewards potentially provide information that a foraging animal can use to decide when it is beneficial to explore versus exploit. However, it is unclear how the predictability of recent experiences can shape an animal's future exploration after the world changes such that they have no option but to search, such as when previously available rewards vanish. We tested how experience with unpredictability related to two types of environmental change, unpredictability in reward location and unpredictability in the daily timing of rewards, shaped the exploratory behavior of honey bees in the absence of a reward. We found that honey bees that experienced unpredictably located or timed rewards were less precise in their exploration after those rewards vanished. Spatially, bees focused their search more broadly within a patch if they had experience with unpredictably located rewards, but did not spend more time searching outside the patch than bees that experienced predictably located rewards. Temporally, bees that experienced unpredictably timed rewards distributed their search effort more broadly outside of the time they had last received a high-quality reward; also, bees that experienced any change in reward searched more intensively regardless of how predictable the reward had previously been. In contrast to the differences in search precision in both space and time, a bee's previous experience with spatial or temporal unpredictability of rewards did not change her search persistence, though it is possible that differences would have been seen over larger spatial or temporal scales. This study provides

a novel perspective on how the predictability of previous experiences can shape exploratory behavior even in the absence of rewards.

INTRODUCTION

Foraging animals are faced with the repeated decision of whether to exploit currently available resources or explore their environment to gain information and locate new resources. The choices animals make can have large impacts on their fitness, as each decision has its own costs and benefits (Stephens and Krebs 1986; Kotler et al. 2004; Berger-Tal and Avgar 2012). Exploiting currently available resources provides a guaranteed reward, but animals may miss better options available in the environment. Exploration helps animals acquire experience with the current state of the environment and can result in locating novel resources, but is energetically costly, especially if a better option is not found (Stephens 1987; Lima and Dill 1990; McNamara et al. 2013; Mehlhorn et al. 2015). Foraging animals must therefore constantly assess how best to balance exploration and exploitation to maximize the benefits of each option while minimizing their costs.

Balancing the tradeoff between exploration and exploitation is complicated by the fact that the environment may change continually, with resources varying in their quality, the ease of accessing them, the timing of their availability throughout the day, and their distribution in the landscape. These variations can be observed from season to season, week to week, even from hour to hour (Delaney et al. 2015; Anjos et al. 2017; CaraDonna et al. 2017; Pope and Jha 2018; Wood et al. 2018; Treanore et al. 2019). Some variation occurs in predictable patterns to which species are highly adapted, such as seasonal differences in resource quality (e.g., abundant floral resources in early summer versus a dearth of resources in fall [Delaney et al. 2015; Wood et al.

2018]). Even within a given season, many fluctuations in resource availability and quality are highly predictable, particularly in time (e.g., daily patterns in nectar production and sugar accumulation [Edge et al. 2012; Anjos et al. 2017; Kaur et al. 2020]) allowing animals to learn and respond to the variability in ways that maximize their exploitation of the rewards. However, much of the variation is unpredictable, especially if affected by competition (Gill 1988; Ohashi et al. 2008; Balfour et al. 2015; Ferretti et al. 2019) or weather (Peat and Goulson 2005; Anton et al. 2017; Ferretti et al. 2019). Animals must therefore be plastic in their foraging behavior to adaptively respond to both predictable and unpredictable factors influencing resource availability and quality.

To deal with this mix of predictable and unpredictable variation, and thus respond to the expected benefits of exploration, animals can potentially rely upon their recent experience in monitoring when and where they have found food, how variable the rewards are, and how predictable the variation is (Wei et al. 2002; Doligez et al. 2003; Gil et al. 2007; Ohashi et al. 2008; Dunlap et al. 2017). Mathematical models and experimental studies of animal exploratory behavior and learning suggest that animals do use their recent experiences with environmental resource variation to guide their sampling behavior, a common metric of exploration. Animals generally are more persistently exploratory (i.e., sample more) when the cost of missing a good reward is large (Dunlap and Stephens 2012; Keasar et al. 2013; Dunlap et al. 2017), when the environment changes at a moderate rate (where information gained during search is both necessary and helpful for predicting the future state; [Stephens 1987; Shettleworth et al. 1988; Koops 2004; Dunlap et al. 2017]), when they are more likely to get a reward after sampling (Zeiler 1968), or when they are more certain that a reward exists (Leadbeater and Chittka 2009; Townsend-Mehler et al. 2012). These previous studies have primarily studied exploration in laboratory operant tasks using the two-armed bandit paradigm (but see Townsend-Mehler et al. 2012 for an exception). In this paradigm, animals are presented with two resources, one that is of mediocre quality and one that fluctuates between high and low quality, and are given multiple opportunities to choose between the two. The frequency with which an animal samples the fluctuating resource and how it changes over time demonstrates their investment in exploration and their learning, respectively, and can provide information on how factors such as the predictability of change of a fluctuating reward and the relative quality of multiple rewards shape the decisions animals make regarding exploration. These choice patterns can also be compared to the theoretical optimal behavior for maximizing resource acquisition to determine the rules being used to make behavioral choices.

Less attention has been paid, though, to the related questions of how an animal's previous experiences with environmental change affects their exploratory behavior outside the scenario of the experimental two-armed bandit problem, how experiences with environmental changes unrelated to rewards that fluctuate in quality affects exploratory behavior, or how experiences shape exploration in free-flying foraging scenarios where animals can decide where and when to search. One open question is how previous experience with environmental variation can inform future exploratory behavior after the world an animal was used to has fundamentally changed, such as when rewards that were previously available vanish entirely and animals are forced to search. This scenario is one that foragers may face in naturalistic contexts, where rewards can vanish suddenly due to resource fluctuations that are predictable (e.g., seasonal resource turnover; daily phenological patterns) or unpredictable (e.g., competition [Edge et al. 2012; Delaney et al. Wood et al. 2018; Anjos et al. 2017; Kaur et al. 2020; Ohashi et al. 2008; Balfour et al. 2015; Ferretti et al. 2019]). The disappearance of a specific resource does not necessarily imply that resources cannot be found in another location or at another time of day. Thus, there should be an incentive to the forager to persist in looking for the food, not only where it was previously found, but also in other locations. But the animal may face decisions about the search strategy it should use: How long should it search before giving up? Should it restrict its search to the immediate vicinity of the previous reward, or range more widely? Should it restrict itself to the time that the food was available or explore at other times of the day?

We expect that these decisions should be based not only on the costs and benefits associated with exploration and exploitation but also on the animal's recent experience with resource variation, especially whether the environment changed in a predictable or unpredictable way. While there are many ways in which rewards can be predictable or unpredictable, we focused specifically on two kinds of unpredictability that we expect should commonly be experienced by foraging animals: unpredictability in reward location and unpredictability in the daily timing of reward.

We conducted this study using honey bees as a model due to their experimental tractability and their reliance on floral resources, which typically vary in space and time. Flowering plants often occur in patches that endure for days or weeks, and often produce rewards on a daily schedule, and thus provide rewards that are fairly stable in time and space (Edge et al. 2012; Delaney et al. 2015; Anjos et al. 2017; Wood et al. 2018; Kaur et al. 2020). However, floral resources are also subject to spatial and temporal variation that may be predictable or unpredictable in ways outlined earlier (Gill 1988; Peat and Goulson 2005; Ohashi et al. 2008; Balfour et al. 2015; Anton et al. 2017; Ferretti et al. 2019). Thus, we expect honey bees to have evolved the ability to be sensitive to variability in resources according to whether it is predictable or unpredictable.

Honey bees are well-known for being able to learn the locations and timing of rewards quickly and accurately. Significant learning may require just one training experience, with increasing experience leading to more accurate and longer-lasting memories even in the absence of rewards (Moore et al. 1989; Couvillon et al. 1991; Lehrer and Collett 1994; Wei et al. 2002; Wei and Dyer 2009; Moore et al. 2011; Al Toufailia et al. 2013; Tsvetkov et al. 2019). Differing amounts of experience, then, seem to change a bee's expectations of rewards, and thereby affects the precision of their search in space or time. As has been shown through operant conditioning in the two-arm bandit paradigm, experience with more variable environments or rewards often leads to increased sampling and exploration (Neuringer et al. 2000; Dunlap and Stephens 2012; Keasar et al. 2013; Dunlap et al. 2017). An animal's experience of being rewarded in a predictable way, then, similarly seems to change their expectations regarding the payoffs of search, and thereby shapes how often search occurs. For those reasons, we hypothesized that honey bees that have experienced a predictable reward in space or time (a fixed pattern of reinforcement) would, when confronted by the unexpected absence of reward, search differently from honey bees that have experienced unpredictably located or timed resources (a variable pattern of reinforcement). Specifically, we hypothesized that it would be adaptive for honey bees with experience in a spatially or temporally unpredictable environment to focus their search more broadly (i.e., less precisely) across a patch or across the course of a day, respectively, as they would have had less reinforcement at that location or time of day. Behaviorally, decreased precision could be seen through decreased attention paid to previously rewarded locations or times (especially ones that had been most recently rewarded), increased search outside of a resource patch, or broadly distributed search throughout an entire day. Additionally, we hypothesized that it would be adaptive for honey bees with experience in unpredictable environments to be more persistent in their exploration, as previous experience with a variable resource should have shown them that the failure of the resource to occur where or when it had previously been found means that search should be extended. Behaviorally, increased persistence could be seen through higher total sampling, slower extinction of search, or increased bouts of high sampling throughout the day.

Here we present the results of two experiments investigating the effects of previous experience with environmental predictability on honey bee exploratory behavior in the absence of rewards. In the first experiment, we show how experience with unpredictability in reward location shaped both investment in search and how search was conducted in space. In the second experiment, we show how experience with unpredictability in reward timing shaped both investment in search and how search was conducted across time.

MATERIALS AND METHODS

General Setup

We conducted these experiments on the Michigan State University Campus in East Lansing, Michigan during the summers of 2018 and 2019. The experiments were conducted inside a 25 m (l) x 5.6 m (w) x 2.3 m (h) outdoor flight cage, which allowed for experimental control of food resources and experiences while maintaining naturalistic foraging conditions. We used a colony consisting of roughly 4,000 incubator-reared honey bees for each experiment and housed the colony in a four-frame observation hive in the middle of the flight cage. We monitored colony state regularly, and provided pollen as needed.

Spatial Predictability

Experimental Setup and Training

In our first experiment we examined the effect of the predictability of reward location on honey bee exploratory behavior. We first pre-trained foragers to a feeder providing a constant source of low-quality sucrose solution (0.5M). The feeder consisted of an inverted jar on a grooved Plexiglass plate 1 m off the ground. After bees were reliably visiting the "constant" feeder, we individually marked bees by gluing a numbered tag to the thorax of each bee. We created a 0.75 m (w) by 1.25 m (l) testing array that contained 12 artificial flowers, which were made using 4cm diameter blue cardstock circles with a blue birthday candle holder in the middle (Figure 2.1). We numbered the positions of each flower such that position 1 was in the top left corner of the testing array and position 12 was in the bottom right corner of the testing array. We set up the testing array 10 m from the low-quality constant feeder in front of a large green blind. During both the training and testing periods, all observers stayed behind the blind to minimize the variability of landmarks available for bees between visits to the testing array.

To begin training for an experiment, we captured an individually marked forager at the constant feeder, transported it to the testing array, and introduced it to a rewarding artificial flower within the array (Townsend-Mehler et al. 2012). Once a bee arrived at the testing array without assistance, we allowed it 6 unassisted training visits where the location of the rewarding artificial flower was either predictable or unpredictable. In the predictable condition, high quality sucrose solution was available in one artificial flower in the training array and remained in the same location for the duration of the training period. In the unpredictable condition, the location of the one rewarding flower changed pseudo-randomly between each successive training visit such that no location was rewarded more than once during the training period. After the final successful training visit, we began the test. We caught any unmarked bees that were recruited to the testing array and released them from the flight cage.

Figure 2.1. Testing array for spatial unpredictability experiment. The array consisted of 12 artificial flowers composed of a blue cardstock circle (4 cm diameter) with a blue birthday candle holder in the middle. Flowers were numbered according to their position, with position 1 in the top left corner of the board and position 12 in the bottom right corner of the board.

Test Period

After a bee's sixth training visit (a sufficient time for learning to have occurred [Wei et al. 2002]), we removed all rewards from the testing array, replaced the current flowers with clean ones, and rotated the board to disrupt any scent cues. A test began when the test bee arrived at the now unrewarding testing array. We recorded each test using a Panasonic 4K Ultra HD Video Camcorder (Model VX981K) for later analysis, with one observer narrating the test bee's movements on the testing array. Observers also noted if and when the test bee left the testing array to explore other parts of the flight cage, while another observer watched the constant feeder to record if and when the test bee arrived at it during her search. A test trial was terminated after 10 minutes, whereupon the test bee was caught and released from the flight cage to prevent retesting. We analyzed the recordings to determine the behavior of each bee, including the location of every contact the bee made to the artificial flowers in the testing array, the time spent on and off the testing array, and the number of transitions made between any two artificial flowers. For each bee, the environmental predictability condition was noted (predictable or unpredictable) as were the locations of all rewarding artificial flowers and the location of the last rewarding artificial flower during the training period.

Statistical Analyses

We analyzed the data using R version 4.0.2. To examine whether and how a bee's search behavior changed during the training period, we analyzed how long bees searched and how many times they contacted the testing array before finding the rewarded location during each training visit. We used generalized linear mixed effects models for both analyses, using a gaussian and a negative binomial distribution respectively ('glmmTMB' function). In each model, we considered the fixed factors of predictability condition (predictable or unpredictable), training visit (1-6), their interaction, and the random factors of bee identity, first training position (1-12), and last training position (1-12) in the full model. Both final models included only the fixed factor of predictability condition and the random factor of bee identity.

To examine how a bee's past experience shaped the persistence and the precision of her search within the testing array when rewards were no longer available, we analyzed the total number of contacts made at the testing array and the number of contacts made at the last rewarded training location, respectively. We analyzed each response variable using a generalized linear mixed effects model with a negative binomial distribution (‘glmmTMB’ function), with the full model testing the fixed factors of predictability condition, time block (first or second 5 minutes of test), and their interaction, plus the random factor of bee identity nested within time block. Both final models included the fixed factors of predictability condition and time block, and the random factor of bee identity nested within time block. We next used a chi-square goodness of fit test to determine whether bees in each predictability condition were making more contacts at rewarded locations than would be expected if they were searching randomly, and visualized the search patterns of bees to rewarded and unrewarded locations using network analyses (‘igraph’ package). For the network analyses, the three bees from each predictability condition that made the highest number of total contacts were used as representative samples.

To further examine how a bee’s past experience shaped the persistence and precision of her search in the context of the entire environment available for search, we analyzed the amount of time bees spent searching throughout the environment using a generalized linear mixed effects model. We constructed a model that included the fixed effects of predictability condition, time block, search location (on or off the testing array), all possible two-way interactions, and the three-way interaction between predictability condition, time block, and search location, as well as the random effect of bee identity nested within time block. The final model for how long bees spent searching the environment included all fixed factors considered in the full model. We used Kaplan-Meier survival curves to further investigate the persistence of a bee’s search on the testing array versus in the broader environment by analyzing how quickly she abandoned the testing array to visit or feed at the constant feeder (‘survival’ package). Finally, we analyzed the amount of time bees spent searching at the constant feeder using a generalized linear mixed effects model with a Gaussian distribution (‘glmmTMB’ function). In this model the fixed effects of predictability condition, time block, their interaction, and the random effect of bee identity nested within time block were considered, but the final model included only predictability condition, time block, and the random effect of bee identity nested within time block.

For all generalized linear models, we build models using a forward approach, beginning with null models and adding complexity through the addition of fixed and random factors. Final models were selected according to their AICc values; final models selected for each response variable had the lowest AICc values. After selecting final models, we analyzed them using ANOVA, further analyzing significant factors using post hoc tests with a Tukey correction.

Temporal Predictability

Experimental Setup and Training

In our second experiment we examined the effects of predictability of reward timing on honey bee exploratory behavior. We trained two groups of bees from the observation hive to separate feeders at opposite ends of the flight cage: one was a constant feeder that offered a low-quality reward throughout the day, and the other was a changing test feeder that offered a low-quality reward for most of the day, but occasionally offered a higher-quality reward for a short period of time (30 min). Each feeder consisted of an inverted jar on a grooved Plexiglass plate set on a tripod 1 m off the ground. Each bee coming to each feeder was individually marked using a numbered tag glued to its thorax, with tags differing in color to identify which feeder a bee was trained to. Any bees that drifted between feeders were caught and removed from the experiment.

After being individually marked, we gave the groups of bees 7 days of experience at their respective feeders. The constant feeder provided a low-quality sucrose reward (0.25M) from 900 to 1530 every day during all 7 days, while the changing feeder provided the same low-quality reward from 900 to 1530 every day except for a 30-minute period where the reward was increased to high quality sucrose solution (2M). To examine the effect of the predictability of reward timing on honey bee search, in half of the experimental trials we provided the high-quality reward at the changing feeder at the same time all 7 days (from 1100 to 1130), while in the other trials we provided the high-quality reward at a different time every day. We chose the quality upgrade time in this unpredictable condition pseudo-randomly such that no 30-minute time period experienced an upgrade more than once during the 7 days. We also ensured that the final upgrade in the unpredictable trial occurred in the afternoon (1430 to 1500), to allow for better detection of a contrast between the two conditions. For both of the predictability conditions, we censused the visitation of bees at the constant and the changing feeders every half hour for 10 minutes throughout the 7-day training period. In addition, we recorded all visits to the changing test feeders during the 30-minute upgrade time and the 30 minutes immediately following the ensuing downgrade of the sucrose concentration to 0.25M; the purpose of recording visitation during this one-hour period in its entirety was to examine the response of the bees to a sudden increase and decrease in reward quality. This daily decline in food reward at the variable feeder triggered bees to begin searching, and we paid particular attention to two metrics of search intensity: how long a bee searched following the downgrade before resuming feeding (her latency to resume feeding) and how many contacts she made at the downgraded feeder before resuming feeding. For each bee we also recorded how many days of experience a bee received during the training period, as some bees were not seen every day.

Test Period

After 7 days of training experience, we removed all rewards in the flight cage for the 8th day of the trial. During this test day, both the constant and the changing feeder contained only water from 900 to 1530 (regardless of whether the changing feeder had previously changed in a predictable or unpredictable manner). We censused the number of visits that marked bees made to each feeder for 10 minutes every half hour, recording both the number of visits made by each bee and the timing of those visits. After the water-only test day, all tested bees were collected and removed from the flight cage to prevent retesting. We then trained two new groups of bees to the constant and changing feeders to begin another trial.

Statistical Analyses

We analyzed the data using R version 4.0.2. To examine the exploratory behavior of test bees during the training period, we analyzed Kaplan-Meier survival curves for time spent searching following a resource downgrade ('survival' package). We further examined investment in exploration following a resource downgrade by comparing the number of contacts bees made to the downgraded feeder before resuming feeding using a generalized linear mixed effects model with a Poisson distribution ('glmmTMB' function). We considered the fixed effects of predictability condition (predictable or unpredictable), training day (1-7), their interaction, and the random effects of bee identity and trial number (1-4) in the full model, and all were retained in the final model.

To examine the persistence and precision of searching bees during the water-only test day (when no food was available), we analyzed the total number of visits made throughout the day and the percentage of visits made during the time of interest (the half hour before and during the last experienced upgrade time) to the water feeders. Using the percentage rather than the raw number of visits during the time of interest prevented misleading results if treatment groups differed in the total number of visits bees made. We analyzed both response variables using generalized linear mixed effects models, considering the fixed effects of predictability condition, training feeder (constant or changing), the interaction between predictability condition and training feeder, and the random effects of bee identity and trial number in the full model. The final models for total number of visits and percentage of visits during the time of interest both included the fixed factors of predictability condition and training feeder, and the final model for percentage of visits also included the interaction between predictability condition and training feeder and the random factor of trial number.

To get a better understanding of the distribution of bees' visits throughout the day, we analyzed the number of visits to the water feeder made during each half hour of the water-only test day using a generalized linear mixed effects model with a Poisson distribution. This model used an autoregressive correlation structure since our response variable consisted of count data over a time series. We considered the fixed factors of predictability environment, training feeder, time of day (900 – 1530h in half hour blocks), every possible two-way interaction, and the three-way interaction between predictability environment, training feeder, and time of day, as well as the random factors of bee identity and trial number, in the full model. The final model included predictability condition, training feeder, time period, all two-way interactions, and the random factor of bee identity. We used a Spearman correlation analysis to compare the average number of visits made by bees trained to the constant versus changing feeders in each condition during each half hour time period throughout the water-only test day. Finally, to compare search persistence throughout the entire day we used a Fisher's exact test to analyze if test bees from different predictability conditions differed in how many time periods they visited the water feeder more than would be expected by chance if their visits were spread randomly throughout the day.

We again built all models using a forward approach and selected final models according to their AICc values. After selecting final models, we analyzed them using ANOVA and post hoc tests with a Tukey correction.

RESULTS

Spatial Predictability

In this experiment, bees were trained to a testing array that had one location with a high-quality reward; the location of this reward either stayed the same or changed during the training period. This experience of foraging in a spatially predictable versus unpredictable environment affected the search behavior of bees even during the training period. During training, bees in the unpredictable spatial condition made significantly more contacts (Condition: F^{1}_{207} = 42.929, P = 5.68e-11) and searched for significantly longer (Condition: F^{1}_{207} = 9.37, P = 0.0022) at the testing array before finding the location containing the rewarding flower (Figure 2.2). The pattern displayed in each predictability condition did not significantly change as bees received more training visits (as evidenced by the fact that training visit number was not retained in either final model).

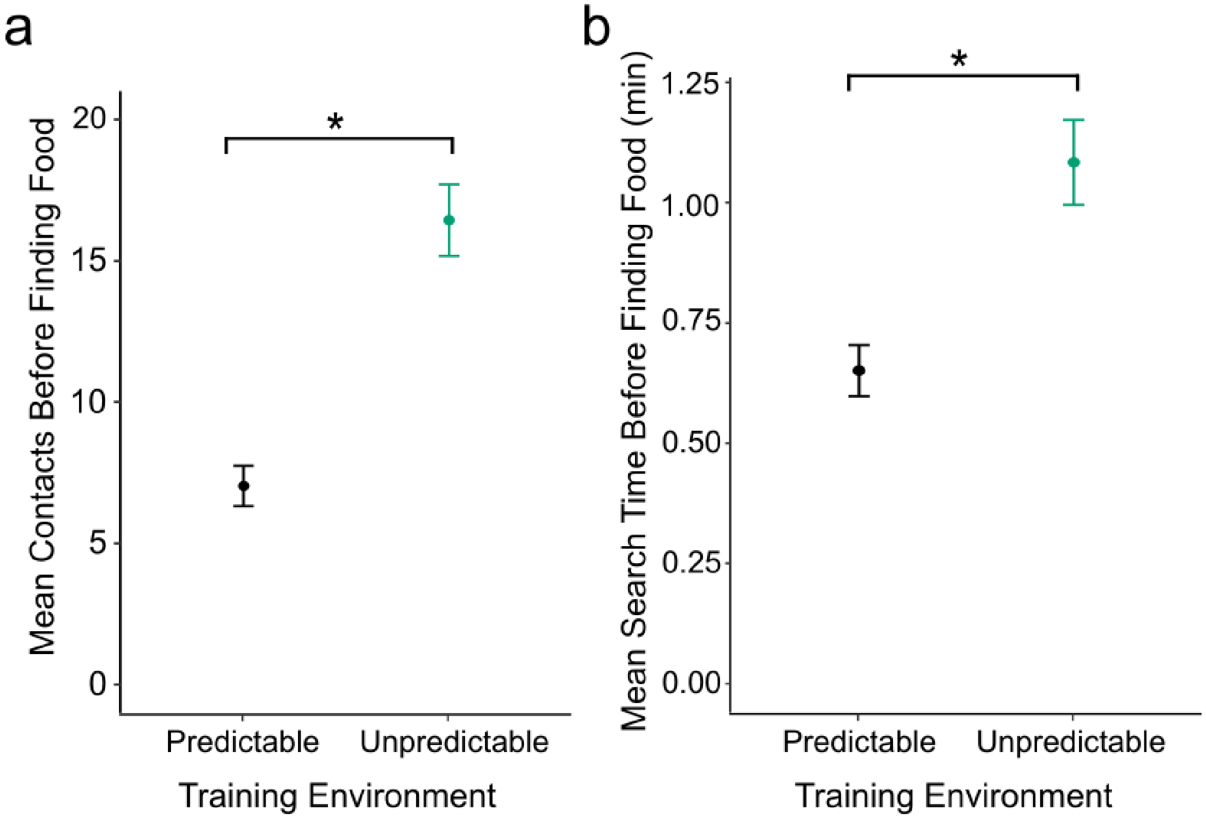

Figure 2.2. Exploratory behavior during the spatial experiment training period. The mean (a) number of contacts bees made and (b) time bees spent searching before finding the rewarding location in the testing array during training in the spatial unpredictability experiment are shown. Bees in the unpredictable spatial environment (a) made significantly more contacts (*P* < 5.68e-11) and (b) searched significantly longer (*P* = 0.0022) before finding the rewarding location. Error bars represent standard error. Significant differences are indicated with *.

The effects of experience with foraging in a spatially predictable or unpredictable environment on search behavior continued even when rewards were no longer present in the testing array. During the test period, bees in the predictable and the unpredictable condition did not significantly differ in how persistently they contacted the testing array (Condition: F^{1}_{91} = 0.0052, $P = 0.94$; Figure 2.3a). In addition, bees in both conditions changed their search patterns over the course of the 10-minute test in the same way, making significantly more contacts on the array during the first half of the test than during the second half (Time Block: F^{1}_{91} = 32.35, $P <$

1.29e-8). However, a bee's previous experience with environmental predictability did shape how they distributed their search spatially. Bees that had previously experienced a predictable training condition made significantly more contacts at the last rewarded training location (the only location at which they had received a reward) than did bees that had experienced an unpredictable training condition (Condition: $F_{91}^{1} = 30.33$, $P = 3.65\text{e-}8$; Figure 2.3b). As was seen for the total number of contacts made on the testing array, bees in both groups focused their contacts more precisely at the last rewarded training location during the first half of the test than during the second half (Time Block: $F_{91}^{1} = 42.94$, $P = 5.65\text{e-}11$).

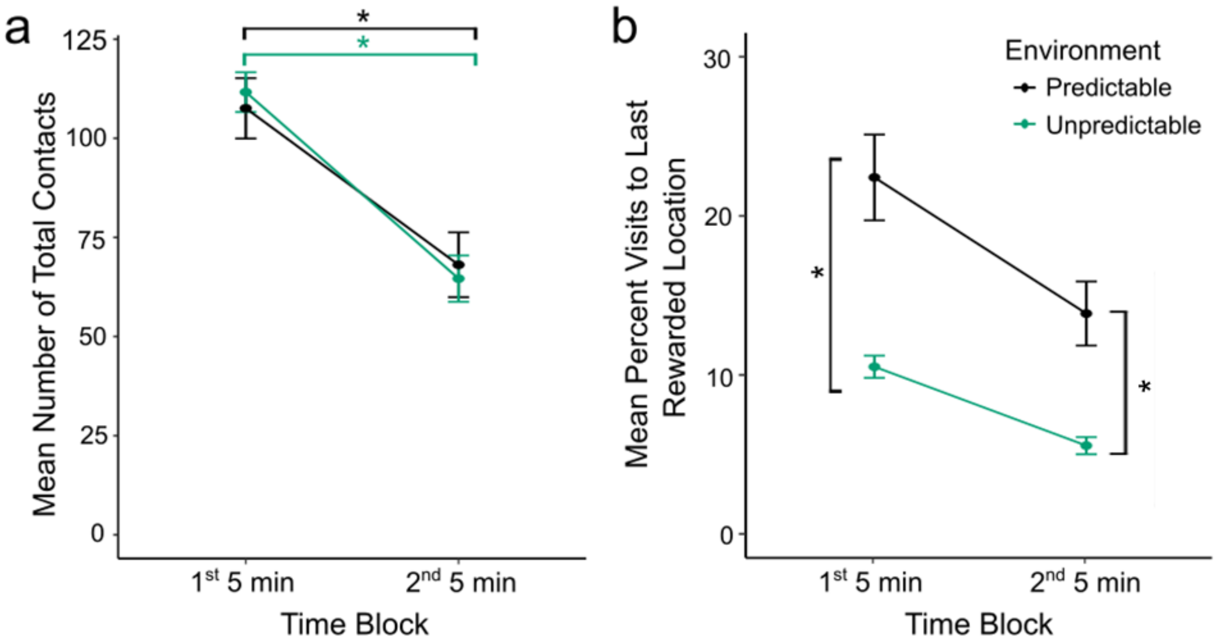

Figure 2.3. Contacts at testing array during spatial experiment test. The mean (a) total number of contacts bees made and (b) percentage of contacts bees made at the last rewarded location across the 10-minute test period in the spatial unpredictability experiment are shown. (a) Bees trained in predictable and unpredictable environments made more contacts during the first 5 minutes of the test than during the second 5 minutes of the test ($P < 1.29\text{e-}8$) but did not differ from each other in the total number of contacts they made ($P = 0.94$). (b) Bees in the predictable spatial environment focused more of their contacts on the last rewarded location than did bees in the unpredictable spatial environment ($P = 3.65\text{e-}8$), and this pattern held throughout the test. Significant differences are indicated with *.

