Drinking from the Magic Well: Studies on Honey Bee Foraging, Recruitment, and Sublethal Stress Responses using Waggle Dance Analysis

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Scientific Abstract:

Anthropogenic landscape changes threaten our ecologically and economically critical honey bees by decreasing the availability of quality foraging resources. Importantly, waggle dance analysis provides a versatile and relatively cost-effective tool for investigating the obstacles that honey bees face, such as habitat loss, in our changing landscapes. While this emerging tool has improved our understanding of honey bee foraging in specific landscape contexts, additional research is needed to identify broad trends that span across landscapes. For this dissertation, I used waggle dance decoding and analysis to investigate honey bee foraging, and sublethal stress responses, across three ecologically distinct landscapes in Virginia. In Chapter 1, I introduce waggle dances as a model study system for investigating honey bee foraging and sublethal stress responses by summarizing modern methodological advances in its analysis and emerging research gaps. In Chapter 2, I tested the effects of sublethal imidacloprid exposure on honey bee foraging and recruitment using a semi-field feeder experiment. In doing so, I report that honey bees decreased their foraging, but not recruitment, to an imidacloprid-laced sucrose solution, compared to a control solution. Together, these effects could potentially harm honey bee health by increasing their exposure to pesticides and decreasing their food intake. In Chapter 3, I compared the foraging distances communicated by waggle dancing nectar and pollen foragers across landscapes to explore the economic forces driving foraging to these resources. I observed higher overall and monthly nectar foraging distances compared to pollen foraging distances. Such results suggest that nectar foraging cost dynamics are driven by supply, while pollen foraging cost dynamics are driven

by demand. In Chapter 4, I used waggle dance decoding to map and quantify foraging to agricultural grasslands in a mixed-use landscape. In doing so, I demonstrate that honey bees recruit to agricultural grasslands throughout the season, but that this land type was not more attractive than the broader landscape after correcting for foraging distance, which is a relevant cost that flying bees must consider. Additionally, I qualitatively observe a foraging hot spot, representing high honey bee interest, over a highly heterogenous section of the landscape. The collective results of this chapter identify agricultural grasslands as a potential management target and support the importance of landscape heterogeneity to honey bees/pollinators. In Chapter 5, I used waggle dance decoding to investigate honey bee foraging spatial patterns in the context of optimal foraging theory. In particular, I explore whether co-localized honey bee colonies forage optimally by converging on the same resource patches, or by partitioning the landscape in to distinct foraging territories. Spatial analysis revealed that the colonies widely distributed their foraging at the landscape-scale, with dances from the same and different colonies being similarly distributed, while also establishing distinct, patch-scale, colony-specific, foraging aggregations. Together, these results suggest that the honey bee foraging system produces an emergent foraging pattern that may decrease both within- and among-colony foraging competition. Finally, in Chapter 6, I place my research findings in the context of historical and current trends in honey bee behavioral ecology. Overall, my dissertation improves our understanding of honey bee foraging ecology across landscape contexts using waggle dance analysis, while demonstrating its versatility and effectiveness as a tool for ecologists.

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General Audience Abstract:

Honey bees collect nectar (carbohydrate source) and pollen (protein source) from flowers as their food for survival and reproduction. Human activities, such urbanization, change landscapes and threaten our critically important honey bees by decreasing the availability of flower-rich habitats. Importantly, honey bees share the location of good food sources with their nest mates using a communication behavior called the waggle dance. Interestingly, scientists can estimate the approximate location of the food sources communicated by waggle dancing bees through close observation and cutting-edge analysis. Therefore, we can "decode" honey bees' waggle dances to map their food collection, or foraging, patterns and investigate the obstacles that they face in our changing landscapes. For this dissertation, I used waggle dance decoding and analysis to investigate honey bee foraging across three different landscapes in Virginia. In Chapter 1, I introduce waggle dances as a tool for investigating honey bee behavior by summarizing the modern improvements in its analysis and areas where research is needed. In Chapter 2, I tested the effects of a sublethal exposure to a pesticide, imidacloprid, by observing the foraging and waggle dance behavior of bees visiting feeders with artificial food. I report that honey bees decreased their foraging, but not recruitment, while collecting an imidacloprid-laced sugar solution, compared to a solution without imidacloprid. In Chapter 3, I compared the foraging distances communicated by waggle dancing nectar and pollen foragers across landscapes to explore the economic forces driving foraging to these resources. I observed higher overall and monthly nectar foraging distances compared to pollen foraging distances. Such results suggest that nectar foraging is driven by supply, while pollen foraging is more driven by demand. In Chapter 4, I used waggle dance

decoding to map and quantify foraging to agricultural grasslands (pastures and hay fields) in a landscape characterized by diverse land uses. In doing so, I demonstrate that honey bees recruit to agricultural grasslands throughout the season, but that this land type was not more attractive than the broader landscape after correcting for foraging distance. Additionally, I qualitatively observe a foraging hot spot, representing high honey bee interest, over a highly heterogenous section of the landscape. The collective results of this chapter identify agricultural grasslands as a potential management target and support the importance of landscape heterogeneity to honey bees/pollinators. In Chapter 5, I used waggle dance decoding to investigate the spatial patterns of honey bee foraging in the context of optimal foraging theory, which attempts to explain efficient resource collection strategies. In particular, I explore whether neighboring honey bee colonies forage optimally by converging on the same resource patches, or by dividing the landscape in to distinct foraging territories. We found that colonies distributed their foraging widely at the landscape-scale, with dances locations from the same and different colonies being similarly distributed, while also establishing distinct, patch-scale, colony-specific, foraging areas. Together, these results suggest that honey bees use a foraging strategy that decreases both within- and among-colony foraging competition. Finally, in Chapter 6, I place my research findings in the context of historical and current trends in honey bee behavioral ecology. Overall, my dissertation uses waggle dance analysis to improve our understanding of honey bee foraging behavior, while demonstrating its versatility and effectiveness as a tool for ecologists.

TABLE OF CONTENTS

Chapter 1: Introduction to waggle dance analysis, recent methodological advances, and their applications

The ecological sciences investigate the complex interactions among organisms and between them and their environments. In trying to make sense of ecological phenomena, ecologists are tasked with developing methods that efficiently produce understandable descriptions of the underlying processes, while sufficiently capturing the complexity inherent in them. To do this, scientists often turn to model study systems, which utilize organisms that are ecologically representative, easily manipulated for experimentation, and can demonstrate broad ecological processes that span across diverse taxa (Leonelli & Ankeny 2013). By placing methodological tractability alongside broad scientific utility, the development of the model organism, such as the honey bee, has been a catalyst for research producing broad, generalizable, concepts in biology and ecology (Duffy *et al.* 2021).