The more widely distributed contacts of bees trained in the unpredictable condition were not simply a result of those bees searching randomly within the array, as bees from both conditions made significantly more contacts at rewarded flowers than would be expected by chance (Predictable: $X^2 = 790.75$, $DF = 1$, $P < 2.2\text{e-}16$; Unpredictable: $X^2 = 13.97$, $DF = 1$, $P = 0.00019$; Figure 2.4). This focus on the rewarded locations was especially present in the predictable condition, where bees made 21.09% of their visits to the lone rewarded flower, much more than the 8.33% that would be expected by chance. While bees in the unpredictable condition also focused more of their visits to rewarded flowers, the difference was much smaller. 52.89% of their visits were made to rewarded flowers, as compared to the 50% that would be expected by chance.

The differences in how bees in the predictable versus unpredictable training conditions focused their search can be seen clearly in the network analyses, which show the number of contacts received by each location as well as the transitions between locations. We illustrate this with data from the three bees from each condition that made the most contacts at the testing array (Figure 2.4). In the predictable condition, the rewarded location is clearly visited most often (as seen by the large size of the circle at that location), whereas the flowers immediately around it tend to be visited the next most times and the flowers farthest away visited the least. In contrast, the visits to rewarded locations by bees from unpredictable environments are much more spaced out across the board. Often, the previously rewarded flowers are not visited much more than the previously unrewarded flowers (if at all), and the last rewarded location and the flowers surrounding it are not generally visited in greater numbers than the other rewarded locations.

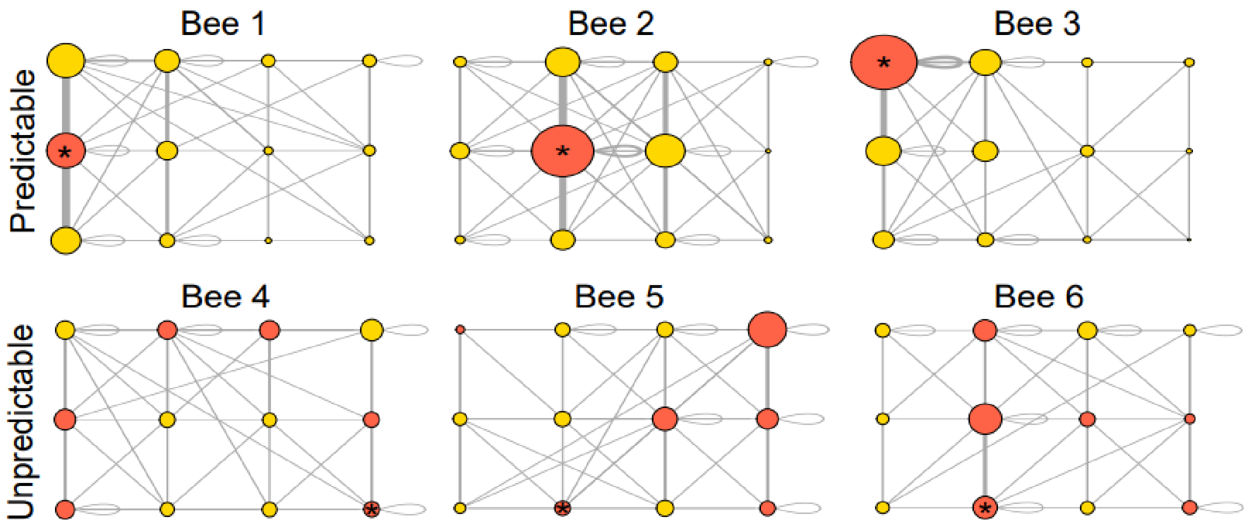

Figure 2.4. Testing array visitation networks. The maps show the movement patterns of representative bees from the predictable (top row) and unpredictable (bottom row) spatial environments on the testing array during the 10-minute testing period. The three bees from each environment with the highest numbers of total contacts were used as representative examples. The diameter of each circle is proportional to the frequency of visits to that location (larger indicates higher visitation). Orange circles indicate the location was rewarded and yellow circles indicate the location was unrewarded; the last rewarded location is marked with an asterisk. Line thickness between two points is proportional to the frequency of transitions between the locations (thicker indicates more transitions). Bees in the predictable environment focused more of their attention on the last rewarded location, but bees in both environments made more visits to the rewarded than the unrewarded locations ($P < 0.0001$ for both).

How persistently and precisely bees searched within the testing array versus outside the array in the boarder environment was influenced by multiple interacting factors. All bees spent more time searching on the array than off of it overall (Search Location: F^{1}_{173} =146.1980, P < 2e-16; Figure 2.5a). While a significant interaction between a bee's predictability training condition and her search location was found in the model (Condition*Search Location: F^{1}_{173} = 4.84, P = 0.028), bees in the predictable and the unpredictable conditions did not differ from each other in how long they searched on versus off the array ($P > 0.10$; Figure 2.5a). However, the amount of time bees searched on the array did change over time (Time Block*Search Location: F^{1}_{173} = 120.61, P < 2e-16; Figure 2.5b). In particular, the amount of time bees searched on the array significantly declined during the second half of the test ($P < 0.001$), while the amount of time they spent searching off the array significantly increased during the second half of the test ($P < 0.001$). Because of this interaction between how long bees had been searching and where they searched, bees searched longer on the array than off of it during the first half of the test ($P < 0.001$) but spent equal amounts of time searching on and off the array in the second half of the test ($P = 0.86$). However, bees from the two conditions did not differ in how the location of their searching changed over time (Condition*Time Block*Search Location: $F^{1}_{173} = 0.071$, $P = 0.79$), or in how much time they searched in general over time (Condition*Time Block: $F^{1}_{173} = 0.50$, $P = 0.48$).

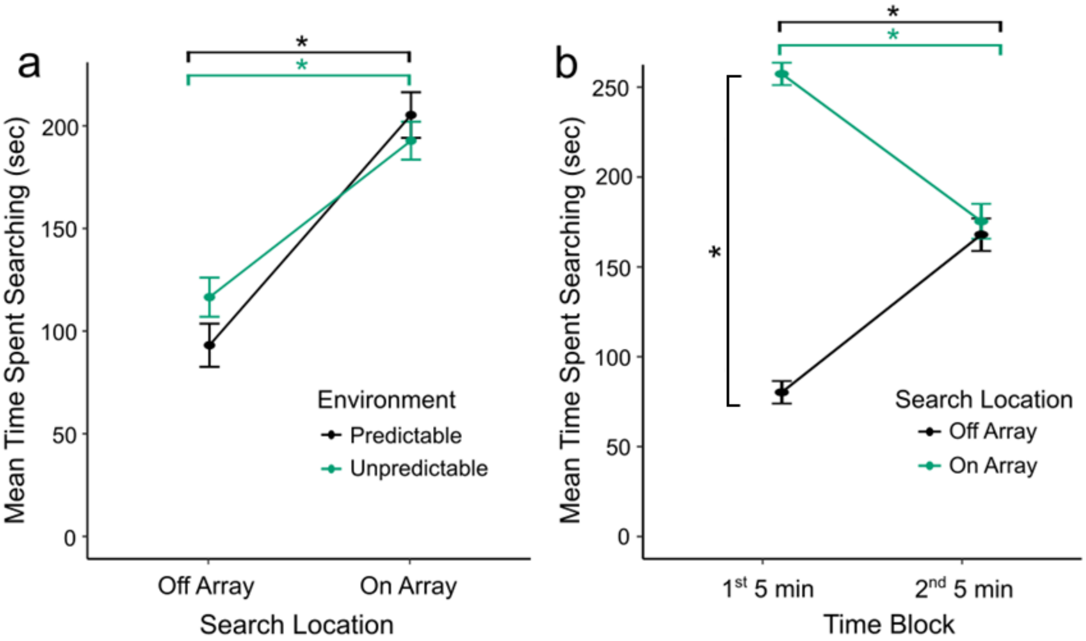

Figure 2.5. Exploratory behavior relative to the spatial testing array. The mean time spent searching on versus off the testing array (a) for the two training conditions and (b) between the first and second halves of the test period. (a) Bees in both environments searched longer on than off the array ($P < 0.001$) and did not significantly differ in how long they searched in either location ($P > 0.10$). (b) Bees searched significantly longer on than off the testing array during the first 5 minutes of the test ($P \leq 0.001$) but searched equally in both locations during the second half of the test ($P = 0.86$). Error bars represent standard error. Significant differences are indicated with *.

Bees had the option to give up on searching for the missing high-quality reward and instead return to the constant feeder (and its low-quality reward) throughout the test period. When we examined how previous experience with spatial predictability shaped how persistently bees searched before giving up and returning to the constant feeder, we found that bees trained in the predictable and the unpredictable conditions did not differ in their latency to return to (X^2 = 0.6, *DF* = 1, *P* = 0.4) or feed at (X^2 = 0.0, *DF* = 1, *P* = 0.9) the constant feeder (Figure 2.6). Similarly, all bees, regardless of their training experience, spent more time at the constant feeder during the second half of the test than during the first half (Time Block: F^1_{91} = 15.43, *P* = 8.57e-5; Condition: F^1_{91} = 0.0023, *P* = 0.96).

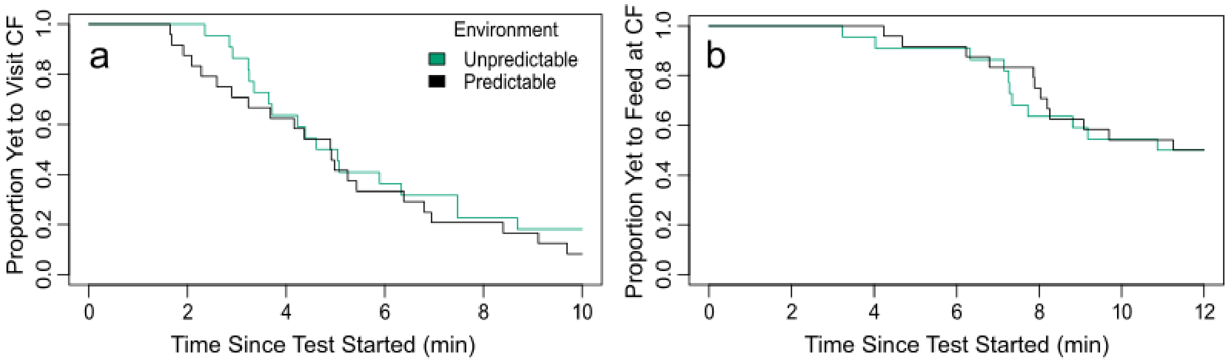

Figure 2.6. Exploratory behavior relative to the constant feeder. The latencies of bees to abandon the testing array to (a) visit the constant feeder for the first time or (b) feed at the constant feeder during the test period are shown. Bees trained in either environment did not differ in their time to visit (*P* = 0.4) or feed (*P* = 0.9) at the constant feeder.

Temporal Predictability

In this experiment, bees at a variable feeder were trained to expect that a low-quality reward available throughout the day would briefly increase in quality at a predictable or an unpredictable time every day. Meanwhile, other bees from the same hive were trained concurrently to a feeder with a constant low-quality reward. For bees trained to a variable feeder, experiencing a temporally predictable versus unpredictable high-quality reward significantly affected their search even during the training period. Bees trained in the temporally predictable condition searched significantly longer (i.e., more persistently; X^2 = 6.2, *DF* =1, *P* = 0.01; Figure 2.7a) following the daily resource downgrade than did bees with experience in a temporally unpredictable condition. Bees did not change how long they searched following a downgrade over the course of the training period (training day was not included in the final model), but they did change the number of contacts they made before resuming feeding over time in ways that depended on their training condition (Trial Day: F_{281}^{1} = 205.098, *P* < 2.2e-16; Trial Day*Condition: F_{281}^{6} = 40.15, *P* = 4.26e-7; Figure 2.7b). In general, bees within a given predictability condition made more contacts during the later training days (days 5-7) than during the initial training days (days 1-2; all *P* < 0.05, excluding day 2 vs day 6 in predictable [*P* = 0.75]). However, bees in the predictable and the unpredictable training environments made equal numbers of contacts on a given training day (e.g., equal contacts on day 5; all *P* > 0.80).

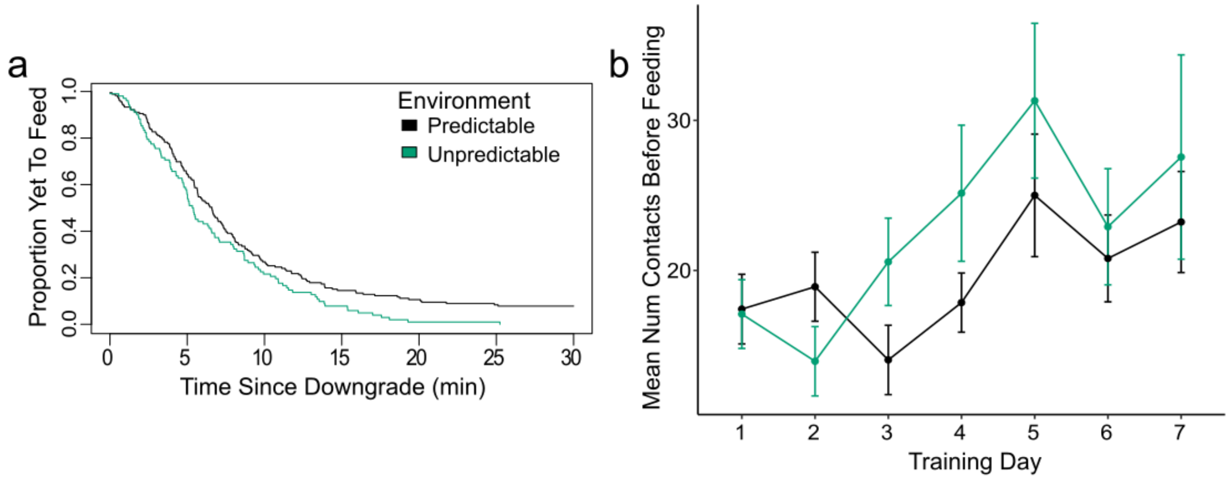

Figure 2.7. Exploratory behavior during the temporal experiment training period. The (a) latency to resume feeding following a downgrade over the training period and (b) mean number of contacts made each day over the training period are shown. (a) Bees trained in predictable environments searched significantly longer following a downgrade than bees trained in unpredictable environments (*P* = 0.01). (b) Bees tended to make more contacts before feeding in the latter training days than the first few training days (*P* < 2.2e-16), but the average number of contacts on a given day did not differ between bees trained in different environments (*P* > 0.8).

On the water-only test day, when all sucrose rewards were absent from the environment, bees previously trained to the changing feeders visited the unrewarding feeder more often (i.e., more persistently) than did bees trained to the constant feeders (Feeder: F_{47}^{1}= 6.91, P = 0.0086), regardless of whether they had experienced reward increases on a predictable or an unpredictable schedule (Condition: F_{47}^{1} = 0.99, P = 0.32; Figure 2.8a). While we observed a trend where bees in the unpredictable condition made more visits to the test feeder and fewer visits to the constant feeder compared to bees in the predictable condition, this interaction was not significant and it was not included in the final model.

Generally, the precision of bees' search on the water-only test day, as measured using proportions of visits during the time of interest, was higher for bees trained in temporally predictable conditions (Condition: F_{50}^{1} = 32.72, P = 1.07e-8) and was influenced by the type of feeder she was trained to (Feeder: F_{50}^{1} = 6.43, P = 0.011). However, a bee's training condition and training feeder interacted to shape how much of her search occurred during the time of interest (Condition*Feeder: F^{1}_{50} = 6.53, P = 0.011; Figure 2.8b), making those general patterns potentially misleading. Within the predictable condition, bees trained to the changing feeder did indeed make a significantly higher proportion of their visits during the reward time of interest as compared to bees trained to the constant feeder ($P < 0.01$). In contrast, within the unpredictable condition bees trained to the changing feeder versus the constant feeder did not differ in the precision of their visits ($P > 0.10$). Between training conditions, bees trained to the changing feeder in the predictable condition focused more of their visits on the time of interest than did bees trained to the changing feeder in the unpredictable condition ($P < 0.01$), but bees trained to the constant feeders did not differ in their precision between conditions (P = 0.48). The high visitation by bees trained to the changing feeder in the predictable condition is therefore the primary driver of the significant main effects.

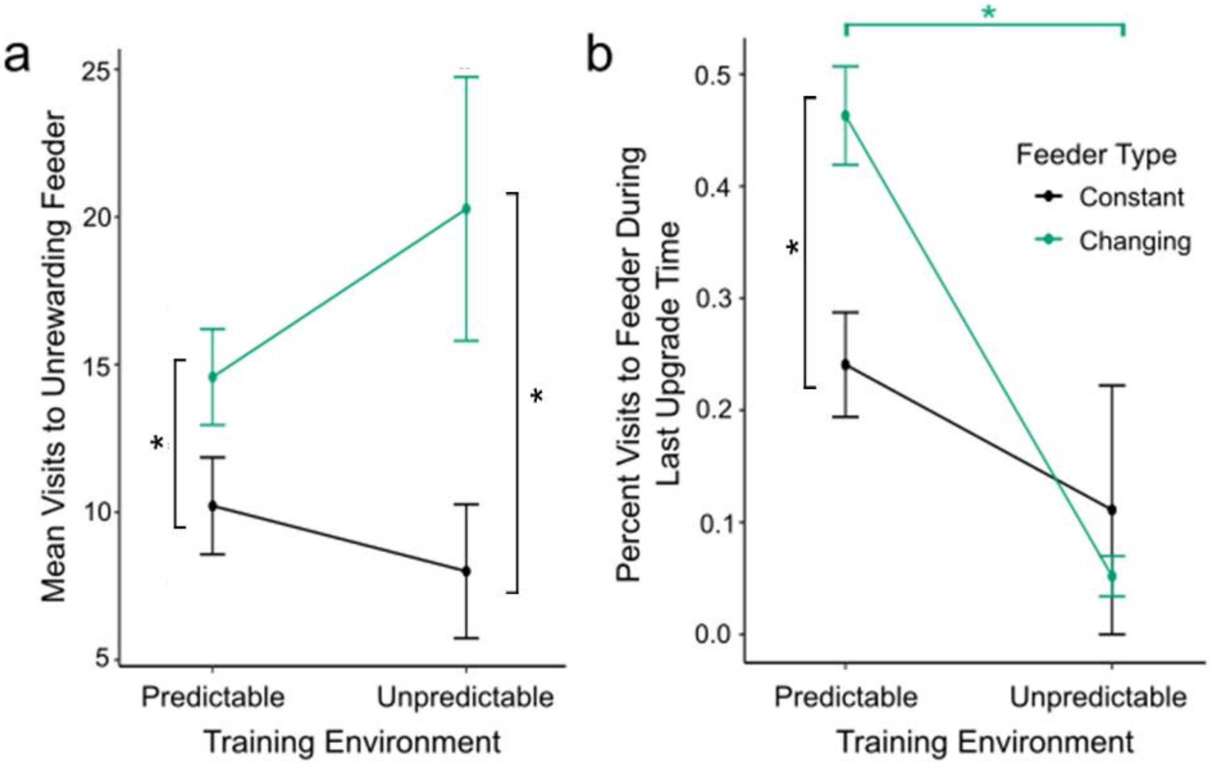

Figure 2.8. Contacts at the unrewarding water feeder during the temporal experiment test. The mean (a) total number of contacts bees made and (b) percentage of contacts bees made during the upgrade time of interest during the water-only test day in the temporal unpredictability experiment are shown. (a) Bees in both environment that were trained to a feeder that changed (experienced an upgrade) searched significantly longer than did bees that were trained to an unchanging feeder ($P = 0.0088$), but bees trained to the same type of feeder did not differ in their visitation based on their training environment ($P = 0.32$). (b) Bees trained to the changing feeder in the predictable environment focused a significantly higher percentage of their visits during the upgrade time of interest than did any other bees (all $P < 0.01$). Bees trained to the constant feeder in either environment or to the changing feeder in the unpredictable environment did not differ from each other in their visitation during the upgrade time of interest (all $P > 0.1$). Significant differences are indicated with *.

When bees' search was considered throughout the entire day in a temporally explicit manner, a bee's search effort during a given time was shaped by many interacting factors. Bees trained in the predictable and the unpredictable conditions showed peak visitation during different times of the day (Condition*Time Period: $F_{650}^{12} = 60.61$, $P = 1.74\text{e-}8$): in the morning from 1030-1200 (i.e., during and after the time of interest) in the predictable condition and in the afternoon from 1300-1430 (i.e., primarily before the time of interest) in the unpredictable condition (Figure 2.9). However, bees trained to the changing feeder did not differ in their visitation throughout the day from bees trained to the constant feeder (Feeder*Time Period: F_{650}^{12} = 8.75, P = 0.72). As seen in Figure 2.9, bees in the same predictability condition unexpectedly showed similar patterns of visitation throughout the day regardless of which feeder they were trained to. This correlation between the number of visits made by bees trained to the changing feeder versus the test feeder was significant in both predictability conditions (Predictable: S = 62.27, r_S = 0.83, P = 0.00046; Unpredictable: S = 72.90, r_S = 0.80, P = 0.0010; Figure 2.10). Generally, the number of visits made by bees in a given predictability condition was influenced by which feeder they had been trained on (Condition*Feeder: F_{650}^{12} = 4.85, P = 0.028), though the pairwise differences between changing and control bees were not strong enough to be detected in posthoc tests (P > 0.05 all).

Bees in the predictable and the unpredictable conditions did not differ in how persistently they searched at the feeders throughout the water-only test day. Using the metric of the ‘number of time periods where visits were more frequent than would be expected by chance if bees visited at all times of day equally’, we found no difference between predictable and unpredictable test bees (P = 0.23).

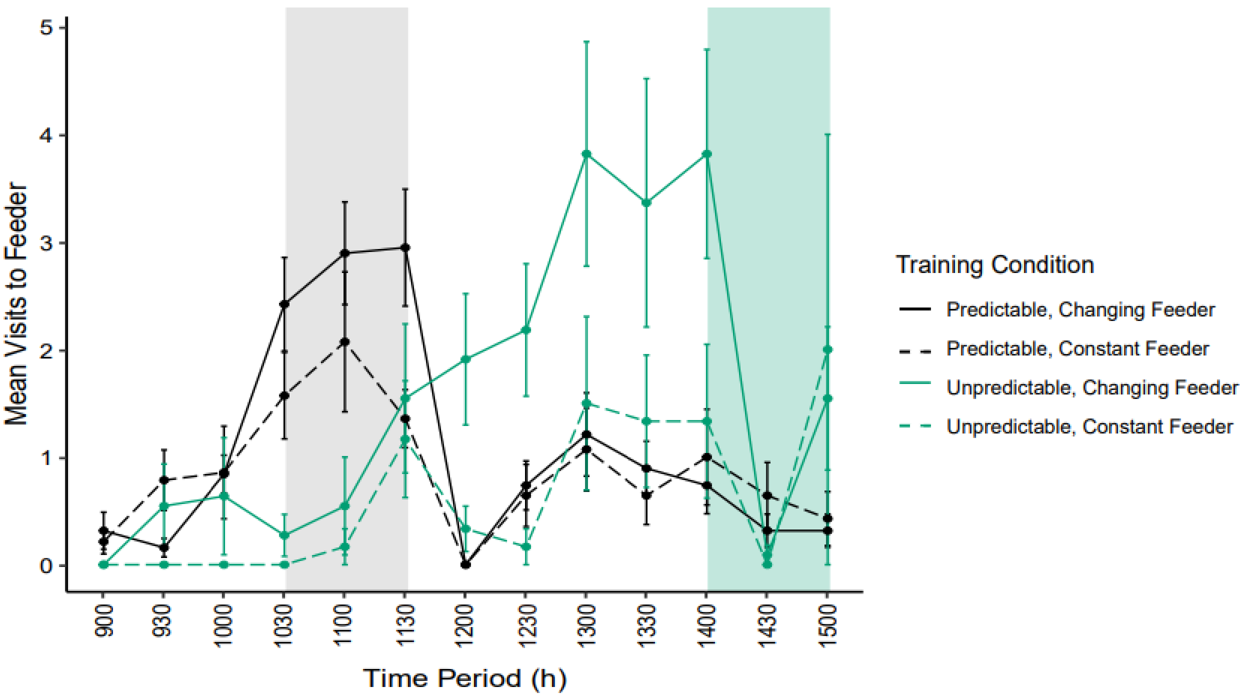

Figure 2.9. Visitation to water feeders throughout the temporal experiment test day. The mean numbers of visits made to the water-only test feeder every half hour throughout the water-only test day is shown for bees trained to changing or unchanging feeders in the predictable and unpredictable environments. Bees trained in the same type of environment exhibited similar visitation patterns regardless of the type of feeder they were trained to ($P > 0.05$ all). Bees trained in the predictable environment showed peak visitation during the upgrade time of interest, while bees trained in the unpredictable environment showed peak visitation primarily before the upgrade time of interest. The gray shaded region indicates the upgrade time of interest for the predictable condition (1030-1130) and the green shaded region indicates the upgrade time of interest for the unpredictable condition (1400-1500).

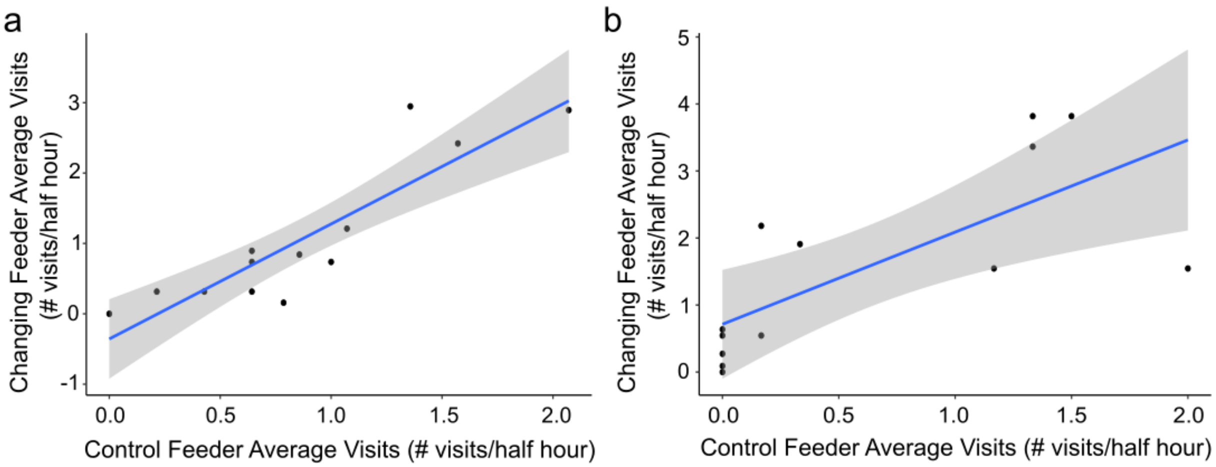

Figure 2.10. Correlations in visitation between bees at changing and control feeders. The average number of visits to the changing and control feeders during the water-only test day for the (a) predictable and (b) unpredictable conditions are shown. (a) A significant positive correlation between average number of visits to the changing and control test feeders during each half hour period was found in the predictably timed rewards condition ($P = 0.00046$). (b) A significant positive correlation between average number of visits to the changing and control test feeders during each half hour period was also found in the unpredictably timed rewards condition ($P = 0.0010$).

DISCUSSION

When and how animals should explore their environment is a classic question (Stephens and Krebs 1986; Stephens 1987; Lima and Dill 1990). We investigated one situation where animals are required to explore their environment: when a previously available resource in the environment suddenly becomes unrewarding. Specifically, we studied how a honey bee's previous experience with unpredictability in reward location or reward timing shapes its subsequent exploratory behavior in space and time when faced with the absence of rewards. We found that a bee's previous experience has a strong impact on the precision of its search behavior but does not seem to influence the persistence of its search, at least not in the environments we created.

Bees are known to be extraordinarily efficient at learning the location and the daily timing of rewards, and are even able to do so in as little as one foraging trip (Moore et al. 1989; Lehrer and Collett 1994; Wei et al. 2002; Wei and Dyer 2009; Moore et al. 2011; Al Toufailia et al. 2013; Tsvetkov et al. 2019). During the training period of both the spatial and the temporal unpredictability experiments we found, as expected, that bees were able to quickly and accurately learn when and where to find high quality rewards when those rewards were predictable (Figures 2.2 and 2.7). Bees trained in predictable spatial conditions appeared to learn the location of the rewarding flower more quickly than did bees trained in unpredictable spatial conditions, finding it rapidly by the third training visit while the search time and effort remained high throughout the training period for bees in the unpredictable condition (Figure 2.2). Similarly, bees that experienced predictably timed rewards searched significantly longer following a downgrade in reward quality during the training period (Figure 2.7a), possibly because they were more certain that high-quality food exists in the environment, and were therefore more willing to pay the costs of unrewarded search to find it. In addition, all bees regardless of condition made more contacts at the test feeder after the daily resource downgrade as they gained more experience (Figure 2.7b), suggesting they were searching more intensely over time. The longer, less precise search behavior in space or time exhibited by bees in the unpredictable environment during the training portion of these experiments supports our underlying assumption that the training condition we created for bees in unpredictable environments prevented bees from being able to rely on previous experience to guide future behavior (i.e., successfully find high-quality rewards).

The key insights from this study come during the test period, when we found evidence that the experiences bees gained during training influenced their search behavior, particularly their search precision, even when there were no rewards present at all. Bees that experienced predictable rewards, either in space or time, focused their search more precisely on the location or time they were accustomed to receiving a reward. In the spatial experiment, bees in the predictable environment focused significantly more of their visits on the last rewarded training flower and concentrated their search more closely around that rewarded flower and its immediate neighbors than did bees in the unpredictable environment (Figures 2.3b and 2.4). However, because bees in the unpredictable environment received rewards at 6 locations, it is possible that their visitation to the last rewarded location was lower because they divided their attention among all locations at which they had received a reward, but they were still visiting rewarded locations at a higher rate than unrewarded locations. Indeed, bees in both conditions spent more time searching at previously rewarded locations than would be expected by chance, but that increase was much more dramatic for bees in the predictable environment, supporting the idea that they are searching more precisely (Figure 2.4). Similarly, bees trained to a predictably timed high-quality reward focused more of their visits more precisely during the time when they had received rewards every day; this contrasts with the temporally more displaced search of bees that experienced unpredictably timed high-quality rewards (Figure 2.8b). Interestingly, bees trained in the unpredictable environment actually showed peak visitation hours before the last rewarded training time (Figure 2.9), again highlighting the relatively imprecise timing of their visits compared to the most recent reward time.

The increased precision exhibited by bees trained in the predictable condition, both in the spatial and the temporal experiments, can be interpreted as the result of more cumulative positive reinforcement of the association between reward and either location or time (Neuringer et al. 2000; Moore and Doherty 2009; Moore et al. 2011; Al Toudailia et al. 2013; Tsvetkov et al.

2019). However, our data suggest a richer picture than would be suggested by a simple interpretation based on the associative strength between stimulus and response. For one thing, since bees are able to learn reward locations and timings in just one training visit (Moore et al. 2011; Tsvetkov et al. 2019), the lack of precision of search that we observed in the bees trained in unpredictable environments suggests that bees are integrating across their prior experiences to make decisions about where and when to search, not just relying on their memory of the last reward. In the spatial predictability experiment in particular, honey bees might be storing multiple rewarded locations in their short-term memory and using those to guide their search at the testing array.

A simple account based on associative strength between stimulus and reward also does not explain the tendency of bees in the unpredictable temporal environment to show up hours earlier than the last rewarded time. This behavior might be an extreme consequence of honey bees' anticipatory foraging behavior. Honey bees often show up to rewarding resources slightly earlier than the time it will be rewarding, a behavior that is probably advantageous by allowing them to be the first to exploit and recruit other workers to the reward (Moore et al. 1989; Naeger et al. 2010; Van Nest 2012). We took that anticipatory behavior into account by examining visitation in the half hour before and during the last rewarded upgrade time as our time of interest, but the behavior exhibited by the bees in the unpredictable temporal environment was much more anticipatory than expected. These bees did focus their visitation during the afternoon hours (Figure 2.9), the time of day they had last received a reward, but appeared to be 'extra anticipatory' as if they were aiming to beat competitors to a reward they are expecting in the afternoon based on their most recent experience. Alternatively, this extra anticipatory behavior might reflect the fact that honey bees tend to be more anticipatory in the afternoon (Moore et al.

1989). As we did not have the time to train bees in each condition to experience their last upgrade during every part of the day, an interesting follow up experiment would be to examine whether similarly extreme anticipatory behavior is seen if the last upgrade time experienced is in the morning for unpredictably trained bees and in the afternoon for predictably trained bees.

While we were primarily focused on examining how unpredictability in reward locations influenced honey bee exploratory behavior within the space of the testing array, we also found that a bee’s experience influenced the precision of her search in the broader environment over time. Specifically, bees in both the spatially predictable and spatially unpredictable conditions searched less precisely over time, spending more time searching outside of the testing array in the latter half of the testing period (Figure 2.5b). Bees trained in both spatial environments showed similar precision in their searching on versus off the testing array (Figure 2.5a) and in relation to the constant feeder (Figure 2.6), spending more time around the constant feeder later in the test. This expansion of search over time suggests that bees first searched locally within the patch, then broadened their search to be less precise over time as they were unsuccessful. This search pattern is frequently seen in honey bees attempting to locate food sources (Reynolds et al. 2007; Reynolds et al. 2009). Furthermore, such an expansion of search over time is widely observed in animals and humans, both in behavioral and cognitive search tasks (Hills & Dukas 2012). The similarly in search expansion observed between bees in the predictable and unpredictable environments is therefore unsurprising.

While we found evidence that the predictability of a bee’s training experience strongly shapes the precision of its search, we found no indications that it also shapes the persistence with which the bee searched. Bees trained in either environment did not differ in their total number of visits to the spatial testing array or the temporal water feeder during the test periods (Figures 2.3a and 2.8a), and bees in the spatial experiment exhibited similar search extinction patterns where they visited the testing array less over time. Similarly, bees in the temporal experiment visited the water feeder more often than expected by chance equally often (Figure 2.9), suggesting they were visiting the feeder at high rates for the same number of time periods. This unexpectedly similar persistence (or lack thereof) between bees trained in different environments could be due to the size of the environment in the spatial experiment. Honey bees can forage up to 6 km away from their hive, with most of their foraging effort concentrated within 1 km of their hive, although foraging distance is highly dependent on resource availability, landscape context, and reward quality (Seeley and Visscher 1982; Seeley 1995; Beekman and Ratnieks 2000; Steffan-Dewenter and Kuhn 2003). Given the small scale of the flight cage and the simplicity of the testing array (consisting of only 12 artificial flowers), it is possible that bees were able to exhaustively search the entire testing array within a few minutes without expending large amounts of energy flying to the array or traveling from flower to flower. After an exhaustive search of the array, there would be no need to continue to search the testing array, leading to the rapid extinction of search that we saw over time. If the testing array had been larger or farther from the colony, or if the rewarding and unrewarding locations within the patch had been farther apart, the costs of search would be significantly higher and might exert stronger pressure on the persistence of search. In such a case where it would be extremely difficult or costly to exhaustively search every flower in a patch, we might expect bees with experience in a predictable spatial environment to give up search faster as they would be more certain that the previous reward was no longer available and therefore less willing to pay the costs of unrewarded search. Future experiments examining the effect of predictability in reward location on search behavior could test this hypothesis to see how the spatial scale at which rewards are located shapes the costs and benefits of unrewarded search.

As with the spatial experiment, the similarity in persistence seen among bees trained in temporally predictable versus unpredictable environments could be due to the specific parameters of the “world” we presented to the bees. While we did not see differences in the persistence of bees that experienced predictably or unpredictably timed reward upgrades, we did find that those bees that experienced any daily upgrade were significantly more persistent than those that did not (Figure 2.8a). In our experiment, all test bees received a high-quality reward once a day; we were testing not how the predictability of a reward in terms of its availability across days affected future exploratory behavior, but how the predictability of a reward’s timing during the day affected future exploratory behavior. We found here that experiencing a high-quality reward in the environment was sufficient to trigger increased persistence in the face of an absent reward on a later day. If, however, the reward was unpredictable in terms of its daily availability then we might expect bees in the unpredictable condition to be more persistent in searching for it throughout the day as evidence of its absence in the past was not a reliable predictor of its future availability. Another possible reason we did not observe differences in persistence between bees trained to changing feeders in the temporal condition may be our short observation period. In the temporal experiment, we did not continue to track the visitation of bees over multiple unrewarded days, as we were interested in the effects of experience on their short-term exploratory behavior. It is possible, though, that bees trained in different environments would differ in their extinction rates across days for visiting the unrewarding feeder. Follow up experiments could therefore examine how the predictability of reward timing influences the monitoring and extinction of search at an unrewarding resource across multiple days, and whether the distribution of visits at time points of interest changes across days.

One interesting pattern seen when examining the distribution of visits throughout the day in the temporal predictability experiment was that control bees trained to the constant feeder and test bees trained to the changing feeder in the same predictability environment exhibited similar activity patterns over the water-only testing day (Figures 2.9-2.10). For example, the control bees in the predictable environment never experienced an increase in reward quality, and yet they showed an uptick and decline in activity during the morning at the same time as the test bees trained in the predictable environment. The control bees in the unpredictable environment showed a similar pattern matching those of the associated test bees. The strong positive correlation in visitation between control and test bees suggests that the control bees were picking up cues within the hive that they should go out and search at certain times, even though they never experienced any change in reward quality themselves. The stronger peak seen in the control bees in the predictable environment (as compared to those in the unpredictable environment) could even indicate that those control bees learned a time cue from the high activity of the test bees in the hive during the upgrade time. Hive activity and excitement has previously been shown to greatly affect the behavior of other bees, even leading to changes in their subsequent reward expectations and exploratory behavior (Dornhaus and Chittka 2004; Dornhaus and Chittka 2005; Renner and Nieh 2008; Incorvaia et al. 2020), supporting the idea that the control bees were responding to the in-hive activity of the test bees. The fact that this seemed to happen even on the water-test day suggests that this influence is not related to the test bees bringing back high rewards.

One major limitation of the temporal experiment was that we were unable to observe the behavior of control bees, test bees trained in a predictable environment, and test bees trained in an unpredictable environment concurrently due to limited space in the hoop house. Similarly, we did not have time to test each group individually in the hoop house (thereby minimizing any potential in-hive effects of the training condition that might affect control bees). Because of these constraints, we had to train and observe two groups at the same time, leading to some control bees being trained in the predictable environment and some in the unpredictable environment. While we did see differences in their distribution of their visits across time in ways that mirror those seen in the test bees of their given environment, we did not find differences in their total visitation during the water day or in the proportion of visits they made to the water feeder during the time of interest. This lack of difference suggests that while control bees were influenced by the test bees of their predictability environment, the experience they had at the feeder (i.e., a constant, unchanging reward) had a larger impact on their behavior in the absence of rewards than did the hive environment they experienced. Future studies should more clearly disentangle the effects of hive environment and feeder experiences for control bees, and aim to test bees experiencing different environments either all at the same time or all completely separately.

Overall, this study suggests that a bee’s past experience with spatial or temporal resource predictability shapes the manner in which she searches, but not her overall investment in exploration when rewards are absent. We show that, at least in the environments we created, experience with unpredictability in reward location or reward timing leads to less precise exploration in honey bees. While we did not find strong evidence that such experience changes how persistently honey bees search in space and time, our study was limited by small spatial and temporal scales. Future studies should examine exploratory behavior in larger patches or over multiple days to determine if experience influences persistence on scales not studied in these experiments. In addition, similar studies examining the effects of previous experience on future exploratory behavior in the absence of rewards should be done in other species to determine if these effects of experience are unique to honey bees or are more broadly applicable.

ACKNOWLEDGEMENTS

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APPENDIX

Table S2.1. Model selection for length of search before finding rewarding location during spatial experiment training period. Final selected model is bolded.

Model	AICc	Delta AICc	Model Weight
TrainingContacts ~ Condition + (1|BeeID)	**480.464**	**0.000**	**0.916**
TrainingContacts ~ Condition + TrainingVisit + (1|BeeID)	485.444	4.981	0.076
TrainingContacts ~ Condition + TrainingVisit + Condition*TrainingVisit + (1|BeeID)	490.929	10.465	0.005
TrainingContacts ~ TrainingVisit + (1|BeeID)	491.712	11.249	0.003

Table S2.2. Model selection for number of contacts made at the testing array before finding rewarding location during spatial experiment training period. Final selected model is bolded.

Model	AICc	Delta AICc	Model Weight
TrainingContacts ~ Condition + (1|BeeID)	**1430.217**	**0.000**	**0.904**
TrainingContacts ~ Condition + TrainingVisit + Condition*TrainingVisit + (1|BeeID)	1435.505	5.288	0.064
TrainingContacts ~ Condition + TrainingVisit + (1|BeeID)	1436.902	6.686	0.032
TrainingContacts ~ TrainingVisit + (1|BeeID)	1467.239	37.022	0.000

Table S2.3. Model selection for total number of contacts made at the testing array during spatial experiment test. Final selected model is bolded.

Model	AICc	Delta AICc	Model Weight
TotalContacts ~ Condition + TimeBlock + (1|Block/BeeID)	**914.379**	**0.000**	**0.703**
TotalContacts ~ Condition + TimeBlock + Condition*TimeBlock + (1|Block/BeeID)	916.473	2.094	0.247
TotalContacts ~ Condition + (1|Block/BeeID)	919.630	5.252	0.051

Table S2.4. Model selection for number of contacts made at the last rewarded training location during spatial experiment test. Final selected model is bolded.

Model	AICc	Delta AICc	Model Weight
ContactsLastRewardedLocation ~ Condition + TimeBlock + (1|Block/BeeID)	**592.891**	**0.000**	**0.669**
ContactsLastRewardedLocation ~ Condition + TimeBlock + Condition*TimeBlock + (1|Block/BeeID)	594.770	1.880	0.261
ContactsLastRewardedLocation ~ Condition + (1|Block/BeeID)	597.408	0.104	0.070

Table S2.5. Model selection for time spent searching during spatial experiment test. Final selected model is bolded.

Model	AICc	Delta AICc	Model Weight
SearchTime ~ Condition + TimeBlock + SearchLocation + Condition*TimeBlock + Condition*SearchLocation + Condition*TimeBlock*SearchLocation	**1973.754**	**0.000**	**1**
SearchTime ~ Condition + TimeBlock + SearchLocation + Condition*TimeBlock + Condition*SearchLocation	2061.937	88.183	0
SearchTime ~ Condition + TimeBlock + SearchLocation + Condition*TimeBlock	2062.998	89.243	0
SearchTime ~ Condition	2127.578	153.824	0
SearchTime ~ Condition + TimeBlock	2129.617	155.862	0
SearchTime ~ Condition + TimeBlock + Condition*TimeBlock	2131.539	157.775	0

Table S2.6. Model selection for time spent searching near the constant feeder during spatial experiment test. Final selected model is bolded.

Model	AICc	Delta AICc	Model Weight
TimeNearCF ~ Condition + TimeBlock + (1|Block/BeeID)	**193.456**	**0.000**	**0.755**
TotalContacts ~ Condition + TimeBlock + Condition*TimeBlock + (1|Block/BeeID)	195.720	2.263	0.243
TotalContacts ~ Condition + (1|Block/BeeID)	205.162	11.706	0.002

Table S2.7. Model selection for number of probes at downgraded feeder before feeding during temporal experiment training period. Final selected model is bolded.

Model	AICc	Delta AICc	Model Weight
TrainingContacts ~ Condition + TrialDay + Condition*TrialDay + (1|BeeID) + (1|TrialNumber)	**2712.095**	**0.000**	**1**
TrainingContacts ~ Condition + TrialDay + (1|BeeID) + (1|TrialNumber)	2739.263	27.169	0
TrainingContacts ~ Condition + (1|BeeID) + (1|TrialNumber)	2934.833	222.738	0

Table S2.8. Model selection for total number of visits to the water feeders during temporal experiment test. Final selected model is bolded.

Model	AICc	Delta AICc	Model Weight
TotalContacts ~ Condition + FeederType	**368.708**	**0.000**	**0.512**
TrainingContacts ~ Condition + FeederType + Condition*FeederType	369.041	0.332	0.434
TrainingContacts ~ Condition	373.200	4.492	0.054

Table S2.9. Model selection for percentage of visits during the time of interest to the water feeders during temporal experiment test. Final selected model is bolded.

Model	AICc	Delta AICc	Model Weight
PercentVisits ~ Condition + FeederType + Condition*FeederType + (1|TrialNumber)	**-4.361**	**0.000**	**0.397**
PercentVisits ~ Condition + (1|TrialNumber)	-4.219	0.142	0.370
PercentVisits ~ Condition + FeederType + (1|TrialNumber)	-3.297	1.064	0.233

Table S2.10. Model selection for number of visits to the water feeders every half hour during temporal experiment test. Final selected model is bolded.

Model	AICc	Delta AICc	Model Weight
NumVisits ~ Condition + FeederType + TimePeriod + Condition*TimePeriod + FeederType*TimePeriod + Condition*FeederType + (1|BeeID)	**1591.395**	**0.000**	**1**
NumVisits ~ Condition + TimePeriod + (1|BeeID)	1667.321	75.926	0
NumVisits ~ FeederType + TimePeriod + (1|BeeID)	1668.488	77.094	0
NumVisits ~ TimePeriod + (1|BeeID)	1668.583	77.188	0
NumVisits ~ Condition + FeederType + TimePeriod + (1|BeeID)	1669.205	77.811	0
NumVisits ~ Condition + FeederType + TimePeriod + FeederType*TimePeriod + (1|BeeID)	1684.077	92.682	0
NumVisits ~ Condition + (1|BeeID)	1752.889	161.495	0
NumVisits ~ FeederType + (1|BeeID)	1753.720	162.326	0
NumVisits ~ Condition + FeederType + (1|BeeID)	1754.530	163.136	0

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CHAPTER THREE:

Comparative analysis of lifespan and activity differences between three honey bee species

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ABSTRACT

Lifespan is a fundamental life history trait that shows striking variation among and within species, raising questions about how lifespan is regulated and how it evolves. Honey bees exhibit large variation in lifespan even among workers in the same colony. However, there also appear to be differences in lifespan among honey bee species that seem to correlate with worker life history patterns related to nest architecture. These species differences provide an opportunity to identify potential selection pressures that may have shaped worker lifespan, and to identify other worker traits correlated with differences in lifespan. Most research to date has examined the lifespan and factors shaping it in the Western honey bee, *Apis mellifera*, with age at the onset of foraging cited as the primary determinant of lifespan. Species differences in how honey bee colonies construct their nests have been hypothesized to lead to selection for increased lifespans and decreased foraging activity, but no study has yet linked individual lifespan and activity across species that differ in nest construction. In this study we tracked the age at first flight, activity rate, and lifespan of individual honey bee workers from three species (*A. mellifera, A. cerana, and A. florea*) to directly examine the relationship between activity and lifespan of species that differ in nest architecture. We found that workers from the open-nesting species *A. florea* live significantly longer than those from either cavity-nesting species we studied, as has been previously reported, and that this extension of worker lifespan is due to their delayed onset of flight and the smaller proportion of workers active outside the nest. In contrast to previous reports, we found no evidence of a species difference in overall activity rate, primarily because of the activity of a few hard-working individuals in each species. One unexpected finding was that *A. cerana* workers had higher survival and generally longer foraging lifespans than *A. mellifera* workers even though both species transitioned to extra-hive activity at the same age;

future studies should examine alternative mechanisms for this better survivorship. This study provides support for the hypothesis that the way a species builds its nest shapes the lifespan of its workers and suggests that regulation of worker activity and age at the transition to foraging are conserved proximate mechanisms through which lifespan is changed.

INTRODUCTION

Western honey bees (*Apis mellifera*) are a commonly used model system for studying plasticity in aging due to the large variability in lifespan they exhibit (Omholt & Amdam 2004; Keller & Jemielity 2006; Münch et al. 2008; Parker 2008; Amdam 2011; Kramer et al. 2016). Honey bee queens in *A. mellifera* can live for multiple years, while the lifespans of genetically similar workers can vary from a few weeks to a few months depending on their social task and the season of their birth, e.g., workers born in winter can live for multiple months while those born in the summer live 4-6 weeks on average (Winston 1987; Page and Peng 2001). Honey bee workers exhibit an age-based division of labor, with younger bees performing in-hive tasks such as cell cleaning, nest maintenance, and taking care of brood while older bees forage for nectar and pollen resources outside of the nest (Lindauer 1953; Seeley 1982).

The age at which a honey bee worker transitions to foraging behavior, commonly referred to as age at first foraging, has consistently been shown to be a major determinant of her lifespan. Because foraging is much riskier than in-hive tasks, bees that have a later age at first foraging generally have longer lifespans (Visscher & Dukas 1997; Rueppell et al. 2007a; Rueppell et al. 2008; Woyciechowski & Moroń 2009). Age at first foraging can be decreased by parasitic infection or chemical exposure (Wang & Mofller 1970; Tustain & Faulke 1979; Malone & Giacon 1996; Nicolas & Sillans 1989; Janmaat & Winston 2000), suggesting that it is responsive to a bee’s life expectancy, such that bees with lower life expectancies take on the riskier task of foraging regardless of their age (Woyciechowski & Moroń 2009). Senescence also plays a role in survivorship and lifespan of *A. mellifera* workers (Remolina et al. 2007; Rueppell et al. 2007a), though studies of honey bee workers foraging in natural settings suggest most workers die before wearing out (Visscher & Dukas 1997). Older worker bees are less resistant to stressors than younger bees though (Remolina et al. 2007), and exhibit age-dependent increases in mortality risk when foraging (Rueppell et al. 2007b). The effort a honey bee expends as a forager generally does not seem to influence her lifespan, except in extreme circumstances when bees experience excessive workloads, such as when they have an unusually high foraging rate or when flying with extra weight (Schmid-Hempel & Wolf 1988; Wolf & Schmid-Hempel 1989; Rueppell et al. 2007a). However, there is evidence to suggest that conducting fewer foraging trips can sometimes prolong a bee’s lifespan even in situations where predation is not a concern (Townsend-Mehler dissertation 2010).

Although factors shaping the lifespan and survival of honey bee workers have been well studied in *A. mellifera*, little attention has been paid to studying survival and its relationship to foraging activity in other honey bee species. We might expect to find differences between species, though, because honey bees species exhibit a wide diversity in body size, colony size, and modes of nest construction (Seeley et al. 1982; Dyer & Seeley 1987; Dyer & Seeley 1991; Oldoyd & Wongsiri 2009). Associated with this variation are differences in the expected costs of worker mortality and foraging (Dyer & Seeley 1987; Dyer & Seeley 1991). Asian honey bee species that construct their nests in the open on a single sheet of comb, such as the dwarf honey bee *A. florea* and the rock bee *A. dorsata*, have significantly lower ratios of brood cell numbers to worker numbers than cavity-nesting species, resulting in more limited space for brood rearing.

This lower ratio effectively means that workers are slower to be replaced in open-nesting species. In addition, bees in these open-nesting species perform the additional task of forming a protective curtain over the comb for part of their lives (Seeley et al. 1982). These factors are expected to increase the cost of worker mortality for open-nesting species because higher worker attrition would compromise the protective function of the curtain. Taking these factors into account, we hypothesized that the increased cost of worker mortality should have increased selection for longer lifespans and decreased foraging activity in open-nesting species (Dyer & Seeley 1991). Meanwhile, the higher metabolic and colony-level foraging rates of cavity-nesting species such as the Eastern hive bee *A. cerana* could lead to increased worker mortality and thereby select for shorter lifespans in cavity-nesting species (Dyer & Seeley 1987). Examining the relationship between survivorship and foraging in honey bee species beyond *A. mellifera* is important because most honey bee species evolved and live in tropical environments (Michener 2000) and therefore do not have to deal with winter periods of cold weather and prolonged periods of food shortages (Singh and Kushwaha 2006; Santhosh and Basavarajappa 2016). The ability of these species to forage throughout the year might then have consequences for their lifespan, the intensity of their foraging efforts, and the relationship between the two.

The few studies that have examined the lifespan and foraging rates of Asian honey bee workers suggest that open-nesting species such as *A. florea* live significantly longer than cavity-nesting species and forage significantly less, at least on the colony level (Dyer & Seeley 1991). Recent work suggests that this prolonged lifespan, much like in *A. mellifera*, is due primarily to a delayed age at first foraging (Unnikrishnan et al. 2021, preprint). Evidence exists that *A. cerana* workers are longer lived than *A. mellifera* workers in mixed-species colonies, though it remains to be seen whether this difference exists in single species colonies or when comparing each species' lifespan in the type of environment they are evolved to forage in (temperate vs tropical; Zhang et al. 2019).

To date, little is known about the direct relationship between survivorship and foraging activity on an individual basis in Asian honey bee species, nor have studies compared survivorship between Asian honey bee species and *A. mellifera* between single species colonies in environments similar to those they evolved in. Gaining a better understanding of how honey bee species vary in lifespan and the behavioral mechanisms underlying that variation could inform future studies on the evolution of plasticity in aging. In this study we compared survival and foraging activity between the cavity-nesting temperate bee *A mellifera*, the open-nesting tropical bee *A. florea*, and the cavity-nesting tropical bee *A. cerana*. We predicted that because of the need to maintain a protective curtain, *A. florea* would have a longer lifespan and a lower rate of foraging than either *A. cerana* or *A. mellifera*. We also predicted that *A. mellifera* workers would have the highest rates of foraging due to their need to accumulate resources to survive overwintering.

MATERIALS AND METHODS

Study Sites and Observation Cohorts

We compared the survivorship and foraging dynamics of three species of honey bees (*A. cerana*, *A. florea*, and *A. mellifera*). We studied three cohorts of *A. mellifera* on the Michigan State University campus in East Lansing, MI, and two cohorts each of *A. florea* and *A. cerana* on the campus of the National Centre for Biological Sciences – Tata Institute of Fundamental Research (NCBS) in Bangalore, Karnataka, India. Observation dates for each cohort studied can be found in Table 3.1.