Honey bees are a highly eusocial insects from the genus *Apis*, which is native to Eurasia and Africa and consists of nine species (Oldroyd & Wongsiri 2009). They live perennially in large colonies that function using division of labor between the morphologically distinct non-reproductive worker and reproductive queen castes (Winston 1987; Robinson 1992). Worker bees construct the nesting substrate by fashioning excreted wax into double-sided combs consisting of many hexagonal cells for food storage and brood rearing (Winston 1987). Additionally, honey bees are one of the most intensively studied insect model organisms due to the practical benefits of established management practices developed through hobbyist and commercial beekeeping; biological characteristics such as their eusocial nesting (Winston 1987; Yan *et al.* 2014) and generalist foraging strategies (Menzel, Greggers & Hammer 1993); their fully sequenced genome (Sequencing Consortium 2006; Toth & Zayed 2021); and their economic and ecological value as pollinators (Klein *et al.* 2007; Potts *et al.* 2010a; Khalifa *et al.* 2021). Indeed, while these characteristics have contributed to their extensive use as a model organism, their unique and utterly enchanting foraging and recruitment behavior (von Frisch 1967; Seeley 1995), along with emerging experimental and analytical advances for analyzing these behaviors (Couvillon *et al.* 2012; Schürch *et al.* 2013; Schürch *et al.* 2019), provide new and emerging applications for this important model organism (Couvillon & Ratnieks 2015).

In recent years, honey bees have been used extensively as models for studying pesticide risk (Thompson & Maus 2007; Henry *et al.* 2012; Barascou *et al.* 2021), physiology (Maleszka 2014; Wang *et al.* 2018; Zheng *et al.* 2018), cognition (Abramson *et al.* 2013; Abramson, Dinges & Wells 2016; Dinges *et al.* 2017; Howard *et al.* 2018), and the molecular and developmental underpinnings of sociality (Robinson, Grozinger & Whitfield 2005; Page Jr, Rueppell & Amdam 2012; Yan *et al.* 2014; Kohno & Kubo 2019). Additionally, honey bees are attractive models for investigating foraging behavior because they are highly efficient social foragers that survey complex landscapes, and selectively exploit quality resources to meet their nutritional demands (Seeley, Camazine & Sneyd 1991; Seeley 1994; Seeley 1995). Selective exploitation is achieved via gradients of individual- and colony-level foraging responses: honey bees adaptively decide whether and how intensively to forage and recruit (Seeley, Camazine & Sneyd 1991; Seeley 1994; Seeley 1995). Successful honey bee foragers communicate the vector location of quality resources in the landscape to their nestmates using a unique communication behavior called the waggle dance (von Frisch 1967; Seeley 1995; Couvillon 2012; Figure 1). Waggle dancing bees move in a figure-eight pattern consisting of repeated waggle phases,

Figure 1: Honey bee forager performing a waggle dance in an observation colony (*R. Schürch*).

whereby they shake their abdomen while moving linearly across the vertical comb, interspersed by a return phase, during which they circle back to perform another waggle phase (von Frisch 1967; Seeley 1995; Couvillon 2012). Importantly, the angle traveled during the waggle phase relative to the vertical comb communicates to following bees the approximate direction, relative to the sun, to travel to find the food in the landscape, and the duration of the waggle phase communicates approximately how far to travel to find it (von Frisch 1967).

Decades of research into honey bee foraging and recruitment have produced an expansive body of literature comprising a detailed description of the processes and a wealth of methodological and analytical tools for applying knowledge of this system to address real-world issues (Couvillon *et al.* 2012; Schürch *et al.* 2013; Couvillon & Ratnieks 2015; Schürch *et al.* 2019; Markell & Spivak 2021). In particular, waggle dance decoding methods have been developed to extract waggle dance spatial information to map honey bee foraging dynamics (Couvillon *et al.* 2012; Schürch *et al.* 2013; Couvillon & Ratnieks 2015; Schürch *et al.* 2019). The evolution of these methodologies reflects a parallel improvement in our understanding of the underlying behaviors. Some of these advances involve new data abstractions that more accurately reflect honey bee behavior/biology (Schürch *et al.* 2013; Couvillon, Schürch & Ratnieks 2014a; Schürch *et al.* 2019), while others simply make dance decoding and analysis easier (Couvillon *et al.* 2012). For example, early waggle dance analytical techniques that mapped waggle dances as single points (Visscher & Seeley 1982; Schneider 1989; Waddington *et al.* 1994; Beekman & Ratnieks 2000; Steffan-Dewenter & Kuhn 2003; Beekman *et al.* 2004) have been replaced by studies that plot waggle dance locations as probability distributions with dispersion parameters that are consistent with the imprecision inherent in the dance information (Couvillon, Schürch & Ratnieks 2014a; Garbuzov *et al.* 2015; Garbuzov, Schürch & Ratnieks 2015; Balfour & Ratnieks

2017; Sponsler *et al.* 2017; Carr-Markell *et al.* 2020; Samuelson, Schürch & Leadbeater 2022; Silliman *et al.* 2022; Steele *et al.* 2022). Additionally, universal calibration models have replaced landscape-specific calibration models to allow researchers to conduct dance decoding studies more easily in novel landscape contexts (Schürch *et al.* 2019; Markell & Spivak 2021), while efficient dance decoding protocols have decreased the labor input needed to decode dances (Couvillon *et al.* 2012). Finally, statistical methods have been developed to correct for biologically relevant costs, such as foraging distance, which allows researchers to more accurately assess patch attractiveness within landscapes (Couvillon, Schürch & Ratnieks 2014a; Balfour & Ratnieks 2017; Ohlinger et al. submitted).