To create each cohort of *A. mellifera*, we removed frames containing brood that were about to eclose from two different mature colonies and allowed them to emerge in an incubator (33-34˚C) for 24 hours. We individually marked day old bees with colored numbered tags and introduced them into an observation hive consisting of two frames and roughly 2,000 bees. The observation hive was housed indoors, and a clear plastic tube running from the hive to the outside of the building allowed bees to forage freely. Similarly, to create each cohort of *A. cerana* and *A. florea*, we obtained brood combs from local beekeepers and allowed them to emerge in an incubator (33-34˚C) for 24 hours. We individually marked day old bees with unique paint combinations on their thorax and abdomen, and then introduced them into existing *A. cerana* or *A. florea* colonies, respectively. All existing *A. cerana* and *A. florea* colonies were either already present on the NCBS campus or were bought from local beekeepers and moved to the NCBS campus. Colonies of *A. cerana* were housed in manmade 6 frame hive boxes, while colonies of *A. florea* were placed in sheltered observation boxes that permitted free flight to the surrounding environment.

Table 3.1. Observation periods for studied cohorts of *A. florea, A. cerana,* and *A. mellifera*.

Species	Cohort	Observation Dates	Number of Days Observed
A. florea	1	October 27, 2018 – December 5, 2018	40
	2	October 28, 2018 – November 29, 2018	33
A. cerana	1	February 15, 2019 – March 31, 2019	45
	2	April 28, 2019 – June 7, 2019	41
A. mellifera	1	June 30, 2018 – August 10, 2018	41
	2	July 11, 2018 – August 22, 2018	43
	3	July 27, 2018 – September 3, 2018	39

Survivorship and Foraging Activity Measurements

To track survivorship and foraging activity of each cohort, we observed each colony every day for two hours in the morning and two hours in the afternoon. For *A. mellifera*, observations were done in person with one observer recording foraging trips of marked bees and another observer recording marked bees observed in the observation hive. For *A. cerana* and *A. florea*, we recorded the colonies during the observation periods using video cameras, aiming the camera at the entire colony for *A. florea* and the hive entrance for *A. cerana*. Because we were unable to house *A. cerana* in observation hives, we collected data only on the activity of bees entering and leaving the hive; this prevented us from taking bees that never foraged into account. We observed each *A. cerana* and *A. mellifera* cohort until at least 95% of introduced bees died. In the case of *A. florea*, both colonies abandoned their nest while many introduced bees were still alive, preventing us from tracking these bees further. The number of days we observed each colony can be found in Table 3.1.

For each individually marked bee, we recorded every date it was observed, the date it was first seen flying (a proxy for the initiation of foraging), the number of trips we observed a bee make each day, and the date it was last observed (and therefore presumed dead). A bee's average activity rate was calculated by dividing the total number of trips we observed it make by the number of observed hours between when it started flying and when it was no longer observed. A bee's foraging lifespan was calculated by subtracting her age at first flight from her total lifespan. A bee was declared "dead" if she was not observed for three consecutive days, and the last day she was observed was recorded as her death date. If a bee had disappeared within two days of the end of the observation period, we recorded her as "alive" even if she was not observed on the final day.

Statistical Analyses

All statistical analyses were conducted in RStudio version 4.0.2. To compare species differences in survivorship over time, we performed Kaplan-Meier survival analyses on bee survivorship with bees still alive at the end of the observation period censored ("survival" package). To determine if different colonies of the same species differed in their survival, we performed Kaplan-Meier survival analyses in the same manner.

We next analyzed how foraging activity influenced a bee's lifespan. To do this, we first subsetted the data to include only bees whose entire lifespans were recorded during our observation periods. Using that subset of data, we fit a separate generalized linear mixed effects model with lifespan (in days) as the response variable for each species ('lmer' function). The fixed factors of age at the onset of flight, activity rate, the two-way interaction between age at the onset of flight and activity rate, plus the random factor of cohort ID were considered in each model during model selection. The final selection models included only the age at first flight and the random factor of cohort ID for *A. florea* and *A. mellifera*, and also included activity rate for *A. cerana*. We then compared the factors shaping a bee's foraging lifespan between *A. cerana* and *A. mellifera*; *A. florea* was not considered in this assay due to the lack of complete foraging lifespan data for most *A. florea* workers. We again fit a generalized linear mixed effects model with foraging lifespan (days) as the response variable and considered the fixed factors of species, age at the onset of flight, activity rate, each possible two-way interactions, and the random factor of cohort ID ('lmer' function). The final model included only species and age at first flight and the random factor of cohort ID.

We then wanted to compare activity among species. Because each cohort was observed for a different period of time and we were unable to follow the marked *A. florea* foragers throughout their entire lives, we did not feel comfortable comparing observations collected from *A. cerana* and *A. dorsata* bees over their entire lives to those collected during only a portion of the lives of *A. florea* bees. For that reason, in subsequent analyses we compared only the activity and survivorship that was observed on or before the last observation day that all cohorts reached; day 33 was chosen as a common cutoff as that was the length of the shortest observation period in this study. We used a chi-square test of independence to compare species differences in the number of bees that did and did not forage. To analyze if species differed in the age they were first observed flying we used a generalized linear mixed effects model with a negative binomial distribution ('glmer.nb' function). In this model, we used a bee's age when they were observed flying as the response variable and a bee's species (*A. cerana*, *A. mellifera,* or *A. florea*) as a fixed predictor variable, with cohort ID also included as a random factor. We similarly inquired whether species varied in their activity rate using a model identical to that used for age at first flying, but with activity rate as the response variable and a Gaussian distribution ('lmer' function). Because we observed some bees with unusually high activity rates, we identified and removed outliers that were above the 75th or below the 25th percentile by a factor of 1.5 times the interquartile range. We then reanalyzed bees' activity rates using the same model as before.

Finally, we analyzed the factors influencing the probability that a given bee survived to a common age (33 days) in two different ways, each time using a generalized linear mixed effects model with a binomial distribution ('glmer' function). The response variable in both models was a binary indicator of a bee's status on day 33 (alive or dead). We considered the fixed factors of species, age at first flight, activity rate, and the interaction between age at first flight and activity rate plus the random factor of cohort ID in the full model. The final model included all considered fixed factors and the random factor of cohort ID. Because we found that outlier bees with especially high activity rates significantly impacted the ability to detect species differences in activity rate, we similarly analyzed the probability of a bee being alive at age 33 days in the same way but using data that did not contain outlier activity rates. The final model included only species, age at first flight, and the random factor of cohort ID.

For all models, we selected final models with the lowest AICc values, with the most parsimonious model chosen when models had delta AICc differences of less than 2. We then analyzed the final models using ANOVA and post-hoc tests with a Tukey correction unless specified otherwise.

RESULTS

We marked a total of 220 *A. florea* bees, 264 *A. cerana* bees, and 754 *A. mellifera* bees. Of those marked bees, 139 marked *A. florea* bees (63%), 169 marked *A. cerana* bees (64%), and 627 marked *A. mellifera* bees (83%) were observed during the study. As we have no data on the bees that were never seen, all subsequent analyses were conducted using only marked individuals that were seen during the observation period.

Variation Among Species in Survivorship

A. florea had significantly higher survivorship than *A. cerana* or *A. mellifera* during the period all three species could be tracked (both $P < 2e\text{-}16$; Figure 3.1), with 69.1% of *A. florea* bees still alive on the last common observation day (day 33) compared to 25.4% of *A. cerana* bees and only 1.9% of *A. mellifera* bees. *A. cerana* had higher survivorship than *A. mellifera* ($P < 2e\text{-}16$), with 95% of marked bees dead by day 29 in *A. mellifera* but not until day 42 for *A. cerana*.

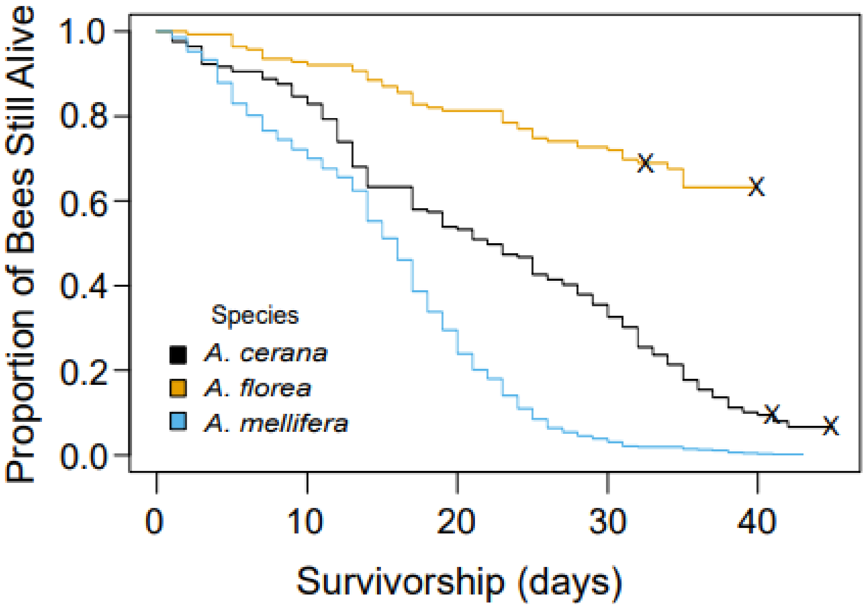

Figure 3.1. Survivorship of honey bee species. *A. florea* had the highest survivorship at the common age of 33 days, followed by *A. cerana*, and then *A. mellifera* (all P < 2e-16). 95% of *A. mellifera* workers had died by the age of 29 days, while 95% of *A. cerana* workers were not dead until day 42. For colonies where observations were stopped before all bees had died, the day in which the cohort was censored is marked with an "X".

Factors Shaping Lifespan of Known Dead Bees

For those bees from each species that died during our observation period and were observed flying, the primary predictor of a bee's lifespan was the age at which it began flying (*A. florea*: F^1_{15} = 5.01, P = 0.025; *A. cerana*: F^1_{156} = 15.97, P = 6.43e-5; *A. mellifera*: F^1_{530} = 51.01, P = 9.17e-13). In each species, bees that started flying later lived longer (Estimate±SE: *A. florea*: 0.66±0.29; *A. cerana*: 0.64±0.15; *A. mellifera*: 0.92±0.13; Figure 3.2a). Although activity rate was included as a predictor in the model for the lifespan of individual *A. cerana* bees that died during our observation period, it did not have a significant effect on lifespan (F^1_{156} = 0.62, P < 0.43).

We further compared the factors shaping a bee's foraging lifespan (the period of time they survived after first leaving the nest to fly) between *A. cerana* and *A. mellifera*, using only bees that died during our observation period. The length of a bee's life once she began flying was dependent on her species (F_{686}^{1} = 5.91, P = 0.015) and her age at first flying (F_{686}^{1} = 9.23, P = 0.0024; Figure 3.2b). Workers from *A. cerana* cohorts tended to have longer foraging lifespans than workers from *A. mellifera* cohorts (P = 0.090). Across species, though, the later a bee began flying, the shorter her lifespan was as a forager (Estimate±SE: -0.27+0.088). We did not include *A. florea* in our primary analysis of foraging lifespan due to the small number of *A. florea* bees that were observed flying and died during our observation period (N = 15). However, results from an analysis of foraging lifespan with *A. florea* included can be found in Table S3.7 and Figure S3.1.

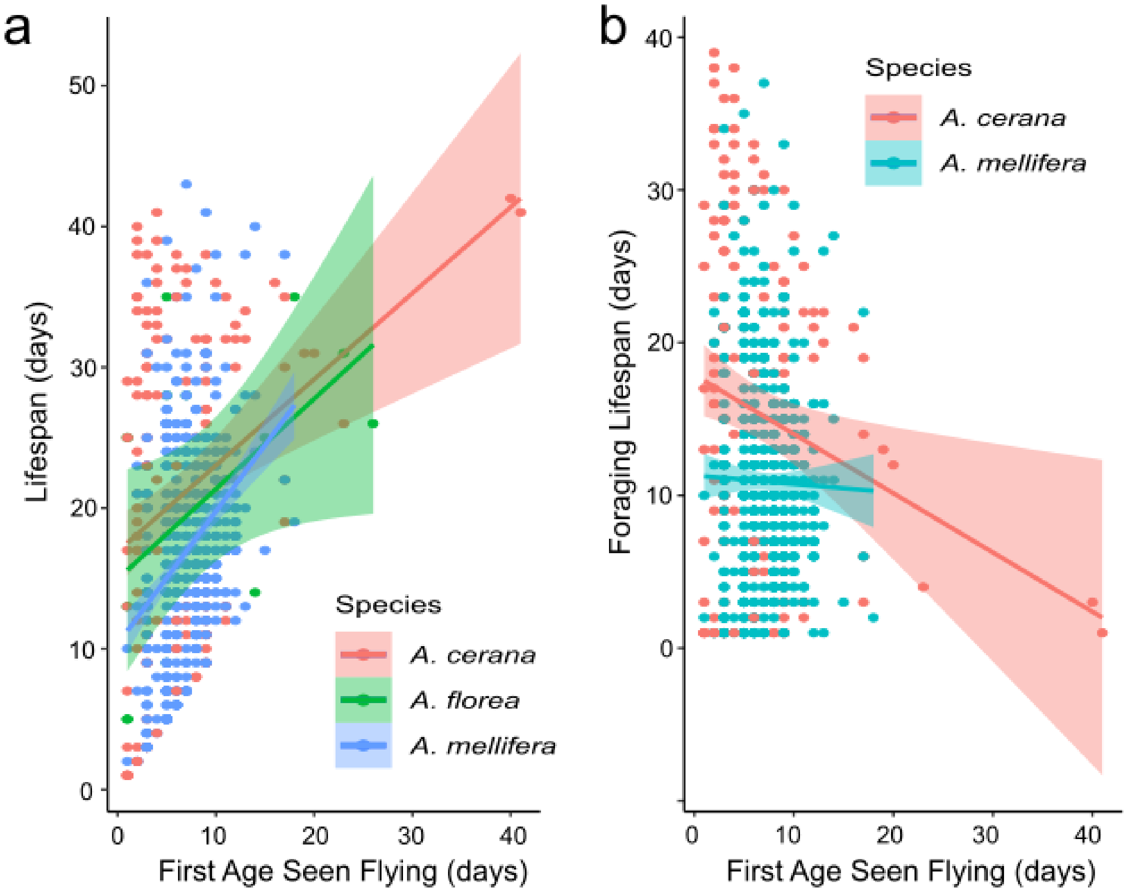

Figure 3.2. Relationship between age at first flight and lifespan metrics. (a) Regressions between a bee's age at first flying and her lifespan are shown for *A. cerana*, *A. florea*, and *A. mellifera*. For all species, lifespan increased with an older age of first flying ($P < 0.03$ all). (b) Regressions between age of first flying and the length of bee's lifespan as a forager are shown for *A. cerana* and *A. mellifera*. *A. cerana* workers tended to have longer foraging lifespans than *A. mellifera* ($P = 0.09$) For both species, bees that started flying earlier had longer foraging lifespans ($P = 0.0024$).

Species Differences in Behavior and Survivorship Among Bees Observed Flying

To compare behavior between species across an equivalent time scale, we performed the next analyses using data from observation days 1 to 33 for each cohort (the period of time in which all cohorts of bees were observed). Species significantly differed in the number of bees that were and were not observed flying ($X^2 = 281.56$, $DF = 2$, $P < 2.2\text{e-}16$). Fewer bees were observed flying in *A. florea* than in *A. cerana* ($P = 2.48\text{e-}40$) or *A. mellifera* ($P = 6.24\text{e-}44$), and fewer bees were observed flying in *A. mellifera* than in *A. cerana* (*P* = 4.32e-7; Figure 3.3; it is important to note however that only flying bees could be observed for *A. cerana*, and so this difference should be treated cautiously). The subsequent analyses focus on bees from each species that were observed flying, in order to determine how factors related to activity differ between species and influence survivorship.

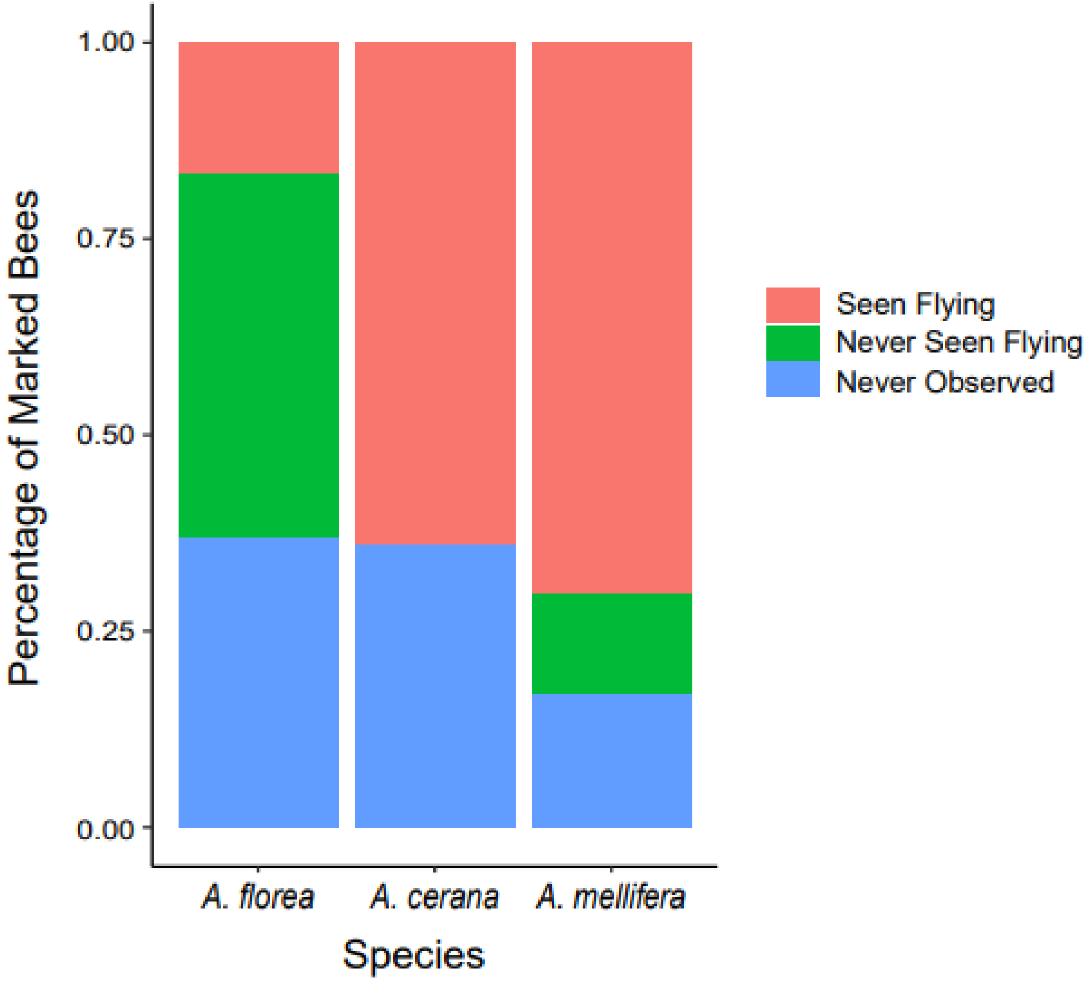

Figure 3.3. Percentage of marked bees observed and not observed. The percentage of marked bees from *A. cerana*, *A. florea*, and *A. mellifera* bees that were never observed, observed but never seen flying, and observed and seen flying are shown. Significantly fewer *A. florea* workers were observed flying than *A. cerana* or *A. mellifera* workers (*P* < 3e-40 all).

Species significantly differed in the age in which bees first flew (F^{2}_{732} = 14.83, *P* = 0.00060; Figure 3.4). This significant difference in age at first flying was driven entirely by *A. florea*, which generally began flying later than either *A. cerana* (*P* = 0.0009) or *A. mellifera* (*P* = 0.0043). On average, *A. florea* bees that were observed flying were almost twice as old when they began flying off the nest as bees from *A. cerana* or *A. mellifera* (Mean±SE: *A. florea*: 12.05±1.61 days; *A. cerana*: 6.17±0.35 days; *A. mellifera*: 7.21±0.11 days). *A. cerana* and *A. mellifera* did not differ in their age at first flying (*P* = 0.68).

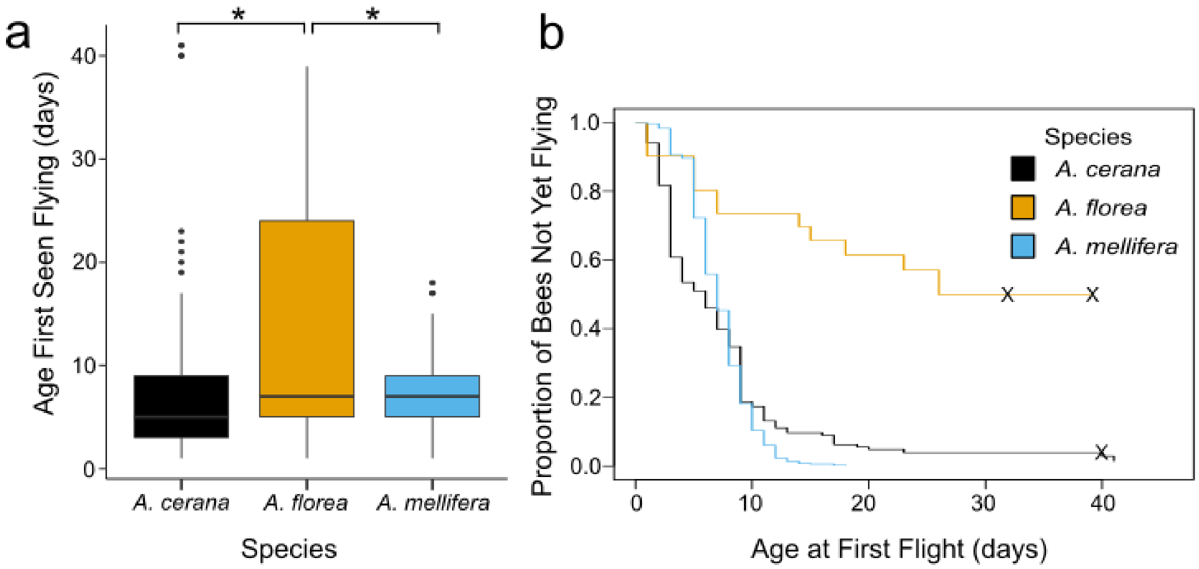

Figure 3.4. Age at first flight of honey bee species. (a) *A. florea* started flying at a significantly older age than *A. cerana* or *A. mellifera* (*P* < 0.01 all), that began flying at the same age (*P* = 0.68). (b) Fewer *A. florea* workers were observed flying as compared to *A. cerana* or *A. mellifera*. For colonies where observations were stopped before all bees had begun flying, the day in which the cohort was censored is marked with an "X". Significant differences are indicated with an *.

In contrast to the differences found in age at first flying, when comparing bees that were observed flying we found that species did not significantly differ in their activity rates during our observation periods (F^{2}_{784} = 1.46, *P* = 0.48; Figure 3.5a). All three species were observed to have low activity rates, though some individuals exhibited unusually high rates of activity. To determine if the unusually high rates of activity seen for some bees were preventing species differences in activity rate from being detected, we reanalyzed the data after removing outlier bees. Using data without outlier activity rate values, a significant difference in activity rate between species was indeed found (F^2_{778} = 12.79, P = 0.0017; Figure 3.5b). In particular, *A. florea* had a significantly lower rate of activity than did *A. mellifera* (P = 0.045). No other species differed in their activity rates (P > 0.20).

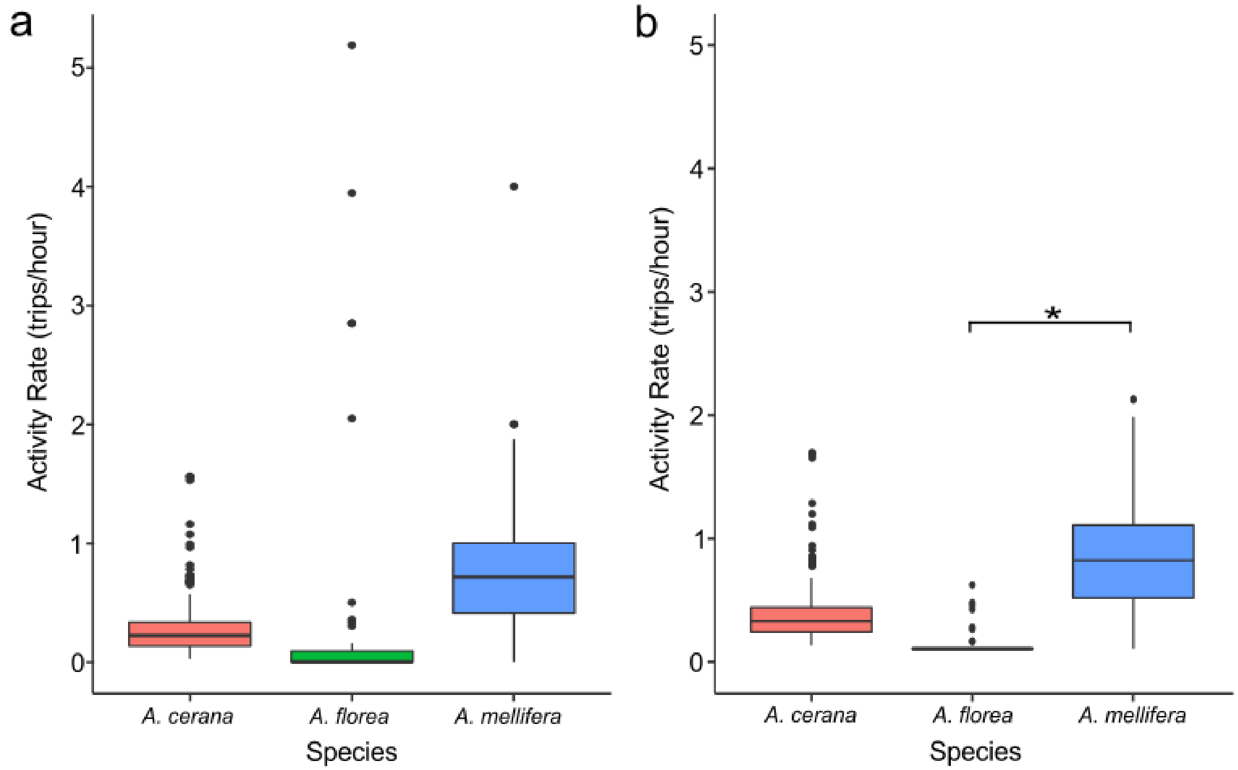

Figure 3.5. Foraging rates of honey bee species. The foraging rates of *A. cerana*, *A. florea*, and *A. mellifera* (a) with and (b) without unusually active workers are shown. (a) No differences in foraging rate were observed between species when unusually active workers were considered (P = 0.48). (b) *A. florea* had a marginally significantly lower foraging rate than *A. mellifera* when workers with outlier activity rates were excluded (P = 0.045). Significant differences are indicated by an *.

For bees that were observed flying, the probability that a bee survived to the age of 33 days depended on the species to which they belonged (F^{2}_{732} = 26.68, *P* = 1.61e-6; Figure 3.6a), the age at which they started flying (F^{1}_{732} = 6.42, *P* = 0.011; Figure 3.6b), and their activity rate (F^{1}_{732} = 4.23, *P* = 0.040; Figure 3.6c). *A. florea* and *A. cerana* bees were both more likely to survive to age 33 days than *A. mellifera* (both $P < 0.001$), though they did not differ from each other ($P = 0.14$). It is important to note, however, that only bees that were observed flying were included in this analysis. As very few *A. florea* bees were observed flying but many bees were still alive at the age of 33 days (Figure 3.1), the lack of species difference seen between *A. cerana* and *A. florea* should be interpreted cautiously. Bees that started flying later were more likely to survive to age 33 days (Estimate$\pm$SE: 0.061$\pm$0.037), while bees that had a higher activity rate were less likely to survive to age 33 days (Estimate$\pm$SE: -1.64$\pm$0.74). While it was included in the final model, the interaction between age at first flight and activity rate was not significant ($P = 0.26$).

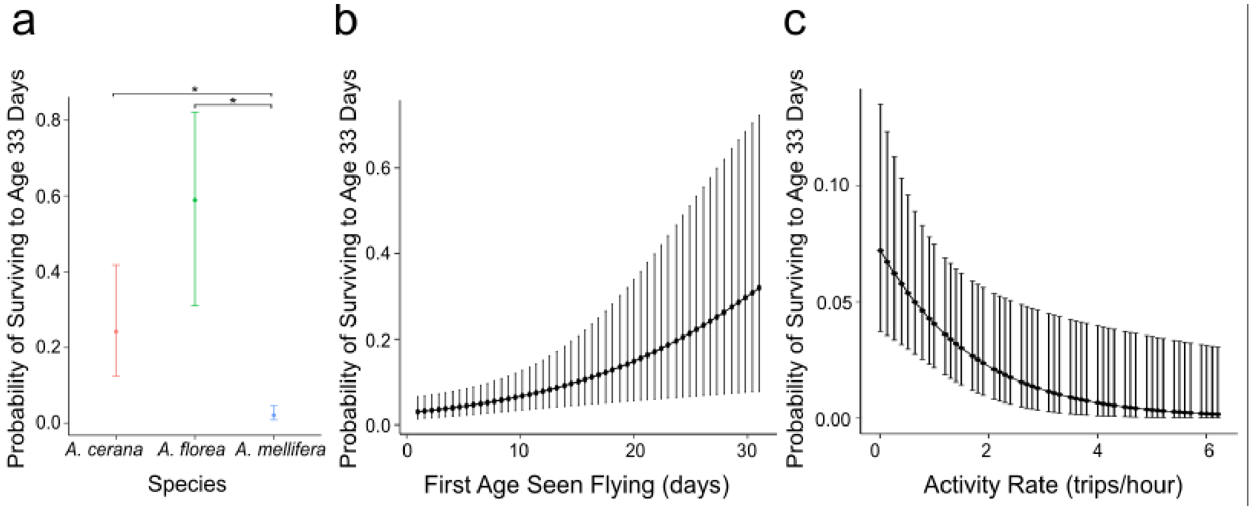

Figure 3.6. Factors shaping the probability of surviving to 33 days. (a) *A. florea* and *A. cerana* workers were both more likely to survive to 33 days than *A. mellifera* workers ($P < 0.001$ all). (b) The probability of a bee surviving to 33 days increased as the age at which a bee was first seen flying increased ($P = 0.011$). (c) The probability of a bee surviving to 33 days decreased as the bee's foraging rate increased ($P = 0.040$). Significant differences in the probability of surviving to 33 days between species are indicated with an *.

Because of the significant effect that bees with unusually high activity rates had on a species' average activity rate, we reanalyzed the probability that a bee survived to 33 days after removing those outliers. Without the unusually high activity rates, a bee's likelihood to survive to the age of 33 days depended only on her species ($F^{2}_{726} = 47.98$, $P = 3.82\text{e-}11$; Figure 3.7a) and her age at first flight ($F^{1}_{726} = 4.82$, $P = 0.028$; Figure 3.7b). *A. florea* workers were again more likely to survive to age 33 days than *A. mellifera* worker ($P < 0.0001$) and tended to be more likely to survive than *A. cerana* workers ($P = 0.065$), and *A. cerana* workers were again more likely to survive than *A. mellifera* workers ($P < 0.0001$). As was seen previously, bees that began flying later in their life were more likely to survive to 33 days (Estimate±SE: 0.070±0.032). As in the analysis for the probability of surviving to 33 days with outlier activity rates, species differences or lack therefore should be interpreted cautiously due to the small sample size of *A. florea* workers.

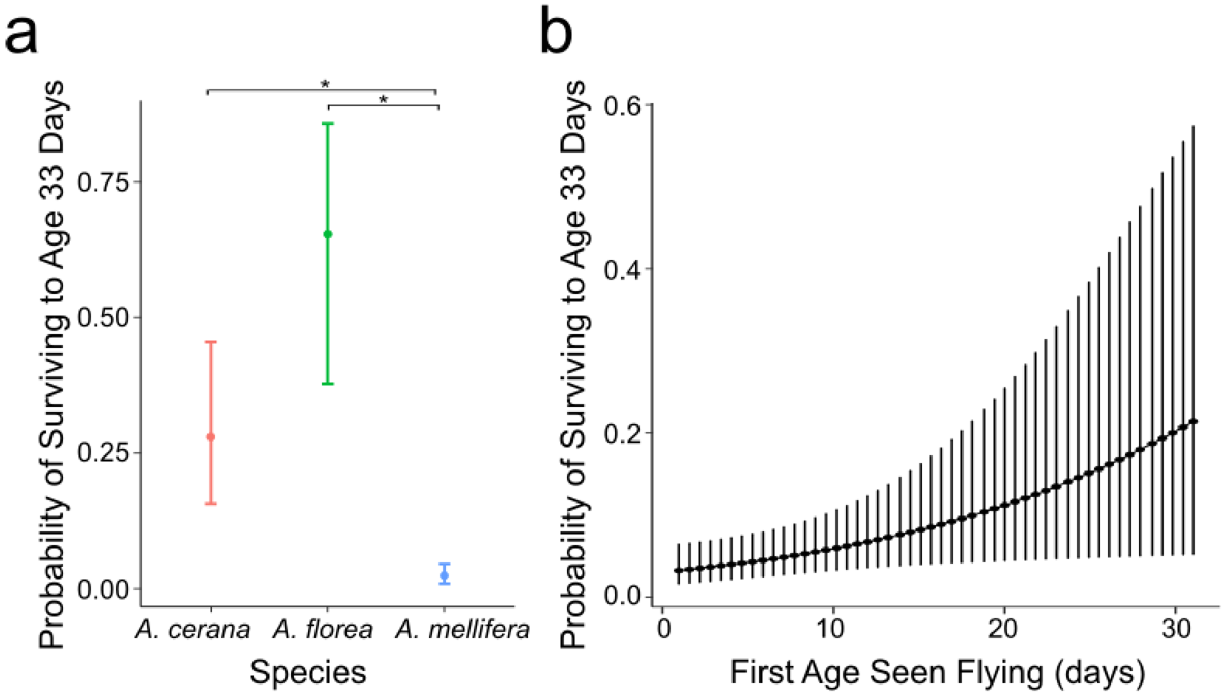

Figure 3.7. Factors shaping the probability of surviving to 33 days if highly active bees are not considered. If bees with unusually high foraging rates are not considered, the probability of a bee surviving to the age of 33 days depended on her (a) species and (b) age at first flight. (a) *A. florea* and *A. cerana* workers were more likely to survive to 33 days than *A. mellifera* workers (*P* < 0.001 all), while *A. florea* workers tended to be more likely to survive than *A. cerana* workers (*P* = 0.065). (b) The probability of a bee surviving to 33 days increased as the age at which a bee was first seen flying increased (*P* = 0.028). Significant differences in the probability of surviving to 33 days between species are indicated with an *.

Inter-Colony Variation in Survival and Flight Activity

When we examined how survivorship differed between cohorts of the same species, we found that the two *A. florea* cohorts studied exhibited similar survivorship (*P* = 0.74; BH adjustment; Figure 3.8a), while bees in *A. cerana* cohort 2 lived longer than those in cohort 1 (P = 0.0036; BH adjustment). We also found that two of the *A. mellifera* cohorts had similar survivorship (cohorts 1 and 3, *P* = 0.16; BH adjustment), but cohort 2 had shorter survivorship (*P* < 7e-6 for both; BH adjustment). Survivorship between cohorts of different species was never the same (*P* < 8e-6 for all comparisons), validating the differences observed when comparing survivorship across cohorts between species.

Age at first flying often differed between cohorts in the same species (Figure 3.8b). Bees in *A. cerana* cohort 1 had an earlier age at first flying than did bees in *A. cerana* cohort 2 (*P* < 0.0001), whereas *A. florea* cohorts did not significantly differ in their age at first flying (*P* = 0.066). All three cohorts of *A. mellifera* differed in their age at first flying (all *P* ≤ 0.0001); cohort 1 had the latest age at first flying, followed by cohort 3, then cohort 2.

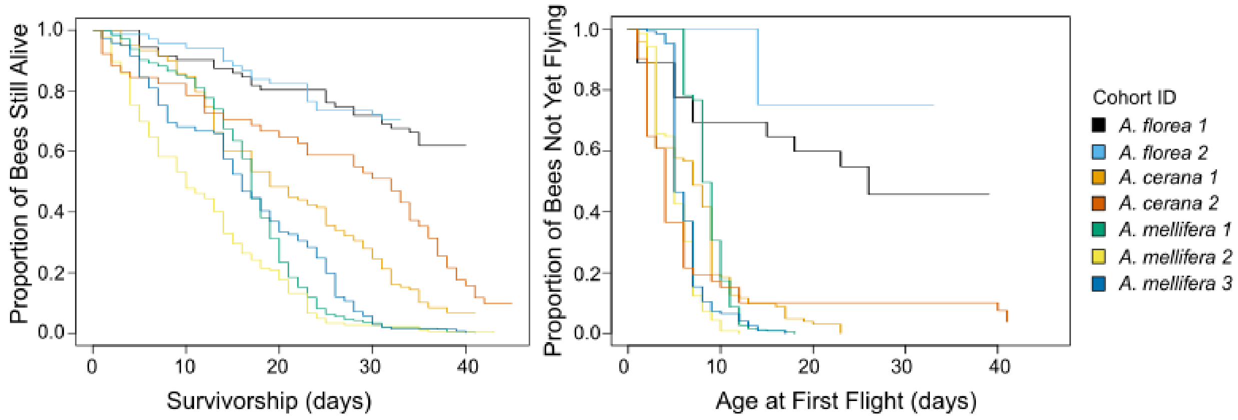

Figure 3.8. Cohort survivorship and age at first flying. (a) Cohorts of *A. florea* did not differ in their survivorship (*P* = 0.74), while *A. cerana* cohort 1 had decreased survivorship than *A. cerana* cohort 2 (*P* = 0.0036). Cohorts 1 and 3 of *A. mellifera* had higher survivorship than *A. mellifera* cohort 2 (*P* < 7.e-6 all). Cohorts of different species differed in their survivorship (*P* < 8e-6 all). (b) Cohorts of *A. florea* also did not differ in their age at first flying (*P* = 0.066), while *A. cerana* cohort 1 had a later age at first foraging than *A. cerana* cohort 2 (*P* = 0.0001). All three *A. mellifera* cohorts differed in their ages at first foraging with cohort 1 having the latest age followed by cohort 2 and then cohort 3 (*P* ≤ 0.0001 all).

DISCUSSION

Of the three honey bee species studied here, *A. florea* workers had the highest survivorship of any species when workers were compared at common ages (Figures 3.1, 3.7a). Over half of the *A. florea* workers observed in this study were still alive at the age of 33 days, whereas almost no *A. mellifera* workers and only a quarter of *A. cerana* workers were still alive at that same age. We conclude therefore that the lifespan of *A. florea* workers is much longer than those of workers from the other species studied; it seems conservative to estimate that these workers live over twice as long as *A. cerana* and *A. mellifera* workers. These results support those found in a previous study where *A. florea* was found to be longer lived than *A. cerana* (Dyer & Seeley 1991). The high survivorship seen in *A. florea* (and the longer lifespans that it implies) are likely due primarily to the delayed age at first flight of its workers (Figure 3.4). Although the age at first flight was highly variable in *A. florea* (as has also been seen in other studies of the behavioral maturation of *A. florea*, e.g., Unnikrishnan et al. 2021), on average bees were twice as old when they were first observed flying in *A. florea* as in *A. cerana* or *A. mellifera*. In addition, many *A. florea* workers that were known to have survived to the age of 33 days were never observed flying at all (Figure 3.3), suggesting that the average age of first flight recorded here is likely an underestimate. In *A. mellifera*, a positive correlation between age at first foraging and lifespan is well established (Visscher & Dukas 1997; Rueppell et al. 2007a; Rueppell et al. 2008; Woyciechowski & Moroń 2009); our results suggest that this correlation holds true for other honey bee species as well and thus represents a conserved proximate mechanism controlling honey bee lifespan (Figure 3.2a).

The evolution of delayed foraging and increased survivorship observed here and in other studies of *A. florea* has been hypothesized to be a consequence of selection pressures associated with nest architecture (Dyer & Seeley 1991). Because colonies of *A. florea* (and the giant honey bee *A. dorsata*) build single comb nests in the open protected by a curtain of bees, they have a restricted brood comb area and workers have the additional task of forming the protective curtain. With these restrictions, *A. florea* colonies cannot replace workers that die as rapidly as can colonies of cavity-nesting species such as *A. mellifera* and *A. cerana*, increasing the cost of worker mortality for the colony (Seeley et al. 1982; Dyer & Seeley 1991). Since foraging is the riskiest task a worker honey bee can perform, selection is expected to select for reduced amounts of foraging, delayed foraging, or both in open-nesting species compared to cavity-nesting species (Dyer & Seeley 1991). In support of this hypothesis, we observed that *A. florea* began flying at a significantly older age and that a reduced number of workers flew at all in *A. florea* as compared to the two cavity-nesting species.

Differences in survival between *A. mellifera* and *A. cerana*, or between those species and *A. florea*, were not due to differences in activity rate once bees began flying (Figure 3.5). All three species had similar rates of activity, which differs from both our prediction that *A. mellifera* would have the highest rates of foraging and from previous research into the foraging patterns of honey bee species (Dyer & Seeley 1991). Previous studies have found cavity-nesting species like *A. cerana* to have higher foraging rates than open-nesting species like *A. florea* (Dyer & Seeley 1991), and even higher foraging rates than fellow cavity-nesting species *A. mellifera* (Zhang et al. 2019). The differences in nest construction between open- and cavity-nesting species are also expected to select for decreased foraging rates in open-nesting species to minimize worker mortality (Dyer & Seeley 1991), but our data do not support that prediction. The lack of difference found in this study could be a result of only monitoring the activity rates of marked individuals over a few hours every day, as we likely missed some of their activity, especially in the early morning. If workers were primarily active during certain periods of the day (e.g., *A. cerana* is often highly active at sunrise [Young et al. 2021]), then our counts of their activity could be underestimates. However, we recorded bees' activity during periods of the day when their species was known to be active, so this is likely not the only reason for the lack of difference. Another possible reason we did not observe species differences in activity rates is because of the efforts of a few hard-working individuals in each species. When examining the activity rates of each species in Figure 3.5a, the rates of *A. mellifera* and *A. cerana* appear to be higher than that of *A. florea*. However, a few highly active workers in each species can also be seen, which might have prevented species differences from being detected. Indeed, when we removed outlier bees (identified by statistical criteria) we found a significant species difference where *A. florea* had a lower foraging rate than *A. mellifera*. Although this difference provides limited support for the idea that cavity- and open-nesting species differ in their activity rates, no difference was seen between the activity rates of *A. florea* and *A. cerana*, undermining that support. In addition, it is also possible that this difference could be misleading due to the low number of *A. florea* workers that were observed flying since we were not able to track the foraging lifespans of many *A. florea* workers due to the colony absconding. Future work should aim to track the activity rates of these species (especially those of *A. florea*) over the entire day and across their entire lifespans.

While our study found little support for the idea that differences in lifespan between cavity- and open-nesting species were partially due to differences in worker activity rates, our study was not able to compare the physiological cost of flight for workers from each species. Differences in the metabolic rates between cavity- and open-nesting species suggest that flight is physiologically less costly to open-nesting species (which fly at a lower mass-specific metabolic rate). This reduced metabolic cost of flying might allow for a longer lifespan and specifically a longer lifespan as a forager (Dyer & Seeley 1987). Even without differences in overall activity rate, then, the cost of the same activity could differ between these species. When we compare between all three studied species, our results suggest that the foraging lifespan between open- and cavity-nesting species does not differ, though, which does not support the idea that flight costs are lower in open-nesting species (Table S2.7, Figure S3.1). However, as we recorded the foraging lifespan of only a few *A. florea* workers, these results should be regarded with a high degree of caution and not used to definitively make statements about the foraging lifespans of open- versus cavity-nesting species. Future work should prioritize tracking the foraging lifespans of *A. florea* to allow for tests of the prediction that this species should have lower flight costs and therefore longer foraging lifespans as compared to cavity-nesting species.

That the interpretation of the foraging lifespan results warrants caution is highlighted by the fact that when *A. florea* was not considered, a difference in the foraging lifespans between *A. cerana* and *A. mellifera* was seen. In particular, *A. cerana* workers had longer lifespans (Figures 3.1, 3.7a) and tended to have longer foraging careers (Figure 3.2b) than did *A. mellifera* workers. The lengthened survival and foraging careers were notable as both species began flying at roughly the same age on average (Figure 3.4), suggesting that another factor besides the transition to foraging is responsible for the extended lifespans of *A. cerana* workers, such as physiological differences or differences in the overall number of flights made over their foraging career. The increased lifespans seen in *A. cerana* workers support the results found in a previous study that compared lifespan between *A. cerana* and *A. mellifera* workers in mixed species hives, suggesting that their observations of extended lifespans in *A. cerana* workers are not simply a result of their experimental manipulations (Zhang et al. 2019). One possible explanation for these differences could be the different environments these species evolved in. The tropical environment in which *A. cerana* evolved and was observed does not have long resource dearth periods as are seen in temperate environments (e.g., dearth periods in the height of summer in Michigan). While resource abundance fluctuates over the year in tropical environments, there are generally always resources available (Singh and Kushwaha 2006; Santhosh and Basavarajappa

2016), which could select for an increased foraging lifespan to take advantage of those long periods of resource availability. More research is needed comparing tropical and temperate honey bee species that are similar in body size and nest construction, such as *A. mellifera* and *A. cerana*, to further investigate the robustness of this lifespan difference and the molecular mechanisms that underlie it.

In addition to comparing the survival curves of our three study species over their lifespans (or the portion of their lifespans that we were able to record in the case of *A. florea*), we wanted to specifically compare survival to a common age in these species (33 days, defined by the time until the first *A. florea* colony abandoned its hive and could no longer be observed), and determine the factors that correlate with survival over this period. We found marked species differences (Figure 3.6a), but while both *A. florea* and *A. cerana* were both more likely to survive to age 33 days than *A. mellifera*, we no longer detected a difference in survival between *A. florea* and *A. cerana* (Figure 3.6a). This lack of difference does not align with our prediction that open-nesting species should have longer lifespans than cavity-nesting species, nor does it match the differences in survivorship seen in the survivorship analyses. This apparent contradiction is likely due to differences in which bees are being analyzed by each of these tests. In the survival analyses, the survival of all observed bees for each species was compared. When analyzing the probability of surviving to 33 days, however, we were focusing specifically on bees that were observed flying. Because very few *A. florea* bees were observed flying by day 33, the analysis did not include many *A. florea* bees that were still alive at day 33 but not yet flying outside the hive. The probability of survival to 33 days for *A. florea* was therefore likely non-representative of the survival of the *A. florea* cohorts we marked, and so the differences seen (or not seen) between open- and cavity-nesting species in this analysis are not as informative as those observed in the survival analyses.

However, the analyses are informative for understanding how a bee’s activity shaped her probability of surviving to 33 days. In particular, we found, as previous research on *A. mellifera* has shown, that bees that became active later were likely to survive to age 33 days (Figure 3.6b). We also found a negative effect of activity rate, where bees that were more active were less likely to reach 33 days (Figure 3.6c). This negative correlation is consistent with prior research on *A. mellifera* which suggests that the foraging effort exerted by honey bees generally does not significantly impact their lifespans except in the cases of extremely high effort (Schmid-Hempel & Wolf 1988; Wolf & Schmid-Hempel 1989; Rueppell et al. 2007a). When we examined our negative effect more closely, we found that the effect was indeed due to a few extraordinarily hard-working bees. Once those bees were removed, we found that a bee’s activity rate no longer affected its probability of surviving to age 33 days, matching previous studies, and the expected species differences in survival were observed, with the open-nesting *A. florea* workers having a higher probability of survival than workers from both cavity-nesting species (Figure 3.7). The presence of especially hard-working individuals in both cavity- and open-nesting species suggests several future lines of study, for example on the percentage of bees that are hard-working, the contributions of these workers to the colony compared to other bees, and relationships between foraging rate and survival in hard working bees considered alone, as well as if any of these factors differ between species that vary in nest architecture.

While we focused our attention on between-species differences in activity and survivorship, we found some indications that cohorts of the same species can vary in their survivorship and activity (Figure 3.8). Although both *A. florea* cohorts had similar survivorship and ages at first flight during our observation period, the two observed *A. cerana* cohorts had significantly different survivorship and ages at first flight and one *A. mellifera* cohort had lower survivorship than the other two. The differences seen in *A. cerana* might be due to seasonal differences in age at first flight, possibly related to resource availability. One *A. cerana* cohort was studied during the winter period, while the other was studied during the summer. Though resources are available throughout the year in our study location, resources do tend to be more abundant in winter in Bangalore (Singh and Kushwaha 2006; Santhosh and Basavarajappa 2016), which corresponded to the longer-lived *A. cerana* cohort (Figure 3.8). Possibly, then, the *A. cerana* cohort with shorter lifespans and younger ages at first flight were foraging earlier due to resource shortages in the colony. In contrast, the three *A. mellifera* cohorts were studied at roughly the same time of the summer, but all three had different ages at first flight (Figure 3.8b); the differences observed in this study might therefore be due simply to inter-colony variation. The species differences seen here highlight the importance of studying multiple cohorts across multiple seasons to account for colony level differences in behavior and obtain more broadly generalizable pictures of species survival and behavior. However, the lack of similarity in survivorship between any cohorts of *different* species supports the species-specific patterns we found in this study.

In conclusion, our study supports and builds on previous studies examining the lifespan, survivorship, and foraging behavior of honey bee species. We have shown through linking an individual bee’s survival to its activity that species differences in survivorship are due in part to varying ages at first flight, though additional factors likely play a role in the prolonged lifespan seen in *A. cerana* workers compared to *A. mellifera* workers. Our results also align with the hypothesis that the increased mortality risks associated with an open-nest construction led to increased lifespan. Our results suggest that this longer lifespan is primarily due to a delayed onset of activity outside the nest and not to differences in the activity rates between workers of different species, though more data are needed on the activity and foraging careers of *A. florea* in particular. Future studies examining the survivorship and foraging behavior of *A. florea* over a longer period of time, as well as of more species (especially the open-nesting giant bee, *A. dorsata*), would be helpful in addressing this hypothesis. This work highlights the conserved relationship between a bee's transition to foraging and her lifespan across honey bee species with different evolutionary histories and nest architectures, and sets the stage for future studies that can investigate how other factors such as a high activity rate and inter-colony differences can also play a role in shaping lifespan.

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APPENDIX

Table S3.1. Model selection for lifespans of bees that died during the observation period. Final selected models are bolded.

Model	AICc	Delta AICc	Model Weight
A. cerana			
Lifespan ~ Age1stFlight + ActivityRate + (1|CohortID)	**1184.056**	**0.000**	**0.539**
Lifespan ~ Age1stFlight + ActivityRate + Age1stFlight*ActivityRate + (1|CohortID)	1185.267	1.210	0.294
Lifespan ~ Age1stFlight + (1|CohortID)	1186.425	2.369	0.165
Lifespan ~ ActivityRate + (1|CohortID)	1195.189	11.132	0.002
A. mellifera			
Lifespan ~ Age1stFlight + ActivityRate + Age1stFlight*ActivityRate + (1|CohortID)	3512.676	0.000	0.360
Lifespan ~ Age1stFlight + (1|CohortID)	**3512.777**	**0.102**	**0.342**
Lifespan ~ Age1stFlight + ActivityRate + (1|CohortID)	3513.051	0.375	0.298
Lifespan ~ ActivityRate + (1|CohortID)	4253.161	740.486	0.000
A. florea			
Lifespan ~ Age1stFlight + (1|CohortID)	**115.920**	**0.000**	**0.601**
Lifespan ~ Age1stFlight + ActivityRate + (1|CohortID)	117.021	1.101	0.346
Lifespan ~ Age1stFlight + ActivityRate + Age1stFlight*ActivityRate + (1|CohortID)	120.774	4.854	0.053
Lifespan ~ ActivityRate + (1|CohortID)	347.842	231.922	0.000

Table S3.2. Model selection for foraging lifespans of *A. cerana* and *A. mellifera* bees that died during the observation period. Final selected model is bolded.

Model	AICc	Delta AICc	Model Weight
ForagingLifespan ~ Species + Age1stFlight + ActivityRate + Species*ActivityRate + (1|CohortID)	4754.420	0.000	0.429
ForagingLifespan ~ Species + Age1stFlight + ActivityRate + (1|CohortID)	4756.153	1.733	0.180
ForagingLifespan ~ Species + Age1stFlight + (1|CohortID)	**4756.321**	**1.901**	**0.166**
ForagingLifespan ~ Species + Age1stFlight + ActivityRate + Age1stFlight*ActivityRate + (1|CohortID)	4756.942	2.522	0.122
ForagingLifespan ~ Species + Age1stFlight + ActivityRate + Species*Age1stFlight + (1|CohortID)	4757.276	2.856	0.103
ForagingLifespan ~ Species + (1|CohortID)	5489.457	735.037	0.000

Table S3.3. Model selection for age first seen flying. Final selected model is bolded.

Model	AICc	Delta AICc	Model Weight
Age 1st Flying ~ Species + (1|CohortID), NB distribution	**3619.958**	**0.000**	**1**
Age 1st Flying ~ Species + (1|CohortID), Poisson distribution	3789.449	69.490	0
Age 1st Flying ~ Species + (1|CohortID), Gaussian distribution	3905.605	185.647	0
Age 1st Flying ~ Species, Gaussian distribution	4035.712	315.754	0

Table S3.4. Model selection for activity rate with and without outliers. Final selected models are bolded.

Model	AICc	Delta AICc	Model Weight
Activity Rate Including Outlier Bees			
Activity Rate ~ Species + (1|CohortID)	**1055.678**	**0.000**	**1**
Activity Rate ~ Species	6714.586	5658.908	0
Activity Rate Without Outlier Bees			
Activity Rate ~ Species + (1|CohortID)	**721.468**	**0.000**	**1**
Activity Rate ~ Species	6301.331	5579.863	0

Table S3.5. Model selection for the probability of surviving to day 33 with and without outlier activity rates. Final selected models are bolded.

Model	AICc	Delta AICc	Model Weight
Probability of Surviving to 33 Days With Outlier Activity Rates			
ProbabiltyAliveDay33 ~ Species + Age1stFlight + Activity Rate + Age1stFlight*ActivityRate + (1|CohortID)	333.252	0.000	0.766
ProbabiltyAliveDay33 ~ Species + Age1stFlight + ActivityRate + (1|CohortID)	**335.823**	**2.571**	**0.212**
ProbabiltyAliveDay33 ~ Species + Age1stFlight + (1|CohortID)	340.399	7.146	0.021
ProbabiltyAliveDay33 ~ Age1stFlight + ActivityRate + (1|CohortID)	347.073	13.821	0.001
ProbabiltyAliveDay33 ~ Age1stFlight + (1|CohortID), Binomial distribution	352.046	18.794	0.000
ProbabiltyAliveDay33 ~ Species + ActivityRate + (1|CohortID)	481.806	148.554	0.000
ProbabiltyAliveDay33 ~ Species + (1|CohortID)	484.623	151.370	0.000
ProbabiltyAliveDay33 ~ ActivityRate + (1|CohortID)	497.783	164.531	0.000
Probability of Surviving to 33 Days Without Outlier Activity Rates			
ProbabiltyAliveDay33 ~ Species + Age1stFlight + Activity Rate + (1|CohortID)	330.745	0.000	0.557
ProbabiltyAliveDay33 ~ Species + Age1stFlight + (1|CohortID)	**332.461**	**1.715**	**0.236**
ProbabiltyAliveDay33 ~ Species + Age1stFlight + Activity Rate + Age1stFlight*ActivityRate + (1|CohortID)	332.783	2.083	0.201
ProbabiltyAliveDay33 ~ Age1stFlight + Activity Rate + (1|CohortID)	340.107	9.362	0.005
ProbabiltyAliveDay33 ~ Age1stFlight + (1|CohortID)	347.552	14.300	0.000
ProbabiltyAliveDay33 ~ Species + (1|CohortID)	471.663	140.918	0.000
ProbabiltyAliveDay33 ~ Species + Activity Rate + (1|CohortID)	472.928	142.183	0.000
ProbabiltyAliveDay33 ~ Activity Rate + (1|CohortID)	488.844	158.099	0.000

Table S3.6. Model selection for foraging lifespans of *A. cerana*, *A. florea*, and *A. mellifera* bees that died during the observation period. Final selected models are bolded.