Honey bees are now considered potential bioindicators of pollinator habitat for various reasons: 1) honey bees are generalist foragers with a maximum forage range of about 12 km (von Frisch 1967), 2) honey bees advertise only the best food sources with waggle dances (von Frisch 1967; Seeley 1995), and 3) scientists can map honey bee foraging patterns using dance decoding (Visscher & Seeley 1982; Beekman & Ratnieks 2000; Beekman *et al.* 2004; Couvillon *et al.* 2012; Schürch *et al.* 2013; Couvillon, Schürch & Ratnieks 2014a; Couvillon, Schürch & Ratnieks 2014b; Couvillon & Ratnieks 2015; Garbuzov *et al.* 2015; Garbuzov, Schürch & Ratnieks 2015; Balfour & Ratnieks 2017; Schürch *et al.* 2019; Carr-Markell *et al.* 2020; Lin *et al.* 2022; Samuelson, Schürch & Leadbeater 2022; Silliman *et al.* 2022; Steele *et al.* 2022; Shackleton *et al.* 2023). Indeed, previous work has demonstrated that waggle dance recruitment is a reliable proxy for honey bee foraging quality (Seeley 1995; Couvillon *et al.* 2015a), and that dance decoding is an effective method for mapping foraging patterns over large areas (Couvillon & Ratnieks 2015). As a result, waggle dance decoding has been applied to monitor temporal trends in resource availability (Couvillon, Schürch & Ratnieks 2014b; Couvillon *et al.* 2015b) and to map spatial patterns in honey bee foraging in various landscape contexts: for example, researchers have used dance decoding to investigate honey bee foraging in agricultural (Garbuzov *et al.* 2015; Danner *et al.* 2016; Balfour & Ratnieks 2017; Lin *et al.* 2022; Silliman *et al.* 2022), urban (Garbuzov, Schürch & Ratnieks 2015; Sponsler *et al.* 2017; Samuelson, Schürch & Leadbeater 2022), and semi-natural (Carr-Markell *et al.* 2020) landscapes. The recent proliferation of waggle dance decoding studies has improved our understanding of how honey bees interact with their foraging environment and provided insights relevant to the fields of pollinator conservation (Couvillon, Schürch & Ratnieks 2014a; Couvillon, Schürch & Ratnieks 2014b; Couvillon & Ratnieks 2015) and agroecology (Garbuzov *et al.* 2015; Balfour & Ratnieks 2017; Lin *et al.* 2022; Silliman *et al.* 2022; Steele *et al.* 2022).

Dance decoding lends itself well to answering basic research questions regarding honey bee foraging economics, which can additionally determine how colonies efficiently meet their nutritional needs in changing foraging environments (Ohlinger *et al.* 2022). For example, waggle dance decoding has been used to investigate the influence of landscape structure (Steffan-Dewenter & Kuhn 2003) and colony size (Beekman *et al.* 2004) on honey bee foraging patterns. Moreover, recent dance decoding research suggests that waggle dances help colonies to forage more efficiently by focusing their foraging on smaller foraging areas and at shorter distances (Shackleton *et al.* 2023). Such studies demonstrate the versatility of waggle dance decoding and analysis as a research tool. Additionally, combining waggle dance analysis with semi-field experimental tools, such as feeder experiments, researchers can observe honey bee foraging and recruitment more closely and under more tightly controlled conditions to test predictions about individual and collective foraging decisions (Seeley 1987; Seeley, Camazine & Sneyd 1991; Seeley 1994; Grüter, Balbuena & Farina 2008; Grüter & Ratnieks 2011; Farina, Grüter & Arenas

2012; Al Toufailia, Grüter & Ratnieks 2013; Grüter, Segers & Ratnieks 2013; Howard *et al.* 2018), and for testing the effect of pesticides and secondary plant compounds on colony functioning (Yang *et al.* 2008; Eiri & Nieh 2012; Couvillon *et al.* 2015a; Tison *et al.* 2016).

The advances in our understanding of how bees use landscapes are critically important given the economic and ecological importance of honey bees (Klein *et al.* 2007; Khalifa *et al.* 2021). Honey bees are capable of increasing the yield of 96% of animal pollinated crops worldwide, making them the most versatile and economically valuable commercial pollinator (Klein *et al.* 2007; Potts *et al.* 2010a). Unfortunately, honey bees face a myriad of stressors that have been implicated in regional population declines (Potts *et al.* 2010b; Smith *et al.* 2013). Honey bees are particularly threatened by habitat loss-induced nutrition stress (Naug 2009; Smart, Otto & Lundgren 2019), which decreases their cognitive functioning (Mattila & Smith 2008) and overwintering survival (Smart *et al.* 2016; Döke *et al.* 2019) and increases their vulnerability to other stressors such as pesticides (Wahl & Ulm 1983; Tosi *et al.* 2017; Tong, Nieh & Tosi 2019), pathogens (Alaux *et al.* 2010; Dolezal & Toth 2018; Castelli *et al.* 2020), and parasites (Dolezal *et al.* 2016). Therefore, scientists must take full advantage of the available tools to address the challenges not only to honey bees and other pollinators, but also to the people and industries that depend on their ecosystems services (Genersch 2010). In particular, additional research is needed to identify broad trends in honey bee foraging, and stress responses, that span across landscape contexts.

In this dissertation, I use waggle dance decoding and analysis to answer both basic and applied research questions related to honey bee foraging: 1) What is the effect of a sublethal dose of imidacloprid on honey bee foraging and recruitment? (Chapter 2), 2) Are honey bee cost dynamics consistent with nectar as a supply-driven and pollen as a demand-driven foraging strategies? (Chapter 3), 3) Do agricultural grasslands provide forage for honey bees in a mixed-use landscape in Virginia? (Chapter 4), and 4) Do neighboring honey bee colonies forage optimally by exploiting the same, high-quality, patches, or by partitioning the landscape into distinct, colony-specific, foraging patches? (Chapter 5). In doing so, my research showcases waggle dance analysis as an effective and versatile tool for pollinator ecologists by demonstrating its potential application for both basic and applied research.

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Chapter 2: Honey Bees (Hymenoptera: Apidae) Decrease Foraging But Not Recruitment After Neonicotinoid Exposure

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Abstract

Honey bees are widely used as commercial pollinators and commonly forage in agricultural and urban landscapes containing neonicotinoid-treated plants. Previous research has demonstrated that honey bees display adverse behavioral and cognitive effects after treatment with sublethal doses of neonicotinoids. In laboratory studies, honey bees simultaneously increase their proportional intake of neonicotinoid-treated solutions and decrease their total solution consumption to some concentrations of certain neonicotinoids. These findings suggest that neonicotinoids might elicit a suboptimal response in honey bees, in which they forage preferentially on foods containing pesticides, effectively increasing their exposure, while also decreasing their total food intake; however, behavioral responses in semi-field and field conditions are less understood. Here we conducted a feeder experiment with freely flying bees to determine the effects of a sublethal, field-realistic concentration of imidacloprid (IMD) on the foraging and recruitment behaviors of honey bees visiting either a control feeder containing a sucrose solution or a treatment feeder containing the same sucrose solution with IMD. We report that IMD treated honey bees foraged less frequently (-28%) and persistently (-66%) than control foragers. Recruitment behaviors (dance frequency and dance propensity) also decreased with IMD, but non-significantly. Our results suggest that neonicotinoids inhibit honey bee foraging, which could potentially decrease food intake and adversely affect colony health.