Model	AICc	Delta AICc	Model Weight
Foraging Lifespan ~ Species + Age1stFlight + ActivityRate + Species*ActivityRate + (1|CohortID)	**4859.683**	**0.000**	**0.645**
Foraging Lifespan ~ Species + Age1stFlight + (1|CohortID)	4862.384	2.701	0.167
Foraging Lifespan ~ Species + Age1stFlight + ActivityRate + (1|CohortID)	4862.805	3.122	0.135
Foraging Lifespan ~ Species + Age1stFlight + ActivityRate + Age1stFlight*ActivityRate + (1|CohortID)	4865.573	5.890	0.034
Foraging Lifespan ~ Species + Age1stFlight + ActivityRate + Species*Age1stFlight + (1|CohortID)	4766.811	7.127	0.018
Foraging Lifespan ~ Species + (1|CohortID)	5810.770	951.087	0.000

Table S3.7. ANOVA results comparing foraging lifespans of *A. florea*, *A. cerana*, and *A. mellifera*. F-statistics and associated p-values for the effects of species and age at first flight on foraging lifespan are shown. Cohort ID was included as a random factor in the model. Significant differences are marked with *.

Model Factor	DF	F value	P-value
Species	2	5.71	0.058
Age 1st Flight	1	11.06	0.00088
Cohort ID	6	4.97	
Residual	701	58.98	

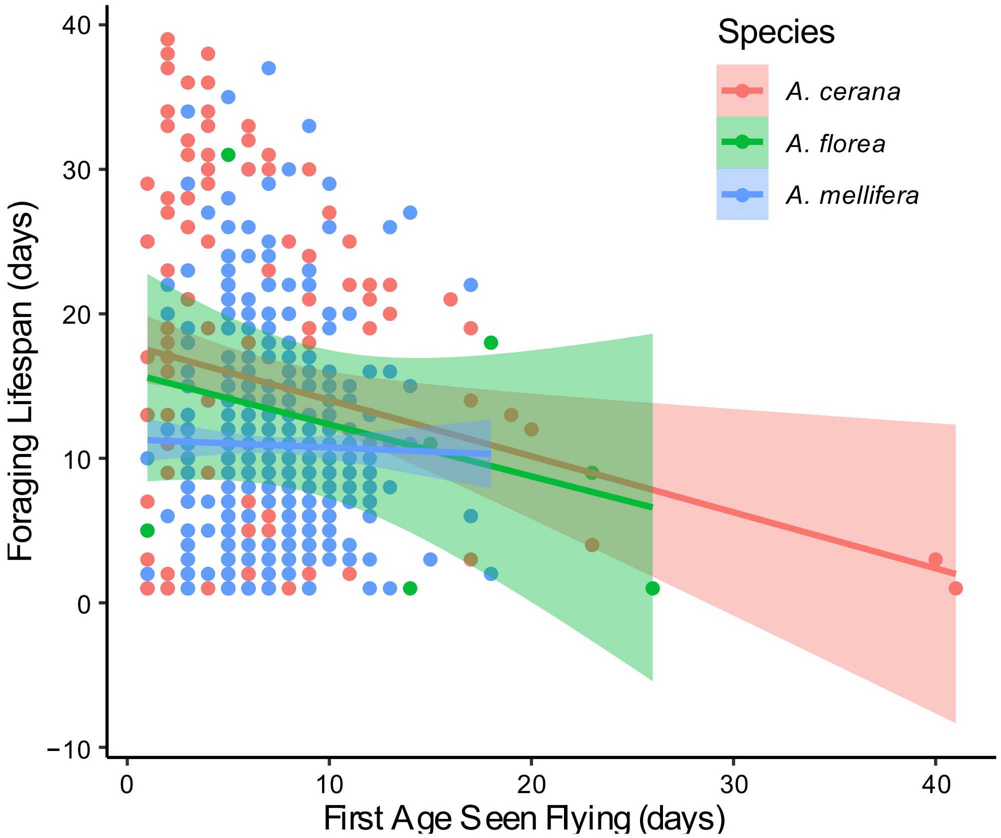

Figure S3.1. Foraging lifespans of *A. cerana*, *A. florea*, and *A. mellifera*. Regressions between a bee's age at first flying and her lifespan as a forager are shown for each study species. There were no species differences in the foraging lifespan of our study species (*P* = 0.058), but a negative relationship between age at first flying and foraging lifespan was seen (Estimate±SE: -0.28±0.083).

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CHAPTER FOUR:

A hard day's night: patterns in the diurnal and nocturnal foraging behavior of *Apis dorsata* across lunar cycles and seasons

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ABSTRACT

The giant honey bee *Apis dorsata* is unusual in being able to forage during both the day and the night. To date, the extent of this unique nocturnal foraging behavior and the environmental factors correlating with it have not been deeply investigated. We conducted the first systematic investigation into the nocturnal behavior of *A. dorsata* by tracking the daily and nightly foraging activity of *A. dorsata* colonies for 8 months, over multiple seasons and lunar cycles. We found strong evidence that *A. dorsata* is “cathemeral” (active over the entire diel cycle) when environmental illumination is sufficient for nocturnal flight. However, workers were not always active even when the environment was bright enough for them to forage, suggesting that their nocturnal foraging behavior is also tuned to seasonal changes in resource availability. The amount of activity observed during the day versus twilight versus night similarly differed between seasons; notably, nocturnal activity was higher than diurnal activity during the winter. We found that *A. dorsata* exhibits both diurnal and crepuscular activity, foraging just as much during the short twilight hours as during the day. The high foraging activity observed during the twilight and nighttime hours shows that *A. dorsata* colonies can extend their foraging beyond the daylight hours and reveals that foraging during these dimly lit hours is an integral part of their foraging ecology. This study highlights the contribution of nocturnal and crepuscular activity to the overall foraging ecology of *A. dorsata* and paves the way for future studies examining the role of *A. dorsata* in nocturnal pollination networks, the contribution of nocturnal foraging to colony-level nutrition and energy budget, and the evolution of this unusual behavior.

INTRODUCTION

Most animal species have evolved to be active only during limited periods of the 24-hour cycle (i.e., the diel cycle). Those periods of activity, often referred to as the species' temporal niche, are primarily the result of the endogenous circadian clock, which has been entrained by environmental cycles and selection pressures such as the light-dark cycle, temperature, humidity, etc. (DeCoursey 2004; Hut et al. 2012; Kronfeld-Schor et al. 2013). An animal's temporal niche presumably evolved to maximize the species' fitness; animals are generally both adapted and limited to their temporal niche through internal (e.g., anatomy, physiology, sensory and thermoregulatory capabilities [DeCoursey 2004; Hut et al. 2012; Shuboni-Mulligan et al. 2019]) and external (e.g., biotic and abiotic environmental conditions, predation [Castillo-Ruiz et al. 2012; Hut et al. 2012; Levy et al. 2012]) factors. However, animals can sometimes extend or switch their temporal niche through changes in the oscillation of their circadian system or through masking, which occurs when environmental factors such as illumination or temperature inhibit or stimulate an animal's behavior in a way that overrides their circadian clock (DeCoursey 2004; Fernández-Duque et al. 2010; Hut et al. 2012; Kronfeld-Schor et al. 2013).

Although much work to date has studied temporal niches and temporal niche switching using mammalian species (Fernández-Duque et al. 2010; Castillo-Ruiz et al. 2012; Hut et al. 2012; but see Kronfeld-Schor et al. 2013), these traits are found throughout the animal kingdom. In bees, for example, evidence that species are adapted to be active only during certain diel periods are shown through adaptations in anatomy (e.g., body size, eye morphology and anatomy, ocelli size [Greiner et al. 2006; Kapustjanskij et al. 2007; Haim & Neuman 2006; Somanathan et al. 2009; Somanathan et al. 2019]) and physiology (e.g., visual sensitivity and resolution, spatial photon summation, thermoregulation [Warrant et al. 1996; Greiner 2006;

Warrant et al. 2006; Levy et al. 2019; Somanathan et al. 2019]). A notable and unusual exception to the general rule that animals are restricted to only one part of the daily light-dark cycle is the behavior of the giant honey bee, *Apis dorsata*. This species, which lives throughout tropical Asia, is highly unusual among animals in being able to actively fly and forage during some nights as well as during the day (Dyer 1985; Kirchner & Dreller 1993).

The ability of *A. dorsata* to extend its temporal niche into the night is likely due in part to traits associated with their large size. As the second largest honey bee species in body size, *A. dorsata* has correspondingly large eyes with more and larger-sized ommatidia, which is correlated with the ability to maintain visual acuity even in dim light (Kapustjanskij et al. 2007; Somanathan et al. 2009; Streinzer et al. 2013). However, large body size alone is not sufficient for nocturnal activity: the largest honey bee species, *A. laboriosa*, does not forage at night, though this might be due in part to the colder nocturnal temperatures it experiences living in higher altitudes (Roubik et al 1985, Underwood 1991, Kirchner et al. 1996). *Apis dorsata* workers also have eyes with a raised vertex so their ocelli are more pronounced, improving their light sensitivity (Wcislo and Tierney 2009). There is also evidence that *A. dorsata* has neural circuitry in the optic lobes that perform photon summation across wide parts of their visual field, which improves light capture and would allow them to discriminate coarse images in moonlight even if the resolution of those images would be lower (Warrant et al. 1996; Warrant et al. 2008). None of these factors can fully explain their ability to fly at night, however, as the eyes of *A. dorsata* remain much less sensitive than those of other bee species that are specialized for crepuscular or nocturnal activity (Somanathan et al. 2009). Apart from vision, another way in which large body size could permit nocturnal activity in an endothermic insect like a honey bee would arise from the ability to maintain a higher body temperature, and thus sustain flight at lower ambient temperatures (Heinrich 1974; Bartholomew 1981). However, *A. dorsata* has been shown to deviate from the expected size-related patterns in flight energetics and is actually less able to fly at low ambient temperatures than the smaller species *A. cerana* and *A. mellifera* (Dyer and Seeley 1987).

While the mechanisms allowing *A. dorsata* to be active during the night are not yet fully understood, previous studies suggest that some level of illumination is necessary for these bees to be nocturnally active. Reports show that worker bees can actively forage throughout the night if the moon is at least half full, suggesting that although they do need some light to be nocturnally active the amount of illumination required is very low (Dyer 1985; Somanathan et al. 2009). There is also recent evidence that *A. dorsata* is capable of flying using artificial light sources such as the sky glow of a city even without any illumination from the moon (Young et al. 2021). This unusual behavior of *A. dorsata* might therefore be an example of positive masking, where the light available from the moon or artificial lights stimulate activity at a time the species would not otherwise be active. Regardless, this ability to be active during the bright daylight hours and the dim nighttime hours is impressive, as light intensity decreases by a factor of one million between a sunny day to a full moon night and is therefore even lower on nights when the moon is not full (Borges et al. 2016).

The ability of *A. dorsata* to fly during the night as well as during the day is all the more intriguing given that while all three closely related species belonging to the *A. dorsata* complex (*A. dorsata dorsata*, *A. dorsata binghami* (Sulawesi), and *A. dorsata breviligula* (Philippines)) can fly during the night, the Himalayan giant honey bee *A. laboriosa* cannot (Sakagami 1980; Roubik et al. 1985; Underwood 1991; Kirchner et al. 1996). Indeed, all other species in the genus *Apis*, with the possible exception of the African bee *A. mellifera adansonii* (Fletcher 1978), are strictly limited to diurnal activity (Warrant 2008). Given that body size and eye morphology alone cannot explain their nocturnal activity, what is it about the biology of *A. dorsata* that has led to the evolution of this ability? This question is challenging to answer because we lack basic information about the importance of nocturnal behavior in the foraging ecology of this species, and the environmental conditions that limit the ability to forage actively at night.

Regardless of the underlying mechanisms that permit crepuscular and nocturnal activity, it is likely that *A. dorsata* benefits from foraging in low light conditions. Nocturnal foraging is often associated with reduced predation and reduced competition (Roubik 1992; Wcislo et al. 2004; Warrant 2008; Smith et al. 2017). There are also potential thermoregulatory advantages for night-active animals in warm locations such as the tropics, as the energy needed to reduce the risk of overheating is decreased (Pereboom & Biesmeijer 2003; Wcislo and Tierney 2009). However, as *A. dorsata* primarily thermoregulates through passive means (e.g., dissipation of heat generated through flight) and does not require high energy expenditure to keep from overheating (Underwood 1991; Stabentheiner et al. 2012), this species probably does not obtain a thermoregulatory advantage by foraging at night. If anything, their lower mass-specific metabolic rate might limit activity during cooler nighttime hours (Dyer & Seeley 1987; Underwood 1991). A particularly significant advantage of nocturnal activity may be related to food gathering. In this view, the nocturnal activity of *A. dorsata* could be seen as an extension of their diurnal activity into the nighttime hours, prolonging their ability to exploit flower species that offer pollen and nectar resources near sunset and sunrise, and giving them access to an entirely new set of night-blooming floral resources (Momose et al. 1998; Somanathan et al. 2009; Somanathan et al. 2020).

Investigating the nocturnal behavior of *A. dorsata* will not only pave the way for investigations into how this unusual behavior has evolved and if it represents an example of positive masking, but will also provide further insight into the role of *A. dorsata* as a critically important pollinator of tropical Asian forests and agricultural crops. Though it is considered to be one of the most important pollinators in tropical Asia (Bawa 1990; Corlett 2004; Hung et al. 2018), we currently have very limited knowledge about the role it is playing in nocturnal pollination networks (but see Momose et al. 1998 and Somanathan et al. 2009 which suggest it is an important nocturnal pollinator). In light of growing concerns about pollinator decline, a full understanding of the foraging ecology of *A. dorsata*, including the ecology of nocturnal foraging, is critical for future assessments of their role as pollinators, possible threats to their population, and their conservation requirements (Walter and Brockmann 2021).

Here we present the first systematic investigation into the nocturnal behavior of *A. dorsata*. Over the course of eight months, we observed colonies of *A. dorsata* in Bangalore, India to examine how their diurnal and nocturnal behavior changed over the course of multiple seasons and lunar cycles. We paid particular attention to correlations between nocturnal activity and light availability, as well as between nocturnal activity and temperature, while also relating their behavior to expected resource availability in the surrounding environment. Overall, our goal is to provide a deeper understanding of the contribution of nocturnal activity to the overall foraging ecology of *A. dorsata* and pave the way for future studies examining the evolution and functional significance of this unusual behavior.

MATERIALS AND METHODS

Study Location

This study was conducted on the campus of the National Centre for Biological Sciences – Tata Institute of Fundamental Research (NCBS) in Bangalore, Karnataka, India, from October 2018 through May 2019. NCBS is located in a primarily residential district in the northern part of the city of Bangalore, adjacent to the University for Agricultural Sciences – Bangalore (GKVK). NCBS has extensive ornamental vegetation on its campus and is surrounded by fields of agricultural vegetation on the GKVK campus, as well as by residential tracts offering ornamental plants (Young et al. 2021).

Apis dorsata Biology

Apis dorsata can be found through southern Asia (Ruttner 1988; Smith 2020). It is one of the largest honey bee species, with a worker mass of ~120 mg when unloaded (Dyer & Seeley 1991). Like all species in this genus, *A. dorsata* is eusocial, with perennial colonies that reproduce by colony fission. Each colony consists of a single queen, a large number of workers, and a substantial but variable number of males (drones) during the swarming season. As foragers, the workers are ecological generalists and exploit a wide variety of floral species for nectar and pollen (Stewart et al. 2018; Mukherjee et al. 2019). Recruitment to food relies upon the dance language, similar to that of the Western honey bee, *A. mellifera* (Dyer 2002). Colonies have up to 50,000 individuals, and nest in the open on a large (≈1m diameter) sheet of comb attached to an overhanging structure such as a tree branch, rock cliff face, or building ledge. The worker bees protect the colony by forming a protective curtain across the entire surface of the comb; foraging bees land on, depart from, and dance on this protective curtain (Seeley et al. 1982; Oldroyd & Wongsiri 2009; Hepburn & Radloff 2011). Colonies of *A. dorsata* can be found in forests, agricultural areas, and even urban areas, and often form aggregations of 10 or more colonies in a single location. *Apis dorsata* is a migratory species, traveling up to 200 km through a series of shorter steps to follow currently available resources (Koeniger & Koeniger 1980; Dyer & Seeley 1994; Robinson 2012). In the study location, *A. dorsata* colonies tend to be found within Bangalore during the dry season (which lasts from October to May), migrating into Bangalore from tropical forests in November and December when resources are scarce in those forests. Colonies then migrate out of Bangalore in May and June before the start of monsoon season (which lasts from June to September) to travel back to tropical forests which exhibit high resource availability during the monsoon season.

Collection of A. dorsata Colonies

Five colonies of *A. dorsata* were observed over the course of this study (Figure 4.1). In October, November, and December, we collected one colony per month from the Bhartiya City apartment complex in Bangalore, which was located approximately 10 km from the NCBS campus. We had to find and transport colonies from this apartment complex to the NCBS campus as no colonies were naturally occurring on the NCBS campus during these months. Colonies were found nesting under the overhanging balconies of the apartment buildings. To collect a colony, we followed the procedure outlined in Crane 1999 and Thomas et al. 2002, where a bamboo stem that had been split down its length is used as a clamp to carry the comb after cutting it from the balcony. Briefly, we first pumped smoke from a bee smoker to drive all the bees from the colony. We then quickly clamped the two halves of the bamboo across the top portion of the comb, which contains honey. After allowing the bees to resettle on the comb, we carefully cut the comb away from the balcony roof while holding it on either side of the bamboo clip and lowered the entire colony into a large cloth bag. We sealed the bag and were then able to transport the colony by truck to the NCBS campus, where it was installed in a large open-faced box on the roof of the NCBS administration building. New colonies had to be collected every month as the transplanted colonies generally absconded (i.e., abandoned their hive) two to three weeks after being moved to the NCBS campus.

During January through May, we used *A. dorsata* colonies that were naturally occurring on the NCBS campus in this study. One colony, located on the administration building, was observed from January through April. After this colony migrated away from the NCBS campus, a second colony which was located on the adjacent laboratory building was observed during May.

Monthly Observations

We recorded the nocturnal activity of *A. dorsata* colonies on nights surrounding the full moons of the months of October through May (Figure 4.1). We used a Panasonic 4K Ultra HD Video Camcorder (Model VX981K) on the infrared setting to record the colonies from shortly before sunset (generally around 1815 hours) to shortly after sunrise (generally around 0630 hours). As much as possible, we recorded the nocturnal activity of each *A. dorsata* colony from five nights before the full moon to five nights after the full moon. It was not always possible to record every night in this period every month, however. During the months of October and November, due to the low number of *A. dorsata* colonies present in Bangalore (because migrating colonies had not returned to the region), we were unable to collect *A. dorsata* colonies until the day of the full moon, and so colonies were only recorded from the night of the full moon onward for those months. Occasionally, the occurrence of rain at night prevented us from recording nocturnal activity, though this was rare. Monthly observations were categorized into seasonal categories; October through December comprised “autumn”, January through February comprised "winter", and March through May comprised "summer" based on the suggestions of local Bangalore residents (Figure 4.1).

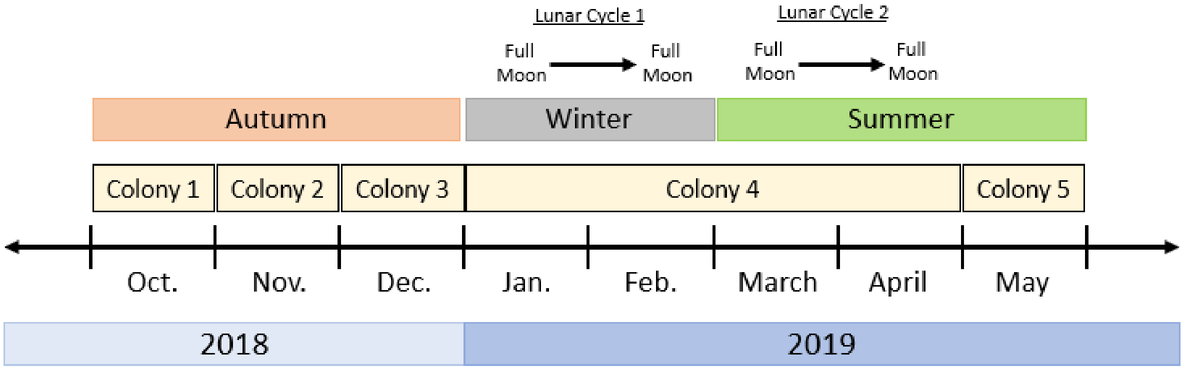

Figure 4.1. Timeline of data collection by month. We recorded nocturnal activity from 5 nights before to 5 nights after the full moon of every month except October and November, where we recorded nocturnal activity from the full moon to 5 nights after the full moon due to delays in obtaining *A. dorsata* colonies. The colony observed each month is shown, as is the season to which each month belongs. We obtained minimum temperatures for every observation day through an online resource, and so do not show it here. In addition to the observations focused on the full moon, we also observed Colony 4 for two complete lunar cycles, indicated by the text above; illumination readings were collected every observation night during the January to February lunar cycle (lunar cycle 1).

Observations Over Lunar Cycles

We recorded the nocturnal and diurnal activity of one *A. dorsata* colony for two complete lunar cycles: January 21 to February 19, 2019 and March 21 to April 19, 2019 (Figure 4.1). For each cycle, we began filming on the night of the first full moon (January 21 and March 21, respectively) and recorded for 24 hours until sunset on the following day. These 24-hour recordings were repeated every four to five days, such that we were able to record behavior on the night of every major moon phase in the lunar cycle (full, third-quarter, new, first-quarter, full) as well as one day in between each major phase.

During the January – February lunar cycle, we also took light measurements during every night when we recorded nocturnal activity. We used a Hagnar Universal Photometer/Radiometer S4 to record the illumination of the environment every hour, starting at sunset and continuing until sunrise. The sensor was on the roof above the studied *A. dorsata* colony and was oriented parallel to the ground and aiming skyward; a reading was recorded when the illumination value displayed was stable for 30 seconds.

Video Analysis

After recording, we analyzed videos to quantify the nocturnal activity of *A. dorsata*. We conducted a ten-minute activity census every half hour, counting the number of arriving bees and the number of waggle dances occurring during the census period. If activity was recorded for 24 hours (as during the lunar cycle observations), we similarly censused the diurnal activity of the colony. Sunset, sunrise, moonset, moonrise, and twilight periods (including the timing of the end of astronomical twilight and the beginning of astronomical dawn) were identified for each night of recording using the website https://www.timeanddate.com/moon/india/bengaluru. The end of astronomical twilight and beginning of astronomical dawn mark the beginning and ending of night, respectively, as light from the sun is not available in the sky and only the moon, stars, and artificial lights provide illumination. Daily temperature data from the Kempegowda International Airport Weather Station in Bangalore, India (14 km from the NCBS campus) was accessed and recorded from the Weather Underground website (https://www.wunderground.com/history/daily/in/bangalore/VOBL).

We characterized the duration of diurnal and nocturnal activity relative to astronomical reference periods. Activity occurring between sunrise and sunset was considered “diurnal activity”; activity occurring from the sunset to the end of astronomical twilight or from the beginning of astronomical dawn to sunrise was considered “twilight activity”; and activity occurring between the end of astronomical twilight and the beginning of astronomical dawn was considered “night activity”.

Statistical Analyses

We conducted exploratory data analyses in R version 4.0.2. As a check of our assumption that bee arrivals reflect ongoing foraging during all diel periods, we ran Spearman correlation analyses on the number of arrivals and number of dances occurring in each 10-minute census period throughout each diel period (day, twilight, night). Our outcome variable of interest, number of bees arriving back to the nest during the census period (hereafter referred to as "arrival rate"), consisted of count data and was found to be over dispersed, so we used a negative binomial distribution in all analyses. We fitted a generalized linear mixed-effect model to determine the effects on arrival rate of season (autumn, winter, summer), minimum temperature, diel period (day, twilight, night), and the interaction between season and diel period, including the random factor of colony ID to account for differences between colonies ('glmmTMB' function). We then subsetted the data to create datasets containing only those measurements collected for lunar cycle or illumination analyses. Separate generalized linear models were fitted to quantify (1) the effect of the lunar cycle and (2) the effect of environmental illumination on bee arrival rates ('glmmTMB' function). The model for lunar cycle included the fixed factors of moon phase (first quarter, full moon, waning gibbous, waning crescent, new moon, third quarter, waxing crescent, waxing gibbous), lunar cycle ID (January, March), diel period (day, twilight, night), the interaction between diel period and lunar cycle ID, and the interaction between diel period and moon phase. The model for illumination included the fixed factors of illumination, time since sunset, and census time. Colony ID was not considered in the models for lunar cycle or illumination, as data were collected from only one colony during these periods. We built models using a forward approach, beginning with null models and adding complexity through the addition of fixed and random factors. We selected final models using a holistic approach based on their AIC values, R^2 values, and performance during model diagnostic tests that checked for outliers, dispersion, and deviation (‘DHARMa’ package). Final models selected were those with the lowest AIC, best model diagnostics, and highest R^2 values. Descriptions of all models tested for each response variable, as well as performance in model selection criteria, can be found in Supplementary Tables 4.1, 4.2, and 4.4. After selecting the final model, we analyzed the models using ANOVA, and analyzed significant factors using post hoc tests with a Tukey correction. Means are reported as the estimated marginal means plus or minus standard error resulting from the final models chosen.

RESULTS

General Description of Patterns of Activity

We found that *A. dorsata* exhibited peak activity in the hours before and just after sunrise (generally 400-600 hours; Figure 4.2). This activity often began before the sun had risen, during dawn, regardless of the phase of the moon. Another peak in activity was seen at sunset (which generally occurred around 1830 hours). Activity tended to be higher during the day when activity at night was low (such as during the new moon). Most bees returned to the hive with pollen, particularly at night. A surprising observation was that nocturnal activity was sometimes seen in the hours after astronomical twilight ended but before the moon had risen. On the nights of the January waning gibbous and third quarter moons in particular high activity was seen during this period. See Figure S4.1 for graphs showing daily activity patterns for every day recorded during the January to February and March to April lunar cycles.

To check our assumption that bees seen arriving at the nest are returning from foraging flights (as opposed to learning flights or defecation flights), we analyzed correlations between the number of bees arriving and the number of recruitment dances during the same census period. Dances are performed only immediately following a forager's return to the nest, and so a positive correlation would mean that arrival rate can be used as a proxy for foraging activity. We observed strongly positive correlations during all parts of the diel cycle (day: $S = 2461556$, $r_s = 0.82$, $P < 2.2e\text{-}16$; twilight: $S = 94819$, $r_s = 0.84$; $P < 2.2e\text{-}16$; night: $S = 5921424$, $r_s = 0.82$, $P < 2.2e\text{-}16$; Figure 4.3). These strong correlations exclude the possibility that nocturnal arrivals are primarily bees that began foraging during the day and found their way back to the nest at night. Thus, we proceeded with the remainder of the analyses using arrival rate as our variable of interest.