Introduction

In any agricultural system, pest management approaches, such as chemical control methods, can adversely affect non-target organisms. Neonicotinoids are widely used to control agricultural and household pests and were responsible for 25% of the total global insecticide market in 2012 (Bass et al. 2015). However, recent reports demonstrating the adverse non-target effects of neonicotinoids have galvanized efforts to reduce their use, with the European Union opting to ban neonicotinoids in open field crops in 2018 (Jactel et al. 2019). Such insecticides are highly selective agonists of insect nicotinic acetylcholine receptors, causing overexcitation of the neurons associated with learning and memory (Palmer et al. 2013). Neonicotinoids are applied via various methods such as seed treatments (Krupke et al. 2012, Huseth and Groves 2014), foliar sprays (Kumar et al. 2012), stem application (Kumar et al. 2012) and soil application (Huseth and Groves 2014). Once applied, neonicotinoids are absorbed into the treated plant and then transported throughout its tissues (Bonmatin et al. 2015), where they protect against damage from chewing and sucking insects (Kumar et al. 2012). Neonicotinoids also migrate to the nectar and pollen of flowering plants, where they can then be ingested by beneficial, non-target, insects such as pollinators (Krupke et al. 2012, Long and Krupke 2016). Pollinators are exposed to pesticides while visiting treated crops (Pohorecka et al. 2012, Byrne et al. 2014). Additionally, plants, like wildflowers, growing near treated crops often contain neonicotinoids and provide another route of exposure for pollinating insects (Krupke et al. 2012, Botías et al. 2016, David et al. 2016, Long and Krupke 2016). Overall, the non-target exposure of beneficial insects to pesticides has recently gained the attention of both researchers, who have reported adverse effects on several pollinator taxa (Sandrock et al. 2014, Tan et al. 2015, Arce et al. 2018, Mustard et al. 2020), and the wider public, who are increasingly concerned about the well-documented declines in pollinators (Biesmeijer et al. 2006, Ellis et al. 2010, Potts et al. 2010).

Research into the effects of neonicotinoids on pollinators has often focused on honey bees because of their importance to agriculture (Klein et al. 2007, Aizen and Harder 2009) and their tractability as a research organism (Thompson and Pamminger 2019). Honey bees are the most widely managed, versatile and economically valuable pollinating insects (Klein et al. 2007). Because of their critical use as commercial pollinators (Klein et al. 2007), honey bees are also closely associated with agricultural crops, and they commonly experience chronic exposure to sublethal doses of neonicotinoids (Tsvetkov et al. 2017). The types and severity of effects reported depend on several factors, which we have grouped into extrinsic and behavioral factors. Important extrinsic factors include the duration of exposure (chronic versus acute; (Tosi et al. 2017a)), type of exposure (oral versus topical; (Aliouane et al. 2009)), the dosage (Yang et al. 2008) and co-exposure to other stressors such as poor nutrition (Tosi et al. 2017b) and pathogens (Doublet et al. 2015). Exposure to neonicotinoids can lead to various adverse effects on honey bee behavior (Yang et al. 2008, Eiri and Nieh 2012, Henry et al. 2012, Schneider et al. 2012, Fischer et al. 2014) and cognition (Aliouane et al. 2009, Tan et al. 2015, Wright et al. 2015, Andrione et al. 2016, Mustard et al. 2020). For example, honey bees treated with pesticides take longer to learn olfactory cues (Wright et al. 2015), are less able to discriminate learned odors from novel odors (Mustard et al. 2020) and retain information related to olfactory cues for shorter periods of time (Tan et al. 2015, Wright et al. 2015). Neonicotinoids have also been reported to impair navigational abilities (Fischer et al. 2014, Tison et al. 2016) and to increase rates of homing failure (Henry et al. 2012, Tison et al. 2016). As a result, honey bees treated with pesticides require more time to complete foraging trips (Yang et al. 2008, Schneider et al. 2012), forage less frequently (Yang et al. 2008, Schneider et al. 2012, Tison et al. 2016, Tison et al. 2020) and perform less waggle dances (Eiri and Nieh 2012, Tison et al. 2016, Tison et al. 2020), leading to possible colony-level effects (Henry et al. 2012).

Additionally, behavioral factors of the honey bees themselves, especially in the field, also play a critical role in determining the level of exposure and the severity of the resultant adverse effects. For example, (1) honey bees could forage preferentially and suboptimally on food sources containing neonicotinoids (Kessler et al. 2015), leading to high exposure, by increasing the rate at which they visit and recruit foragers to neonicotinoid treated food sources. (2) Honey bees could also forage indiscriminately and neutrally on foods containing neonicotinoids versus those not containing neonicotinoids. In this case, exposure to pesticides is determined by the relative availability and quality of the accessible plants containing versus those not containing pesticides. Finally, (3) honey bees could forage optimally by actively avoiding food containing pesticides, leading to lower levels of exposure. Given the range of possible responses to neonicotinoids, research looking at the foraging choices of bees between foods containing neonicotinoids and foods not containing neonicotinoids is necessary to more accurately assess exposure and potential risk for honey bees.

Lab studies report that both honey bees and bumble bees might actually prefer sucrose solutions containing field-realistic concentrations of neonicotinoids over control solutions (Kessler et al. 2015, Arce et al. 2018). Additionally, despite consuming more of the neonicotinoid-laced solution, neonicotinoid treated honey bees and bumble bees consumed overall less total solution to some concentrations of certain neonicotinoids (Kessler et al. 2015). These results indicate that particular doses of neonicotinoids elicit preferential feeding, while also inhibiting total foraging activity. Such results are reflected in semi-field studies showing that single doses of neonicotinoid decrease foraging motivation in bumble bees (Lämsä et al. 2018) and sucrose responsiveness in honey bees (Aliouane et al. 2009, Eiri and Nieh 2012, Démares et al. 2016, Démares et al. 2018, Jiang et al. 2018) and field studies showing decreased dancing for natural forage (Tison et al. 2016). Taken together, the above effects could potentially lead to increased pesticide exposure and decreased food intake, a foraging scenario that could harm honey bee colonies.