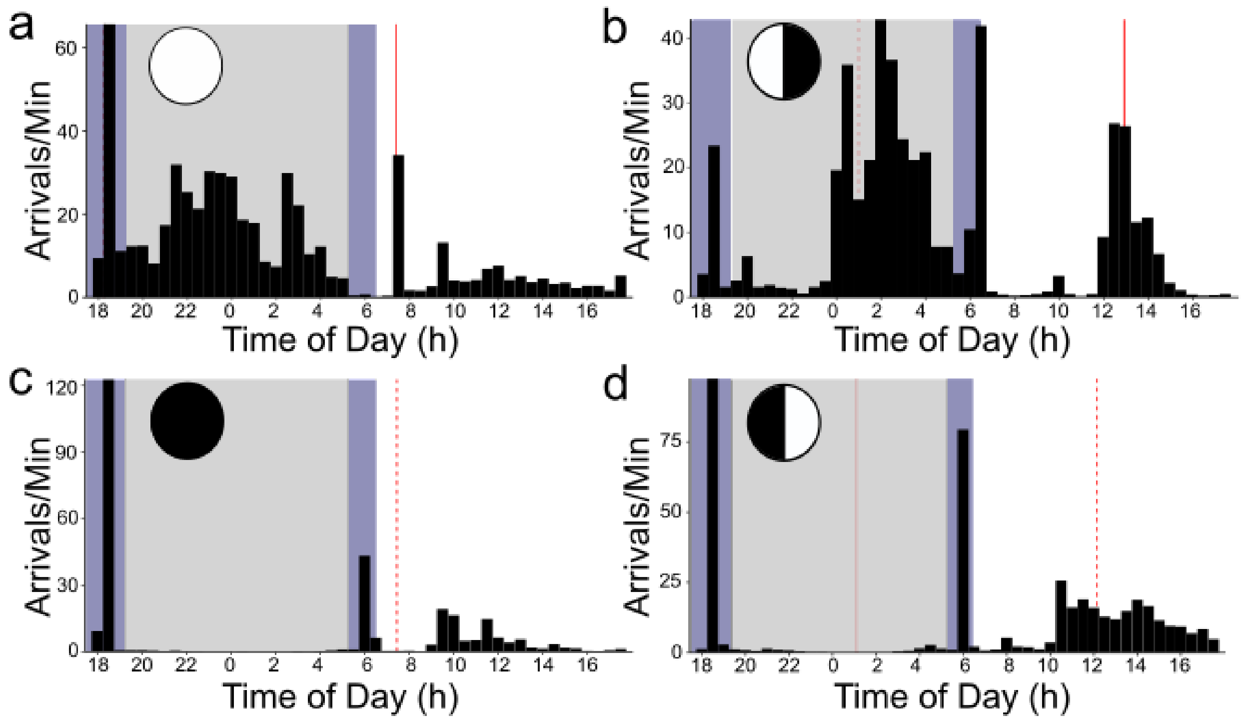

Figure 4.2. Daily foraging activity patterns of *A. dorsata*. Daily foraging activity on the days of the (a) full moon, (b) third quarter moon, (c) new moon, and (d) first quarter moon for the January – February lunar cycle are shown as representative examples of *A. dorsata* daily activity patterns. Purple shaded regions indicate twilight, gray shaded regions indicate night, and white indicates daytime. The red dotted line indicated moonrise, while the solid red line indicates moonset. Arrival rates are shown here as arrivals per minute instead of arrivals per census period. Figures depicting the daily foraging activity for every day of the January – February and March – April lunar cycles can be found in Supplementary Figure 4.1.

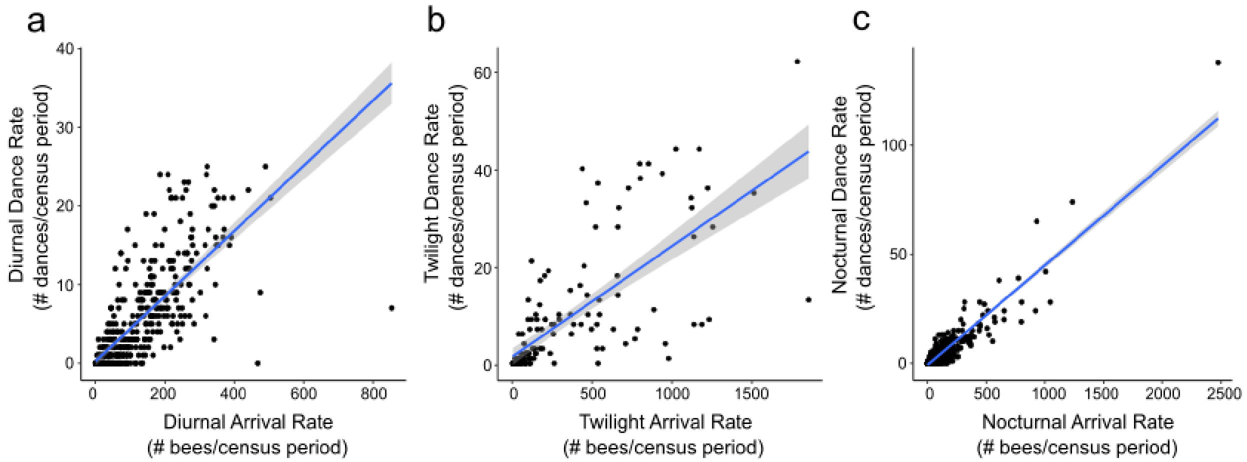

Figure 4.3. Relationship between bee arrival rates and dance rates. Regressions of number of bee arrivals against number of bee dances per census period are shown for (a) day time periods, (c) twilight time periods (both morning and evening), and (c) night time periods. Strong correlations were found between arrival and dance rates for all diel periods ($r_s > 0.82$ for all).

Activity in Relation to Illumination

We found that during the night, illumination had a significant positive effect on bee arrivals ($F^{1}_{86} = 6.94$, $P = 0.0084$): as illumination increased the arrival rate also increased (Figure 4.4). Surprisingly, we also found that arrivals occurred even when illumination was recorded as 0 cd/m^2 (i.e., at the limit of the photometer's sensitivity). Neither time since sunset nor census time significantly influenced the arrival rate.

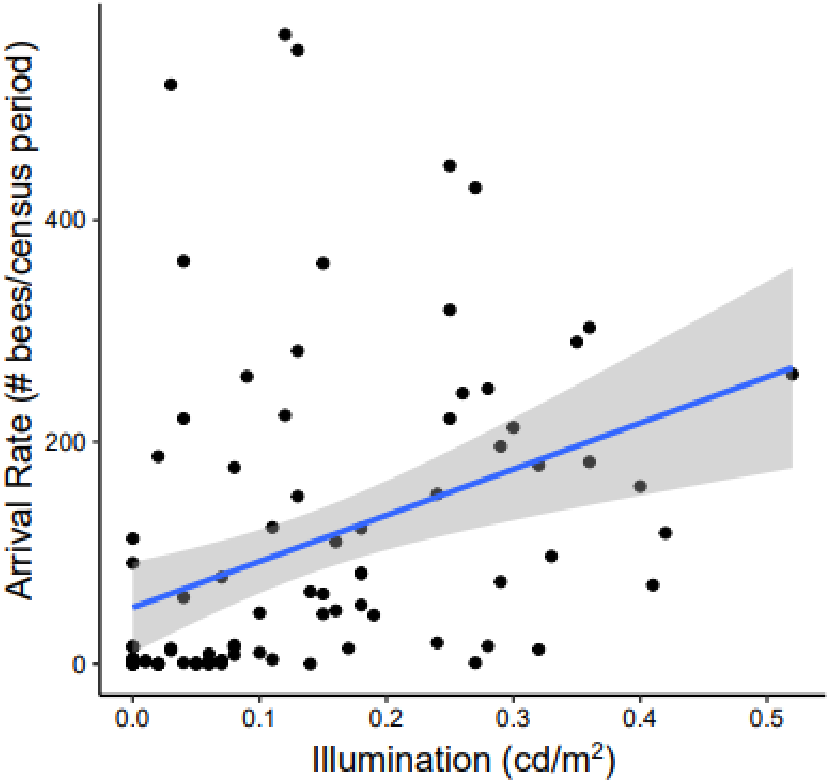

Figure 4.4. Relationship between nighttime illumination and bee arrival rates. A regression between environmental illumination at night and number of bee arrivals to the nest per census period is shown. Illumination had a significant positive effect on arrivals, such that as illumination increased arrivals also increased (*P* = 0.0084). Arrivals were seen even when illumination was recorded as 0 cd/m^2 at the limit of the photometer's sensitivity.

Activity Patterns Over the Lunar Cycle

When examining the effects of moon phase on bee arrival rates, we found that arrival rates varied greatly depending on diel period (F^{2}_{807} = 437.58, *P* < 2e-16) and moon phase (F^{7}_{807} = 291.9286, *P* < 2e-16), and that the activity seen during a given diel period varied depending on the moon phase (Diel Period*Moon Phase: F^{14}_{807} = 317.0116, *P* < 2e-16; Figure 4.5). In particular, we found that all bee arrival rates during the day (all *P* > 0.9) and during twilight (all *P* > 0.5) were constant across the moon phases. Arrivals rates during the day and during twilight on a given moon phase generally did not differ (*P* > 0.05), except for on the night of the waning gibbous moon, where twilight arrival rates were higher than daytime arrival rates ($P < 0.05$). In contrast to the generally steady arrival rates seen during the day and twilight across the moon phases, arrival rates at night were highly dependent on proximity to the full moon (Figure 4.5). Arrival rates during the night were generally either high and close to the daytime arrival rates or extremely low and close to zero (see Table S4.3 for all estimated marginal means). High arrival rates at night that were equal to rates during the same day were seen only within a week of the full moon, namely during the full moon, third quarter moon, waning gibbous moon, and waxing gibbous moon phases (all $P > 0.1$; Figure 4.5). Arrival rates at night were often lower than arrival rates during twilight (all $P < 0.05$) but did match twilight arrival rates on the nights of the full, waxing gibbous, waning gibbous, and the third quarter moons (all $P > 0.05$).

We also found that variation in bee arrival rates at a given diel period was dependent on the lunar cycle (Diel Period*Lunar Cycle: $F_{807}^{2} = 36.77$, $P = 1.04\text{e-}8$), though there was not a difference in total activity between lunar cycles ($F_{807}^{1} = 1.2421$, $P = 0.27$). Arrival rates during the night were higher in the January lunar cycle than the March lunar cycle ($P < 0.001$; X±SE: March = 2.85±0.096, January = 3.41±0.092; Figure 4.6), but arrival rates during the day were higher in the March lunar cycle than the January lunar cycle ($P < 0.001$; X±SE: March = 4.98±0.08; January = 4.43±0.082). Arrival rates during twilight did not differ between lunar cycles ($P = 0.99$), but twilight had the highest rates of activity within both lunar cycles ($P < 0.001$; X±SE: March = 5.84±0.018, January = 5.73±0.16). Diurnal arrival rates were the next highest in both lunar cycles, with nocturnal activity the lowest in both cycles ($P < 0.001$ all).

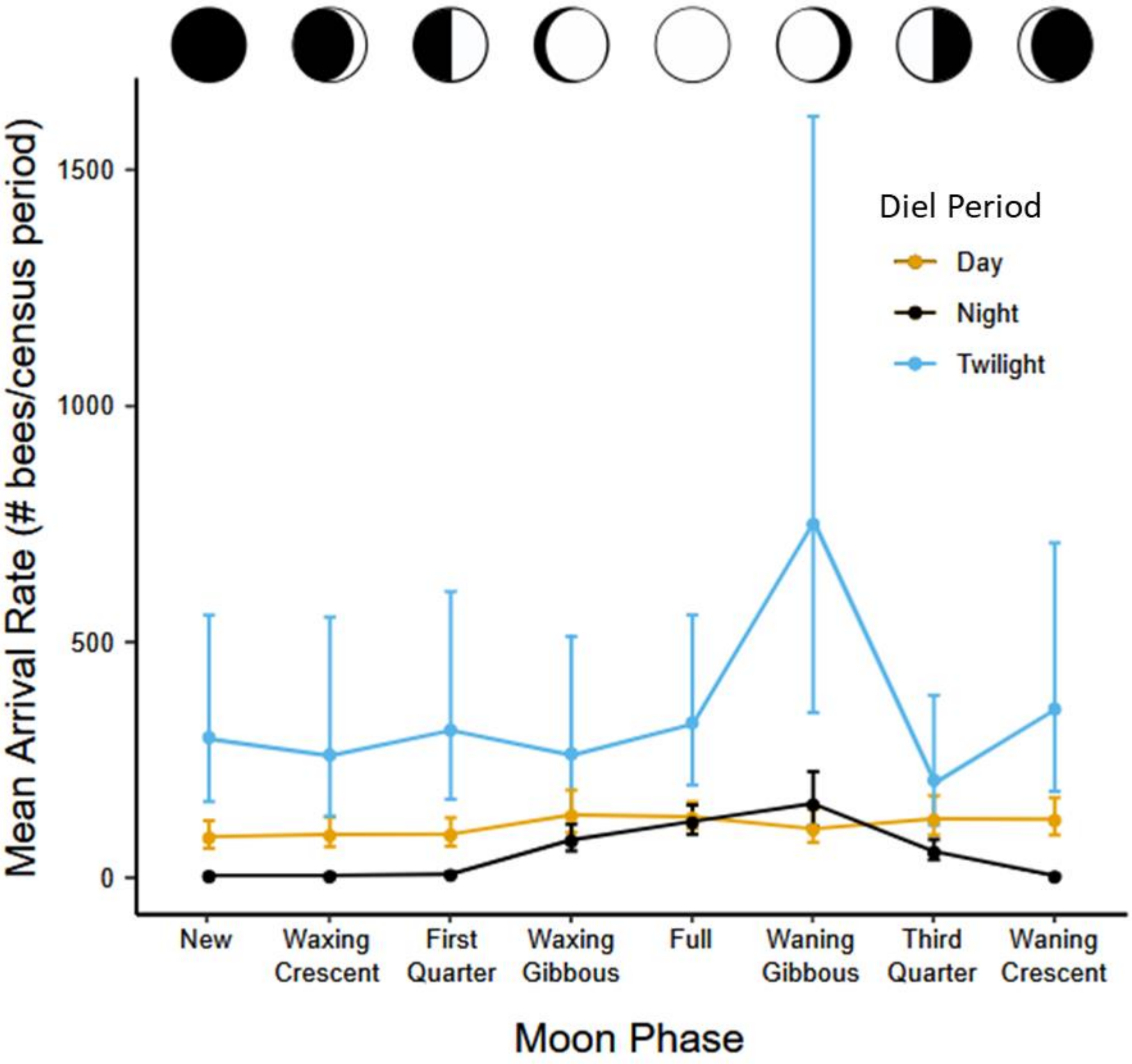

Figure 4.5. Estimated marginal mean arrival rates across moon phases. Estimated marginal mean arrivals rates during different diel periods are shown across moon phases. Activity was consistently the highest and equal during the day and twilight (all *P* > 0.05) except during the waning gibbous and waning crescent moons (*P* < 0.05) where more activity was seen during twilight time periods. Nocturnal activity was generally low compared to activity the rest of the day (*P* < 0.05), except the moon phases including and between the waxing gibbous and third quarter moons, where it was equal to activity seen during the day (*P* > 0.05). Error bars represent 95% confidence intervals.

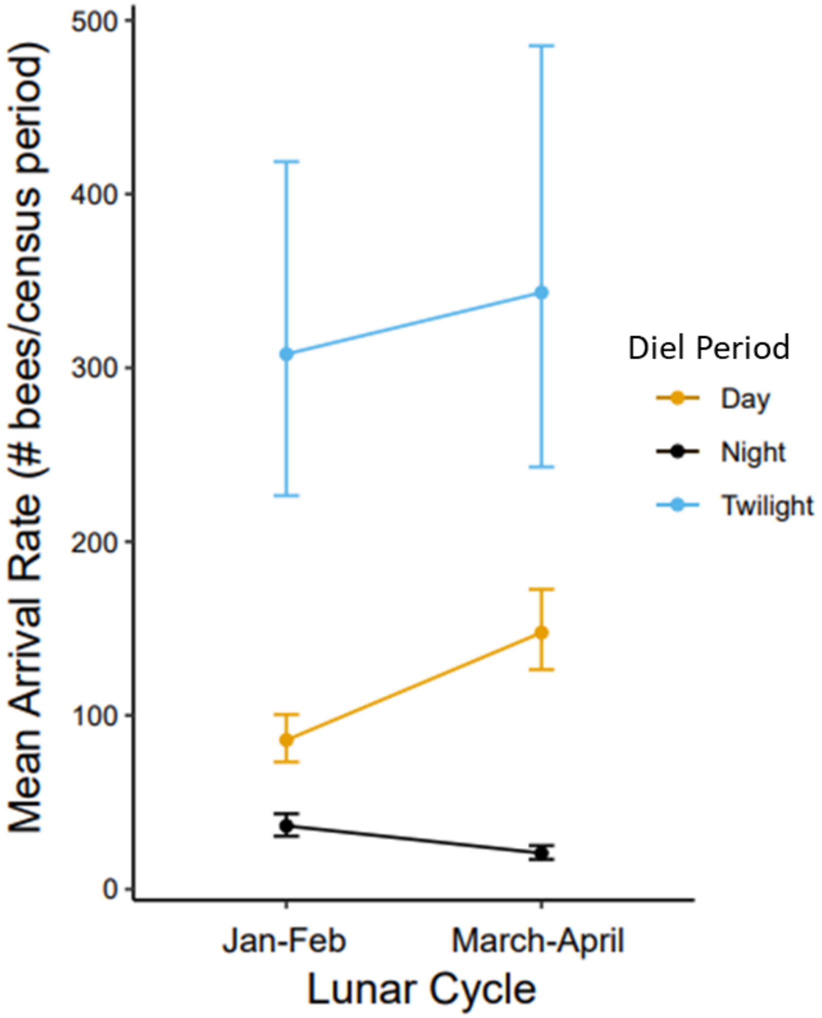

Figure 4.6. Estimated marginal mean arrival rates in each lunar cycle. Estimated marginal mean arrival rates during each diel period over both lunar cycles are shown. In both lunar cycles the highest number of arrivals were seen during twilight (*P* < 0.001), and the least number of arrivals were seen during the night (*P* < 0.001). An interaction was seen where nocturnal activity decreased but diurnal activity increased from the January lunar cycle to the March lunar cycle (*P* < 0.001). Error bars represent 95% confidence intervals.

Activity Patterns Based on Season and Temperature

We found that bee activity varied significantly with diel period (F^{2}_{1237} =151.22, *P* < 2e-16), and that the activity seen at a given diel period differed among seasons (Diel Period*Season: F^{4}_{1237} = 127.37, *P* < 2e-16; Figure 4.7). Specifically, we found that arrival rates during the night were higher in winter than in summer (*P* < 0.001; X±SE: winter night = 4.58±0.63; summer night = 3.30±0.63), but arrival rates during the day were lower in winter than in summer ($P < 0.001$; winter day = 4.08±0.63; summer day =4.87±0.62). Arrival rates during the day did not differ between autumn and summer or between autumn and winter; similarly, arrival rates during the night did not differ between autumn and summer or between autumn and winter ($P > 0.50$ for all; autumn night = 2.98±0.54; autumn day =4.86±0.73). While a trend towards increasing arrival rates during twilight is seen across the seasons, we did not find a significant difference in those rates between any season (all $P>0.75$; winter twilight = 5.40±0.644; autumn twilight = 4.35±0.567; summer twilight = 5.85±0.64). During autumn, arrivals rates were the same regardless of diel period (all $P > 0.05$), except for higher arrival rates during twilight than during the night ($P < 0.05$). During the winter season, nocturnal arrival rates were higher than diurnal rates ($P < 0.05$), while during the summer season it was reversed with higher diurnal arrival rates than nocturnal ($P < 0.05$). In both winter and summer though, there was more activity during twilight than during the day or the night (all $P < 0.05$).

Minimum daily temperature over the course of the study ranged from 10° - 24˚ Celsius. We found no relationship between minimum temperature and bee arrival rate in this experiment ($F^{1}_{1237} = 2.04$, $P = 0.15$; Figure 4.8).

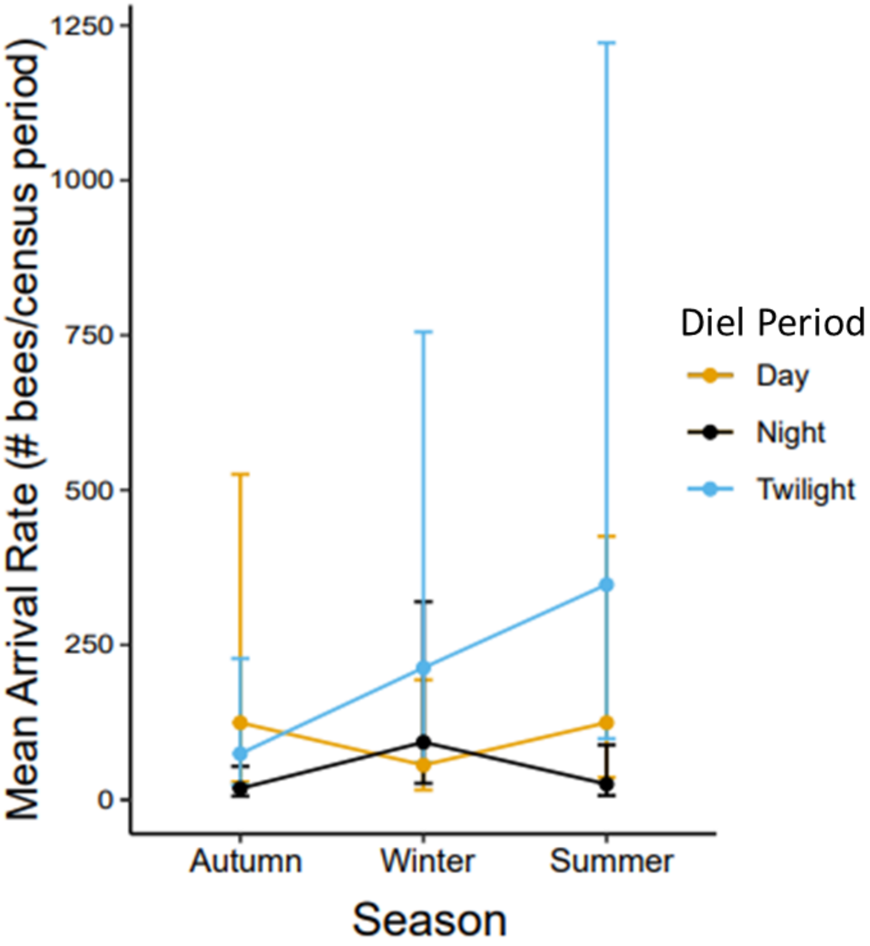

Figure 4.7. Estimated marginal mean arrival rates across seasons. Estimated marginal mean arrival rates during each diel period across seasons are shown. Nocturnal arrival rates were higher and diurnal arrival rates were lower in winter compared to summer ($P < 0.05$), but did not differ between autumn and summer or autumn and winter ($P > 0.05$). In both winter and summer arrival rates were significantly different between every diel period, with twilight having the highest rates. Arrival rates were higher at night than during the day in winter ($P < 0.05$), which was not seen in any other season. Arrival rates during twilight was also higher than the nocturnal arrival rate in autumn ($P < 0.05$), but the arrival rates during twilight did not significantly differ between seasons ($P > 0.6$). Error bars represent 95% confidence intervals.

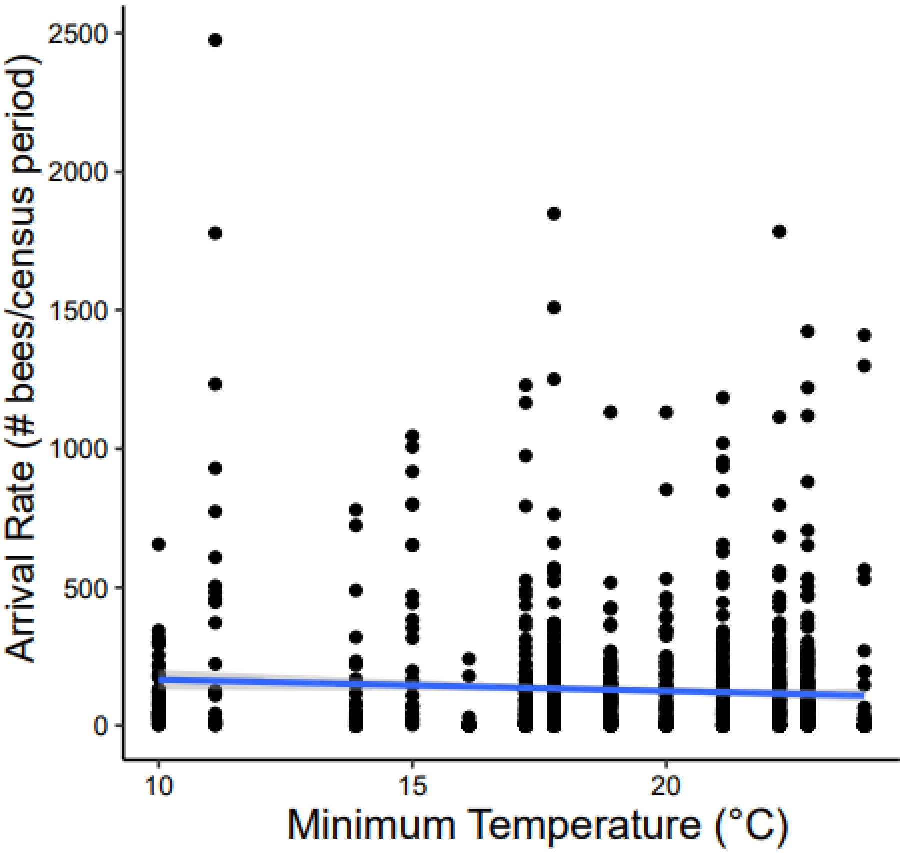

Figure 4.8. Relationship between minimum temperature and arrival rate. A regression between minimum daily temperature and bee arrival rates throughout the day are shown. There was no relationship between minimum temperature and bee arrivals, with high activity seen even during the coldest nights in winter ($P > 0.05$).

DISCUSSION

In this study, we provide the first systematic investigation into the nocturnal foraging behavior of the giant honey bee, *A. dorsata*, across multiple seasons. We observed the activity of *A. dorsata* over a period of eight months to examine environmental correlates of their diurnal, crepuscular, and nocturnal activity. Changes in activity during these time periods were dependent on the season, moon phase, and illumination of the environment, but did not seem to be influenced by temperature. Our results indicate that foraging during crepuscular and nighttime periods make up a substantial portion of a colony's daily foraging activity.

Activity during crepuscular and nighttime periods could be the result of bees actively foraging for resources or simply due to bees returning to their nest at the end of their day's work. Bee arrival rates and dance rates were highly and similarly correlated during the day, twilight, and night in this study (Figure 4.3), providing strong evidence that bees are activity foraging during the crepuscular and nighttime hours. In addition, the majority of bees arriving during those nocturnal hours returned with pollen, further supporting our underlying assumption that arrival rate is an accurate metric for assessing foraging effort. Arrival rates and dance rates were not perfectly correlated, presumably either because bees observed arriving were foragers that returned to the nest and did not dance or because they were away from the nest for some other reason (e.g., learning flights). An additional source of noise could be due to the additional flights *A. dorsata* colonies perform during certain times of the day. Drones frequently perform mass mating flights at sunset (Koeniger & Wijayagunasekera 1976; Rinderer et al. 1993; Woyke et al. 2005), and workers often perform mass orientation and defecation flights early in the morning and during the hottest parts of the day (Woyke et al. 2004; Woyke et al. 2005). However, mass flights are highly distinctive in open-nesting species and can be characterized by the formation of wide gaps in the bee curtain, chains of bees, and increased general activity (Kastberger et al. 1996; Bhagavan et al. 2016). During each census period we paid close attention to the structure of the comb and did not observe such signs of mass flights during any of our census periods, nor did we observe many drones, making it unlikely that the periods of high activity we observed were due to mass flights of non-foraging bees. While we could not perfectly distinguish between foraging versus other types of bee flight activity, the strong and consistent correlations between arrival and dance rates suggest that most arrivals are workers returning from foraging flights.