Although lab-based assays provide important opportunities to measure responses in a controlled treatment, semi-field and field studies are necessary because ecological consequences of a treatment might not be immediately obvious in the lab. Additionally, lab-based studies fail to incorporate the complexities of honey bee foraging and provide only limited insight into the responses of honey bees foraging in the field. Instead, semi-field studies, such as feeder experiments, include additional factors that better simulate a typical honey bee foraging scenario (Couvillon et al. 2015). Most semi-field studies looked at the effects of acute exposure on individual foraging and recruitment behaviors (Yang et al. 2008, Eiri and Nieh 2012, Schneider et al. 2012), with the effects of chronic exposure being less well-studied (Tison et al. 2016, Tison et al. 2020). Therefore, additional investigation into how chronic exposure to field-realistic doses of neonicotinoids affects the foraging choices of honey bees in the field is needed.

In this study, we conducted a feeder experiment with freely flying bees to test for the effect of a sublethal, field-realistic, concentration of the neonicotinoid pesticide, imidacloprid (IMD), on foraging and recruitment behaviors. In doing so, we investigated the individual and colony-level behavioral responses to neonicotinoids in a semi-field context.

Materials and methods

Study organism

We studied sequentially seven queenright honey bee colonies of mixed European race, predominantly *Apis mellifera ligustica*, each with brood and approximately 5000 workers. We studied three colonies in the summer of 2018 and four colonies in the summer of 2019. We housed the colonies in Plexiglas-walled observation hives at the bee field laboratory at the Prices Fork Research Center in Blacksburg, Virginia. The hives were each comprised of three American Standard Deep frames and were connected to the outside via a c. 5 cm × 30 cm plastic tube. We worked with one observation hive at a time (2018: Hive A, B, then C; 2019: Hive D, E, F, then G). Colonies were managed throughout the duration of the project to prevent overcrowding and to standardize the number of empty cells. It is important for there to be space available for additional nectar storage. If not, returning foragers could experience a longer wait time in unloading the sugar solution, which decreases their propensity to recruit via the waggle dance (Seeley 1989, Seeley 1995).

Data were collected from 26 July 2018 - 12 August 2018 and 15 July 2019 - 26 August 2019 on days with good foraging weather. We chose to work in high summer because it is easier to train and to recruit bees to feeders when there is a relative forage dearth in the landscape, which we had heard occurs in late July and August in the study location (R.D. Fell and J.M. Wilson, personal communication). Colonies were not given supplemental food for the duration of the experiment.

Training forager bees to feeders and imidacloprid treatment

Worker honey bees from the observation hives in our study were trained using one of two methods: a "box and jump" (2018) and a step-wise (2019) method. In 2018, foragers were trained using standard procedures ("box and jump"), which has been described extensively in the literature (Al Toufailia et al. 2013, Schürch et al. 2013, Couvillon et al. 2015, Schürch et al. 2019)). Our bee lab is adjacent to an apiary, so we encountered an unexpected challenge of accidentally recruited bees from colonies other than our experimental colonies during the 2018 field season. Therefore, for the second year (2019), we modified our methodology and used step-wise training on the day prior to the experiment (Day-1). In this alternative, we began by training foragers to visit a feeder containing 2M scented (10 µl/L, with lavender in Trial 4, lemongrass in Trial 5, peppermint in Trial 6 and linalool in Trial 7) sucrose solution syrup. The feeders were placed on a tripod (1 m in height) that was positioned approximately 1 m away from the hive entrance tube. We then stepped (10-12 times, approximately 10-15m between steps) the feeder across an adjacent open field to a location 5 m in front of and center to the eventual position of the two experimental feeders ("north" and "south"). The feeder was placed at each successive position until every forager demonstrated that they had learned the current position by visiting the feeder at least three times. Training was conducted by two researchers, one at the feeder and one at the observation hive. The researcher at the feeder marked visiting foragers with numbered plastic discs (Opalithplättchen, Christian Graze KG, Weinstadt-Endersbach, Germany), while the other researcher confirmed whether the marked foragers returned to the colony. To avoid including honey bees from a nearby apiary, we removed the marked foragers that returned to the feeder but failed to return to the observation hive. In each trial, 5–15 foragers were confirmed at the observation hive with this method. We then left the feeder at the final location until at least ten confirmed foragers visited it at least ten times, which usually took until mid or late afternoon on Day -1. The ten visits provided a highly rewarding experience for the foraging bees, making them likely to return to the same feeder the following day (Day 0; (Al Toufailia et al. 2013)).

On the following morning (Day 0), we placed two feeders containing a 2M scented sucrose solution syrup, identical to the solution used on Day -1, side by side on a tripod at the final training location from Day -1. We allowed bees trained from Day-1 to visit the two feeders freely. These foragers were considered “committed” after visiting one of the two feeders at least five times. When several (3-5) committed foragers were observed simultaneously drinking, we carefully moved the feeders, along with the drinking bees, to the experimental feeder locations. One feeder was placed on the “north” tripod and the other was placed on the “south” tripod, and we randomly assigned the color cue background (yellow or blue, alternating between trials). The Day -1 training bees were then allowed to forage and recruit to the experimental feeders freely: their purpose was not to be part of our experimental cohort of bees, but rather to recruit the bees that then would be our experimental bees. Newly recruited foragers (Day 0 foragers) were marked with unique numbered plastic discs at the feeders and their membership to the experimental colony was confirmed at the observation hive. The color of the plastic discs on each Day 0 forager indicated the color of the feeder that they visited and also distinguished them from the Day -1 training foragers. Each visit by the Day 0 foragers was recorded as a Day 0 training visit. Once several Day 0 foragers had begun to visit and recruit consistently, we removed the Day -1 foragers from the experiment.

The two training methods (2018 versus 2019) differed in that the step-wise method required an additional day of training prior to the experiment (Day -1) of bees that were not included in the experiment, while the “box and jump” method only required training on the day of the experiment

(Day 0). Importantly, the Day -1 addition did not affect the experiences of the Day 0 foragers, which are the bees included in the experiment. Our goal was to train 10-20 marked individuals to return reliably to their designated tripod (“north” versus “south”), by 11:30 – 13:30, so that we would have sufficient time to complete the treatment phase. However, in several trials, we were unable to recruit the target number in time (see discussion) and instead began the treatment with a smaller number of bees.