These findings strengthen previous claims that *A. dorsata* is actively foraging at night, especially when the moon is bright (Dyer 1985; Kirchner & Dreller 1993; Somanathan et al. 2009). They also provide a starting point for addition studies investigating the proportion of the foraging workforce that forages at night, whether the same individuals forage during different diel periods, and whether the same resources are exploited during different diel periods (as has been seen in resource overlap between diurnal and nocturnal carpenter bees; Somanathan et al. 2020). While we cannot currently interpret the waggle dances observed in this study because the reference point for nocturnal dances is unknown (Dyer 1985; Young et al. 2021), we are in the process of analyzing the dances to determine the reference. With that information, future studies could also examine and compare the number of resources being exploited during different diel periods and the locations of resources being exploited.

Apis dorsata colonies exhibited consistent peaks in activity at sunrise and sunset across the study period (Figure 4.2). The high activity seen from the start of astronomical dawn to sunrise is of particular note as it generally began to increase rapidly hours before and declined soon after sunrise. This early start to foraging is likely to provide a competitive advantage (Roubik 1992; Wcislo et al. 2004; Warrant 2008; Smith et al. 2017), allowing *A. dorsata* foragers to exploit resources before other social bee species that need the light of the sun to forage (Warrant 2008). The continuation of foraging throughout twilight and into the night is likely a similar extension of diurnal foraging, allowing *A. dorsata* foragers to continue exploiting profitable resources with minimal competition while taking advantage of new resources that become rewarding at night (e.g., some *Dipterocarpus* species (Momose et al. 1998), *Bridelia retusa*, *Randia dumetorum* (Somanathan et al. 2009)). While it was not possible for us to identify and assess the types and amounts of resources collected during each diel period, future studies should prioritize quantifying the amount of nectar and pollen brought in during the twilight and night hours and identifying their sources to determine the contribution of activity during these diel periods to a colony's energy budget.

The amount of light available during the nocturnal hours was the primary determinant of nighttime foraging, supporting the idea that nocturnal foraging is an extension of diurnal foraging. As illumination in the environment increased, the activity of bees during the night similarly increased (Figure 4.4). These results suggest that the nocturnal behavior of *A. dorsata* is an example of positive masking, whereby increased illumination stimulated activity outside of the endogenous circadian rhythm, as has been seen in other diurnal species (Vivanco et al. 2010; Shuboni-Mulligan et al. 2019). This illumination was primarily provided by the moon, with high nocturnal activity seen only within the week before to the week after a full moon, the period when the moon was at least half full (Figure 4.5). These periods of high nocturnal activity correspond with and support previous reports of *A. dorsata's* nocturnal activity (Dyer 1985; Somanathan et al. 2009; Young et al. 2021). Interestingly, nocturnal activity was often higher during the waning phase of the lunar cycle as compared to when the moon was waxing (Figures 4.2 and 4.5), even though the total amount of nocturnal illumination should be the same on these nights, all else being equal. It is possible that the higher activity seen during the waning period of the lunar cycle is a result of the *A. dorsata* workers having increased information after multiple nights of nocturnal foraging. When the moon was waxing, it is possible that bees were in the process of learning when and where they could forage at night; by the time the moon was waning they would already know this information and could therefore increase their activity. Alternatively, the increased activity seen during the waning lunar phases could be a result of the delayed moonrise and moonset. While the moon rises before sunset during the waxing phases of the lunar cycle, allowing bees to continue foraging without pause from day to night, it rises continually later during the waning phases of the lunar cycle. Consequently, it sets later during the waning phases of the lunar cycle, allowing bees to forage earlier in the morning before sunrise, which could provide a competitive advantage by allowing bees to forage on floral resource before they have been depleted by competitors. Future studies should further investigate differences in nocturnal activity during the waxing versus waning phases of the lunar cycle, especially if bees appear to be learning over the course of the lunar cycle.

Though nocturnal activity was highly dependent on the illumination provided by the moon, we provide to our knowledge the first evidence that bees were able to forage throughout twilight periods even without the moon's illumination. Even when illumination was recorded to be 0 cd/m^2 (i.e., at the lowest sensitivity limit of the photometer), bees were still actively foraging (Figure 4.4), although it is important to note that some of the activity during the evening twilight period likely included some bees returning to the nest after ceasing foraging for the day. It is possible that the vision of *A. dorsata* workers is more sensitive than the instrument used to record illumination. The photometer used is tuned for human vision, while honey bees have the ability to see farther into the ultraviolet than humans (Menzel & Greggars 1985), making that a distinct possibility. Alternatively, the recording location might have underestimated the light available to foraging bees as recordings could not be taken directly next to the colony due to their nesting position. Regardless of reason for the underestimation, the high activity seen during those twilight periods suggests that the amount of light necessary for *A. dorsata* to be active is lower than previously assumed, as the illumination available during twilight on a night when the moon is not visible is significantly less than what is available when the moon is half full.

One possible reason that *A. dorsata* was able to forage so extensively during the twilight periods is due to anthropogenic light in our study location. As we studied *A. dorsata* in the urban city of Bangalore, this anthropogenic "sky glow" could be providing sufficient illumination for bees to fly during crepuscular and nocturnal time periods, even if our instrument was not sensitive enough to record it. The nocturnal sky brightness in Bangalore is on average 20 to 40 times brighter than the natural nocturnal sky unpolluted by human light; in Bangalore, then, the sky has the same brightness as during nautical twilight and is therefore brighter than during astronomical twilight (Falchi et al. 2016). We found evidence that this high sky glow was sufficient for nighttime foraging even when the moon is not up, as seen by the small amount of foraging activity after astronomical twilight and before moonrise on the nights of the January waning gibbous and third quarter moons (Figure 4.2, Figure S4.1). The presence of sky glow raises the interesting question of how it might impact not just the nocturnal foraging behavior of *A. dorsata*, but also worker mortality. While the sky glow might allow bees to forage on nights that would otherwise be too dark, it could also lead to additional mortality if nocturnally foraging *A. dorsata* workers get 'trapped' by artificial light sources. Mortality via artificial light has been noted as a significant source of mortality for other nocturnally active insects (Owens & Lewis 2018; Owens et al. 2020). We observed numerous *A. dorsata* workers flying repeatedly at and around artificial light sources on the NCBS campus, suggesting that it is a valid concern for this species as well. Artificial light sources might additionally cause mortality by increasing human-bee conflicts when foraging *A. dorsata* workers mistakenly enter peoples' homes at night, drawn by the light. Given the importance of *A. dorsata* as pollinators in India, further investigations into the minimum illumination required for nocturnal foraging from a bee-centric measuring perspective, as well as the effects of anthropogenic light pollution on foraging ability, bee mortality, and human-bee conflict are critical.

We found no evidence that nocturnal foraging on a given night leads to a subsequent change in diurnal foraging. Instead, activity during the day and during twilight remained relatively constant and equal regardless of the amount of nocturnal activity observed (Figures 4.5-4.6). This consistent diurnal and crepuscular activity across the lunar cycle suggests that nocturnal activity is not due to changes in the bees' circadian clock entrainment by increased moonlight, as daily activity patterns would be expected to shift in parallel with the moon phases in that case (Kronfeld-Schor et al. 2013). Instead, these results again support the idea that nocturnal activity is the result of positive masking by environmental illumination that allows bees to extend their temporal niche without shifting their circadian clock (Fernández-Duque et al. 2010; Hut et al. 2012; Kronfeld-Schor et al. 2013).

Although nocturnal arrival rates were generally the lowest among the three time periods studied in each daily cycle, nocturnal arrival rates around the full moon were equal to those seen during the day (Figure 4.5), suggesting that nighttime foraging can contribute a large portion of a colony's daily activity, energy expenditure, and resource collection. The high rates of activity seen across all diel periods suggest that this species could be considered cathemeral (active during both the light and dark portions of the daily cycle), at least during the days of the lunar cycle when the moon is at least half full. However, as the number of colonies followed here was low, additional studies should be conducted to determine how universal high activity during crepuscular periods is, as well as the influence of urban sky glow on nocturnal activity throughout the lunar cycle.

While ambient light availability in the environment seems to be necessary for nocturnal activity, the amount of activity seen during the day, twilight, or night also seems to vary with the seasons. Nocturnal activity was highest in the winter months (Figure 4.7) and particularly high in the winter months of January and February compared to the summer months of March and April (Figure 4.6). This seasonal change in nighttime activity correlates with seasonal variations in resource availability, as many night blooming flower species are in peak season from December to March in southern India (e.g., the *Balsaminaceae* family; Bhaskar & Razi 1974). As we observed colonies during the full moon period every month, light availability must not be the factor restricting bees' nocturnal foraging during autumn or summer; instead, it seems more likely that nocturnal foraging is responsive to the rewards currently available in the environment. Across all seasons, activity during twilight periods was consistently higher than activity during the day or the night (Figure 4.7), supporting the idea that *A. dorsata* is crepuscular and suggesting that resource availability during these diel periods is high regardless of season. In contrast, the higher diurnal activity seen during autumn and summer than during winter suggests that diurnal resource availability was high during these periods. The high activity during summer was surprising because the months of April to June tend to have fewer floral resources than other months in southern India (Singh and Kushwaha 2006; Santhosh and Basavarajappa 2016). However, as most of the resources located within the foraging range of these colonies are cultivated species in the NCBS gardens and botanical garden, the flowering phenology of these plant species might not match that of native forests in the same part of India. In addition, because resources are available throughout the year in India (Singh and Kushwaha 2006; Santhosh and Basavarajappa 2016), it is possible that resources were not limiting in any season. Another possible explanation for the high diurnal activity in summer is that one of the colonies being studied was preparing to migrate, as it abandoned its hive in the beginning of May. Possibly then, the increased diurnal activity might represent both foraging efforts and scouting behavior of bees looking for new nest sites. The seasonal availability of floral resources (diurnal or nocturnal) in urban cities such as Bangalore are virtually unknown but of critical importance to understanding the foraging behavior of pollinator species such as *A. dorsata* that live in these habitats. While it was outside the scope of our study to quantify local resource availability, future studies identifying and quantifying the resources available in Bangalore throughout the year are needed to quantitatively determine the relationship between *A. dorsata's* foraging activity and resource availability.

Although we expected to find an influence of temperature, ambient temperature was not a limiting factor for *A. dorsata's* nocturnal foraging (Figure 4.8). During the 8-month study period, minimum daily temperature varied over a 14˚C range, and never dipped below 10˚C even at night during the colder winter months. Surprisingly, it was on some of the coldest nights in January when *A. dorsata* exhibited their highest nocturnal activity. While thoracic temperature in endothermic insects generally increases with body size, *A. dorsata* workers have a disproportionately low mass-specific metabolic rate which limits their ability to fly in cooler temperatures (Dyer & Seeley 1987; Underwood 1991). However, while 10˚C is on the lower end of *A. dorsata's* reported thermal tolerance, it is still within the normal bounds at which workers can maintain flight (Dyer & Seeley 1987). Thus, temperature did not limit flight at least in part because it simply did not get cold enough to impair the ability to maintain a high enough thoracic temperature to fly. However, the range of *A. dorsata* extends to the northernmost reaches of India (37.1°N), where the temperature can be much cooler than in Bangalore (12.97°N; Ruttner 1988; Smith 2020). An interesting follow-up study would therefore be to examine the nocturnal activity of *A. dorsata* throughout the extent of their range to examine the minimal temperature needed for nocturnal activity.

Our results suggest that *A. dorsata* should not just be considered a diurnal species that can sometimes forage at night, but a cathemeral species when illumination is sufficient to allow for positive masking. We provide support for the idea that nocturnal activity represents legitimate foraging complete with all the hallmarks of diurnal foraging, including recruitment dances and returning to the hive with pollen resources. Daily patterns of activity in *A. dorsata* also seem to be modulated by season, suggesting that seasonal availability of resources is an important determinant of the amount of nocturnal foraging that is conducted even when illumination is sufficient. By studying single colonies over an extended period of time, we provide an initial systematic investigation of the nocturnal foraging behavior of *A. dorsata*, but more research is necessary to draw broad conclusions about the generality of these findings. Future work investigating the nocturnal foraging behavior of *A. dorsata* in other regions and with other colonies is necessary to gain a deep understanding of how nocturnal foraging behavior is modulated in this species, and to understand if and how colonies vary in the extent to which they forage at night. We suggest research efforts also be exerted to gain a better understanding of the resource availability at night versus during the day and across seasons in the range of *A. dorsata* to further clarify the relationship between activity, resource availability, and season.

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APPENDIX

Table S4.1. Model selection for the effects of illumination, census time, and time since sunset on arrivals. Final model selection is highlighted in bold.

Model	AIC	R2	Dispersion	Deviation	Outliers	Standardized Residuals
(Null model) Arrivals ~ 1		0	NS	NS	NS	Good
Arrivals ~ Illumination	**924.55**	**0.30**	**NS**	**NS**	**NS**	**Minor quantile deviations detected**
Arrivals ~ Illumination + CensusTime	926.46	0.30	NS	NS	NS	Major quantile deviations detected
Arrivals ~ Illumination + log(CensusTime)	926.51	0.31	NS	NS	NS	Major quantile deviations detected
Arrivals ~ Illumination + TimeSinceSunset	926.40	0.30	NS	NS	NS	Major quantile deviations detected
Arrivals ~ Illumination + log(TimeSinceSunset)	926.55	0.31	NS	NS	NS	Major quantile deviations detected
Arrivals ~ Illumination + CensusTime + TimeSinceSunset	928.40	0.30	NS	NS	NS	Minor quantile deviations detected
Arrivals ~ Illumination + CensusTime + log(TimeSinceSunset)	928.49	0.31	NS	NS	NS	Major quantile deviations detected
Arrivals ~ Illumination + log(CensusTime) + TimeSinceSunset	927.94	0.33	NS	NS	NS	Major quantile deviations detected
Arrivals ~ Illumination + log(CensusTime) + log(TimeSinceSunset)	928.49	0.31	NS	NS	NS	Major quantile deviations detected

Table S4.2. Model selection for the effects of moon phase, lunar cycle, diel period, and associated interactions on arrivals. Final model selected is highlight in bold.

Model	AIC	R2	Dispersion	Deviation	Outliers	Standardized Residuals
(Null model) Arrivals ~ 1	9366.8	0	NS	NS	NS	Good
Arrivals ~ MoonPhase	9338.3	0.13	NS	NS	NS	Good
Arrivals ~ MoonPhase + TimeOfDay	9133.7	0.57	P=0.00	NS	P=0.003	Major quantile deviations detected
Arrivals ~ MoonPhase + TimeOfDay + MoonPhase*TimeOfDay	8905.1	0.71	NS	P=0.046	NS	Minor quantile deviations detected
Arrivals ~ MoonPhase + TimeOfDay + LunarCycle	9132.8	0.57	P=0.0	NS	NS	Major quantile deviations detected
Arrivals ~ MoonPhase + TimeOfDay + LunarCycle + MoonPhase*TimeOfDay	8906.1	0.71	NS	NS	NS	Major quantile deviations detected
Arrivals ~ MoonPhase + TimeOfDay + LunarCycle + LunarCycle*TimeOfDay	9083.0	0.59	P=0.0	NS	P=0.018	Major quantile deviations detected
Arrivals ~ MoonPhase + TimeOfDay + LunarCycle + MoonPhase*TimeOfDay + LunarCycle*TimeOfDay	**8874.4**	**0.71**	**NS**	**NS**	**NS**	**Minor quantile deviations detected**

Table S4.3. Estimated marginal means for interaction between moon phase and diel period for model of effects of moon phase, lunar cycle, diel period, and interactions on total arrivals.

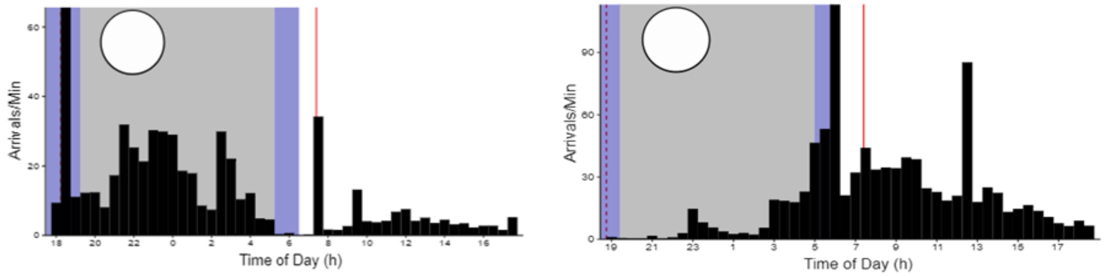
Moon Phase	Diel Period	Estimated Marginal Mean	Standard Error	Df	Lower Confidence Interval	Upper Confidence Interval
First quarter	Day	4.54	0.163	807	4.22	4.85
Full moon	Day	4.87	0.112	807	4.65	5.09
Third quarter	Day	4.84	0.166	807	4.51	5.16
New moon	Day	4.47	0.168	807	4.14	4.80
Waning crescent	Day	4.83	0.160	807	4.51	5.14
Waning gibbous	Day	4.65	0.167	807	4.33	4.98
Waxing crescent	Day	4.53	0.171	807	4.19	4.86
Waxing gibbous	Day	4.90	0.168	807	4.57	5.23
First quarter	Night	2.06	0.188	807	1.69	2.43
Full moon	Night	4.78	0.130	807	4.52	5.03
Third quarter	Night	4.02	0.190	807	3.65	4.39
New moon	Night	1.68	0.193	807	1.30	2.06
Waning crescent	Night	1.48	0.191	807	1.11	1.86
Waning gibbous	Night	5.05	0.183	807	4.69	5.41
Waxing crescent	Night	1.61	0.204	807	1.21	2.01
Waxing gibbous	Night	4.38	0.177	807	4.04	4.73
First quarter	Twilight	5.76	0.329	807	5.12	6.41
Full moon	Twilight	5.80	0.265	807	5.28	6.33
Third quarter	Twilight	5.35	0.317	807	4.72	5.96
New moon	Twilight	5.70	0.315	807	5.08	6.32
Waning crescent	Twilight	5.89	0.345	807	5.21	6.57
Waning gibbous	Twilight	6.61	0.388	807	5.85	7.37
Waxing crescent	Twilight	5.58	0.366	807	4.86	6.30
Waxing gibbous	Twilight	5.59	0.345	807	5.25	6.60

Table S4.4. Model selection for the effects of season and temperature on arrivals. Final model selected is highlighted in bold.

Model	**AIC**	**R2**	**Dispersion**	**Deviation**	**Outliers**	**Standardized Residuals**
(Null model) Arrivals ~ 1	13764	0	NS	NS	NS	Good
(Null model with random intercept) Arrivals ~ 1 + (1|ColonyID)	13737	0.262	NS	P=0	NS	Good
Arrivals ~ Season + (1|ColonyID)	13734	0.169	NS	P=0.01	NS	Good
Arrivals ~ Season + MinTemp + (1|ColonyID)	13731	0.187	NS	P=0.003	NS	Minor Quantile deviations detected
Arrivals ~ Season + MinTemp + TimeOfDay + (1|ColonyID)	13590	0.396	NS	P=0	P=0.001	Major Quantile deviations detected
Arrivals ~ Season + MinTemp + TimeOfDay + Season*TimeOfDay + (1|ColonyID)	**13480**	**0.558**	**NS**	**P=0**	**P=9e-5**	**Major Quantile deviations detected**

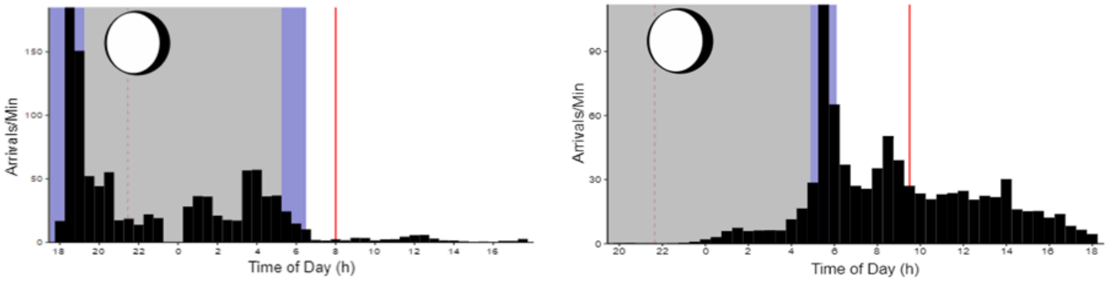
Full Moon – January 21, 2019

Full Moon - March 21, 2019



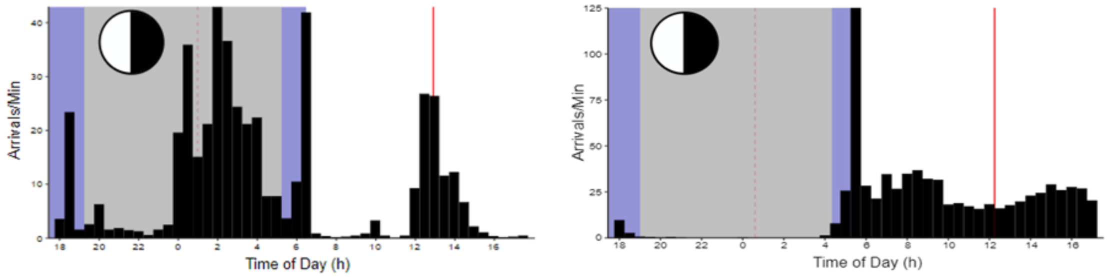
Waning Gibbous Moon – January 24, 2019

Waning Gibbous Moon - March 24, 2019



Third Quarter Moon – January 28, 2019

Third Quarter Moon - March 28, 2019



Waning Crescent Moon – February 1, 2019

Waning Crescent Moon – April 1, 2019

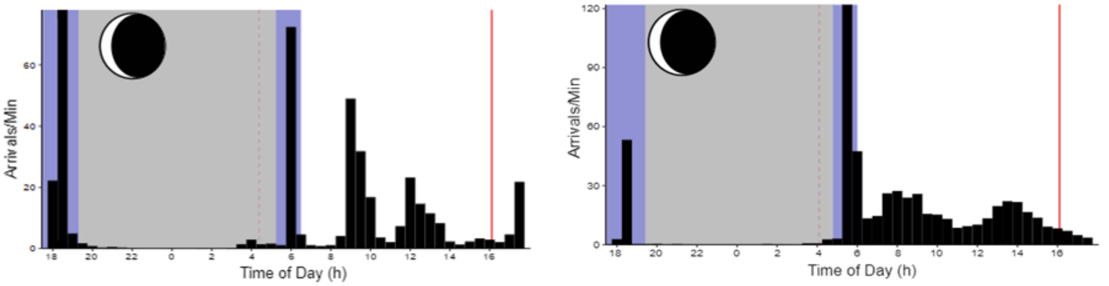
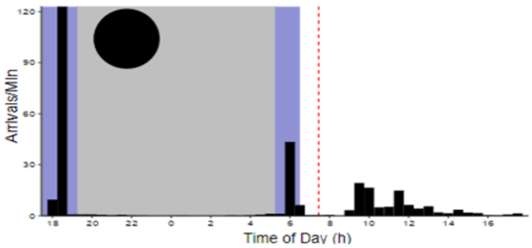


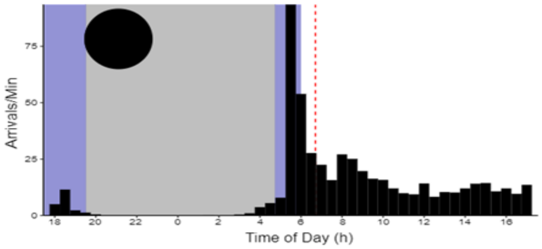
Figure S4.1. Daily foraging activity patterns of *A. dorsata*. Daily foraging activity on each day recorded during the January to February (left) and March to April (right) lunar cycles are shown. Purple shaded regions indicate astronomical twilight, gray shaded regions indicate night, and white indicates daytime. The red dotted line indicated moonrise, while the solid red line indicates moonset. Activity tended to peak during the twilight periods and was only seen at night during the full moons and the weeks before and after them.

Figure S4.1. (cont'd)

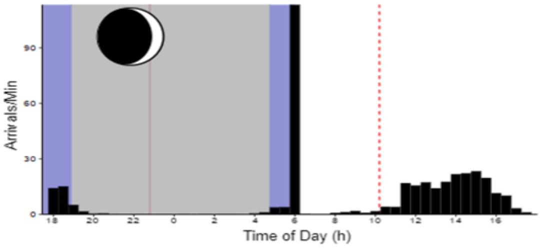
New Moon – February 5, 2019



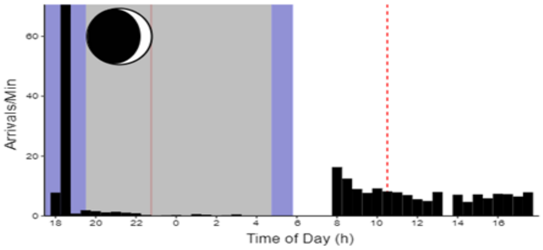
New Moon – April 5, 2019



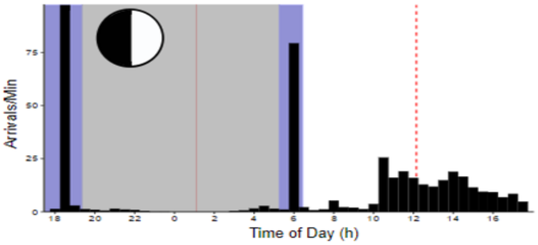
Waxing Crescent Moon – February 11, 2019



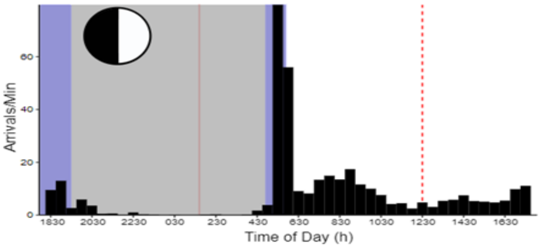
Waxing Crescent Moon – April 10, 2019



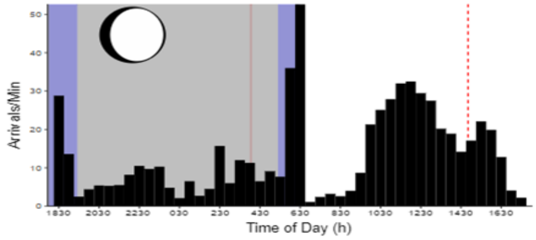
First Quarter Moon – February 13, 2019



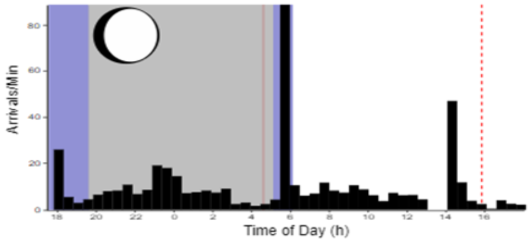
First Quarter Moon – April 11, 2019



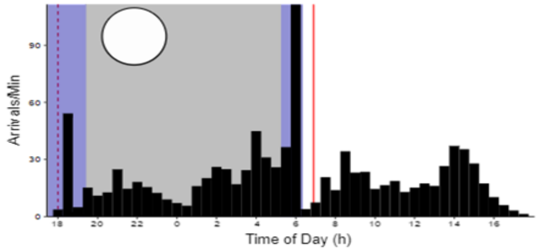
Waxing Gibbous Moon – February 16, 2019



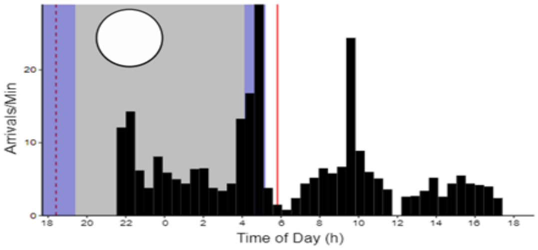
Waxing Gibbous Moon – April 16, 2019



Full Moon – February 19, 2019



Full Moon – April 19, 2019



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