For the treatment phase, we removed the two identical feeders containing 2M scented syrup and replaced them with a new feeder containing 1M unscented solution, either with (treatment) or without (control) IMD (Sigma Aldrich, Reagent Powder). We used a concentration of IMD (100 nM, about 26 ppb) that had previously been shown to generate behavioral effects in the lab (Kessler et al. 2015). In particular, honey bees preferred neonicotinoid laced solutions over control solutions in a two-choice test, with the strongest preference being to solutions containing 26 ppb IMD. Importantly, this concentration has been detected in the nectar of treated plants, such as citrus trees, which are valuable honey producing plants (Byrne et al. 2014), while higher concentrations (60-80 ppb) have been reported in bee-collected pollen from IMD treated cucurbits (Dively and Kamel 2012). Although returning foragers displayed some initial difficulty in orienting towards the new, unscented feeders, they soon began collecting the 1M solution. We chose 1M because we knew, at this time of year for our study location (Schürch et al. 2019) that it would cause good, but not maximum, foraging and recruitment (Seeley 1995), which is important if there is to be an observable effect of treatment on behavior.

Confounding variables of incorrect feeder visits on Day 0

Initially we planned to conduct the entire experiment only in 2018. However, our preliminary analysis of the experiment revealed that some foragers did not forage exclusively on their assigned feeder, either treatment or control, and were therefore exposed to both the IMD solution and the control solution. Specifically, bees made 15.08% of their total foraging visits to the alternative (incorrect) feeder during the 3 h experimental period on Day 0. This behavioral response was unexpected and would therefore alter the amount and type of exposure to IMD that the treatment bees would experience. We decided to rerun the experiment in 2019, with some adjustments aimed to prevent feeder swapping and therefore cross-exposure to the IMD and control solutions: in 2019, we increased the distance between the treatment and control feeders (inter-feeder distance) from two meters to five meters, while still keeping the total distance from the observation hive equal. Despite these adjustments to the experimental design, 7.53% of the visits made by bees during 2019 were still to the alternative (incorrect) feeder (see below for handling issues).

To determine whether the IMD treatment contributed to this unexpected switching behavior, we first assigned the treatment groups according to their trained feeders. The trained feeders were designated as the last feeder that each forager visited during the training phase. We decided on this criterion because of the honey bee's well-documented tendency towards constant foraging, which makes them more likely to visit the same feeder on successive visits than to switch between two different feeders on successive visits (Hill et al. 1997, Grüter et al. 2011). We compared the proportion of total visits that IMD foragers made to their trained feeder (commitment to training feeder) and found that IMD did not affect commitment to the training feeder (mean relative odds: 0.71, 95% CI: [0.33,1.49]). Despite occurring randomly, the switching behavior is a factor that determines the level exposure of individual bees to the treatment and control. We decided to analyze the bees that did not switch at all separately (from here on, the "consistent foragers") because they lacked cross-exposure to the treatment and control solutions and therefore received the treatments as they were described in our initial experimental design. Additionally, the lack of effect of treatment on commitment to the training feeder suggests that the consistent foragers represent a random sample, which is helpful for the experiment because it limits the potential for bias in the subsample.

Data collection – foraging frequency, dance propensity, dance frequency, waggle run frequency, persistency, site specificity

Honey bees are exceptionally sensitive to reward, particularly molarity, and will modulate their behaviors accordingly (von Frisch 1967, Seeley 1995, Couvillon 2012, Couvillon et al. 2015). Therefore, foraging and recruitment reflects how profitable an individual bee has assessed resource quality to be (von Frisch 1967). Of course, the two feeders during the experiment phase possessed identical profitability (1M) and energetic cost (equidistant from the hive), so any differences in the measured behaviors between the treatment and control would indicate an effect of IMD treatment. Once the treatment and control feeders were in place, we counted how many experimental visits an individual bee made to her 1M feeder (either with [treatment] or without [control] IMD) during the 3 h experimental period (foraging frequency). For a visit to be recorded, we used a consistent set of criteria. First, the marked bee must land on the feeder, extend its tongue and drink the solution. Second, visits by individual bees were only counted if three minutes had passed from the previous visit. The second criterion was implemented because foraging bees may visit the feeder without returning to the hive to unload food provisions. Previous studies with similar methods estimated that highly motivated foragers need at least three minutes to travel from the feeder to the colony (145 m) and back to the feeder again (Couvillon et al. 2015).

Concurrent to the recording of foraging frequency, we video recorded waggle dances made by the marked bees within the observation hive during the 3 hr experimental phase. At the start of each experimental phase, a researcher began recording waggle dances with two Canon Vixia HF R82 video cameras, each positioned on either side the glass-walled observation hive. The researcher then focused the view of the camera on the entire area of the frames where waggle dances were observed (typically the bottom two frames of the colony) and adjusted the view of the camera when necessary to ensure that all waggle dances were visible in the video recordings. The videos were recorded to a SanDisk Extreme SD card and then later uploaded to a Google Team Drive (GTD) for analysis. We then converted video files filmed at 30 frames per second to AVI using Ubuntu (v. 2004.2021.222.0) and imported them into ImageJ (version 1.52i) for visual analysis.

The waggle dance is a unique behavior in which a returning bee who is foraging at a profitable food source communicates the distance and direction from the hive to the feeding location (von Frisch 1967, Couvillon 2012). Honey bees, with their exceptional sensitivity to reward quality, are more likely to dance and will dance more if the forager is visiting a highly profitable resource (von Frisch 1967, Seeley 1995, Couvillon et al. 2015). For example, a forager may go back and forth between the hive and the forage site to collect a low or medium valued sucrose solution and still not dance, but she is more likely to begin to dance if the solution is replaced with a higher valued reward (von Frisch 1967, Seeley 1995). Therefore, we monitored the videos for several metrics of the dance. Firstly, we determined the proportion of successful foragers that make any dances at all during the entire 3 hr experimental phase (dance propensity) compared to the foragers that do not dance. Secondly, once a bee had made a dance, we monitored how often she then repeats the dance (i.e. the number of return trips to the hive that she dances, or dance frequency). Finally, because the waggle dance consists of multiple repeated circuits, each containing an information-rich waggle run phase + return phase, we recorded the number of waggle runs that the experimental bees performed per dance. Each of these three behaviors (dance propensity, waggle dance frequency, and waggle run frequency) are expected to increase with a honey bees' perception of food quality and/or adjusted by foraging cost (distance; (Seeley 1994, Seeley 1995, Seeley et al. 2000)). For our experiment, quality and cost were equal, so we monitored these responses to determine the effect of IMD. We allowed the foragers to continue to visit their feeders for the entire 3 hr experimental period, which ended between 14:30 – 16:30. We then removed the tripod, umbrellas and feeders, while carefully noting exactly where each tripod was positioned, so the experimental apparatus could be reassembled in the same location on the following morning for the next phase of the experiment that began on Day 1.

Data collection – persistency and site specificity

Previous research had shown that bees are more persistent (i.e., returning on Day 1 to check if the feeder has become re-rewarding) to a newly unrewarding feeding location if the location had previously been highly rewarding (Al Toufailia et al. 2013), making persistency another honey bee behavior that correlates with the resource quality. Therefore, to determine if there was an effect of IMD on persistency, we set up the tripods and two empty, scent-free feeders on Day 1 at the exact location ("north" versus "south") as Day 0. We then monitored them for 1-2 days with observation periods coinciding with the start and end of Day 0 (≈ 9:00 – 16:30). We aimed to monitor persistency for at least 2 days because our previous research had indicated that these days were the most interesting and informative for these variables (Couvillon et al. 2015). Bad weather shortened our observation period to one day for Trial 4 in Year 2. Persistency was monitored by noting bee number and time of landing on the unrewarding feeder on Day 1-2. Our criterion for counting a persistency visit was that a marked bee should contact the feeder. Simply landing on the tripod or flying near the feeder did not count. Additionally, we surveyed the colonies on the mornings of

Days 1 and 2 to assess mortality. The surveys revealed no significant effect of IMD on mortality on either day (Day 1, mean relative odds = 1.36, 95% CI [0.52,3.49]; Day 2, mean relative odds = 1.11 ,95% CI [0.47,2.69]). During the days following the completion of each trial, we installed a new observation hive at our field site, which we used to house a new experimental colony. We then began the subsequent trial by repeating the training protocol with the new colony.

A bee, upon discovering that the feeder to which she had been trained was now empty, would sometimes remain in the area to investigate the other feeder (e.g., not the tripod/tripod color/feeder where that bee was trained on Day 0). We noted the time and bee ID of these visits to the "incorrect" feeder, using the same criterion as for a persistency visit. Visiting the opposite feeder demonstrated low site specificity (the degree to which honey bees continue to visit a reference feeder), whereas only ever persistently investigating one's own trained feeder shows high site specificity.

We used a different observation hive and training scent for all seven trials. Changing scent reduced the likelihood of attracting robbers from a previously-trained observation hive. Lastly, for each trial we swapped both tripod location (north vs. south) and color of treatment tripod (blue vs. yellow). We tested only three of the four possible 2 x 2 combinations in the 2018 field season. Ideally, we would have continued onto an additional trial, but the blooming of goldenrod, a highly attractive resource for foraging honey bees, made training impossible during the Year 1 field season.

Statistical analysis

The two years' experiments tested the same hypotheses and we increased the distance between the feeders in 2019. However, because we added the second year after the fact, knowing already the broad strokes of the first year's data that we analyzed with Maximum Likelihood Models, we then decided that a Bayesian statistical approach would be most appropriate for our analysis. By using Bayesian models, we were able to generate statistical inferences according to our experimental observations from both years, without pooling the data produced by two slightly different experimental designs. The analyses from the two different statistical paradigms (Maximum Likelihood and Bayesian) yielded similar results. We will present here the results of the (more philosophically correct) Bayesian models, and the Maximum Likelihood Models are available upon request.

The data sets from the two years (2018 + 2019) were assessed together using a Bayesian updating approach, in which the mean parameter values derived from the models of the data from 2018 were used as priors for the models of the data from 2019. Lastly, to address the unexpected issue of foraging bees incorrectly visiting the other feeder (i.e., treatment bees going to control and control bees going to treatment), we decided to include an analysis, which we will call per-protocol, that looked just at "consistent" honey bee foragers, those that visited their correct, trained feeder (either treatment or control) 100% of the time during the 3 h experimental phase (analysis that included all bees, even those that swapped, is referred to as supplementary analysis, see below). We also excluded the data from hive E in the per-protocol analysis: we had begun the experiment the day before and had to stop the trial prematurely. Even though we removed the bees before beginning again, we later considered how the solution collected was likely distributed throughout the colony via unloader bees (Seeley 1989, Seeley 1995) and dance followers (B.D. Ohlinger, personal observation), who might have then visited the feeder on the following day. We report the results of the per-protocol analysis (Control: n = 52, IMD: n = 62) in the main text because those foragers collected either control or treatment under the target experiment conditions (i.e. 100% of the time), while the results of the supplementary analysis (Control: n = 71, IMD: n = 114) can be found in the supplementary information.

All data analysis was done in R 3.6.3 (R Core Team 2020). To determine the effect of IMD on the mean number of experimental visits per bee to the feeders during the 3 h experimental window (foraging frequency), we used Poisson Generalized Linear Mixed Models (GLMMs) with log-link, and with treatment (Control solution versus IMD solution) as a fixed effect and random intercept for hive and for individual bees. Additionally, we used binomial GLMMs with logit-link to model treatment as a fixed effect and with random intercepts for hive and for individual bees to determine the effect of IMD on commitment to the training feeder during day 0. To determine the effect of IMD on recruitment, we first analyzed dance propensity with binomial GLMMs (logit-link) on the proportions of dancing to non-dancing bees per treatment with treatment as a fixed effect and a random intercept for hive. Then we used Poisson GLMMs (log-link) to determine the effect of IMD on the number of dances per bee per treatment (dance frequency), with a random intercept for hive and for individual bees. Additionally, we used Poisson GLMMs (log-link) to determine the effect of IMD on the number of waggle runs per dance per treatment (waggle run frequency), with a random intercept for hive and for individual bees. To determine the effect of IMD on the number of visits per bee per treatment to the empty feeders during Day 1 and Day 2 feeders (persistency), we used Poisson GLMMs (log-link) with treatment as a fixed effect, random intercepts for hive and for individual bees. Finally, we use binomial GLMMs (logit-link) with treatment as a fixed effect and random intercepts for hive and individual bees to determine the effect of IMD on proportion of persistency visits that were made to the trained feeders (site specificity).

The Bayesian statistical analysis was done using the Rstan (Stan Development Team 2020) and Rethinking packages (McElreath 2018) in R 3.6.3 (R Core Team 2020). The ulam function was used to build the models in R and to run Hamiltonian Monte Carlo sampling of the resultant posterior distributions (McElreath 2018). For the Bayesian models, we report the mean and 95% credibility interval for the mean response and the odds ratio (binomial models) and proportional mean difference for the treatment versus the control (Poisson models). We used the position of the posterior distributions for the odds ratio and the proportional mean difference to indicate the type (significant versus non-significant) and direction of the observed effects. Credibility intervals on the response scale containing 1 indicate non-significance, while those distributed entirely above 1 indicate a positive treatment effect and those below 1 indicate a negative treatment effect for Poisson and binomial models.

Results

IMD decreased foraging frequency

There was a significant, negative effect of IMD on foraging frequency (Control: mean = 30.94, 95% CI [15.17,57.20]; IMD: mean = 21.98, 95% CI [10.59,40.59]; proportional mean difference = 0.72, 95% CI [0.51,0.95]). In other words, IMD foragers displayed a 28% [-49 to -5%] decrease in mean foraging frequency compared to control foragers (Figure 1).

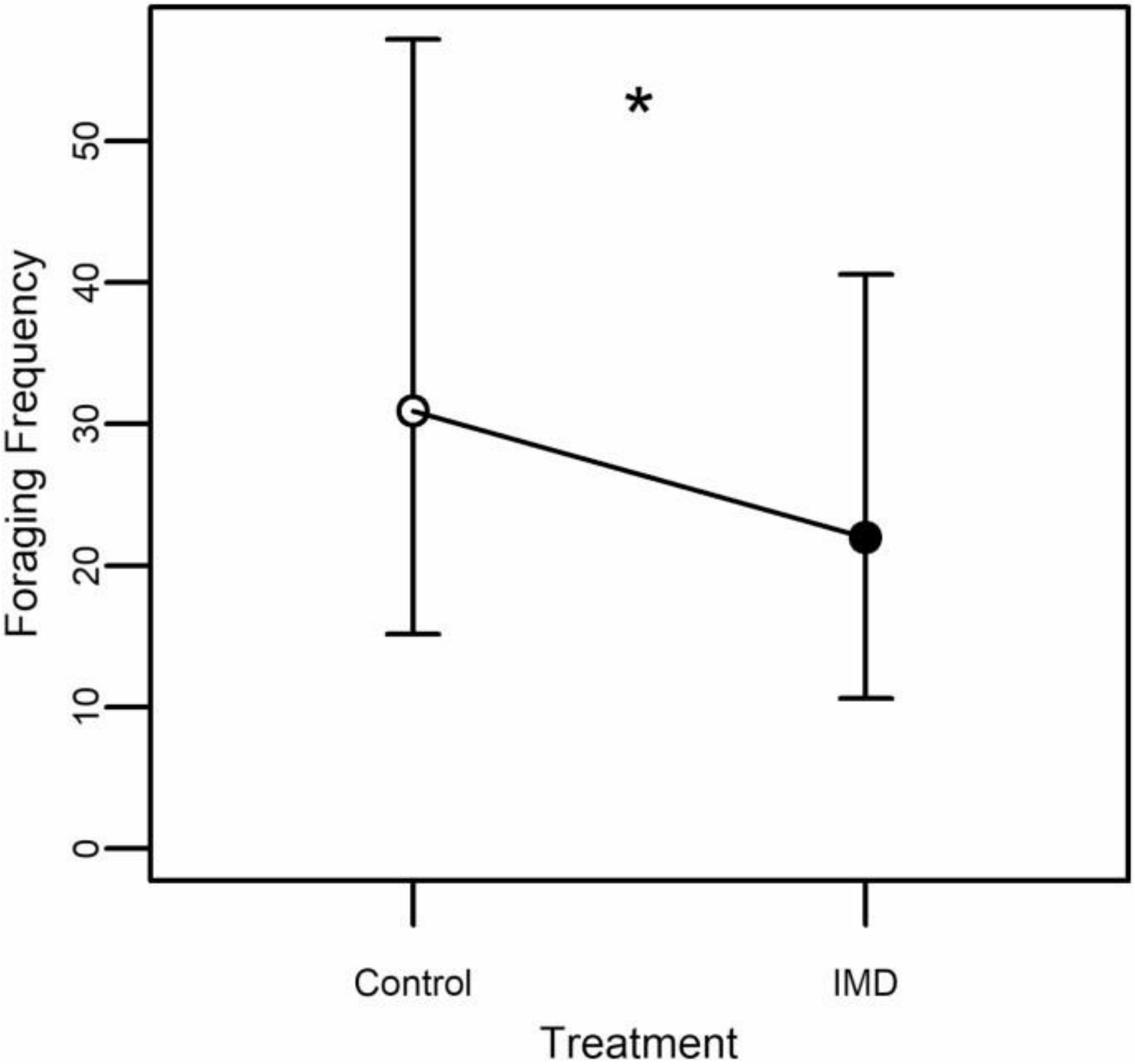

Figure 1: IMD decreased foraging frequency between control and treatment bees during the 3 hour experimental period (*). The vertical line represents the 95% credibility intervals sampled from the posterior for the mean foraging frequency, while the white and black points indicate the mean value from the posterior for the control and IMD foragers, respectively. IMD treated bees foraged c. –28% [–49% to –5%] compared to control bees.

IMD treated foragers displayed consistent numerical decreases in dance propensity, dance frequency and waggle run frequency

IMD treated per protocol foragers displayed a numerical decrease in dance propensity (Control: mean = 0.79, 95% CI [0.56,0.92], IMD: mean = 0.61, 95% CI [0.28,0.87]; mean relative odds: 0.40, 95% CI [0.16,1.02]), with IMD foragers displaying a 60% [-84 to +2%] decrease in their odds of dancing compared to control foragers (Figure 2A). Likewise, IMD treated per protocol foragers displayed a numerical decrease in dance frequency (Control: mean = 11.10, 95% CI [4.46,22.94]; IMD: mean = 7.86, 95% CI [3.54,15.41], proportional mean difference = 0.73, 95% CI: 0.50 to 1.04) with IMD foragers displaying a 27% [-50, +4%] decrease in dance frequency compared to control foragers (Figure 2B). Despite being non-significant, these numerical decreases suggest that IMD foragers tended to be less likely to both dance and, if they did dance, to dance less frequently compared to control foragers across the 3 hr experimental phase. Finally, there was a small numerical decrease in waggle run frequency in IMD treated foragers compared to control foragers (Control: mean = 15.44, 95% CI [9.85,21.43]; IMD: mean = 14.42, 95% CI [8.99,20.26]; proportional mean difference = 0.94, 95% CI [0.74,1.17]) of 6% [-26,+17%] (Figure 2C).

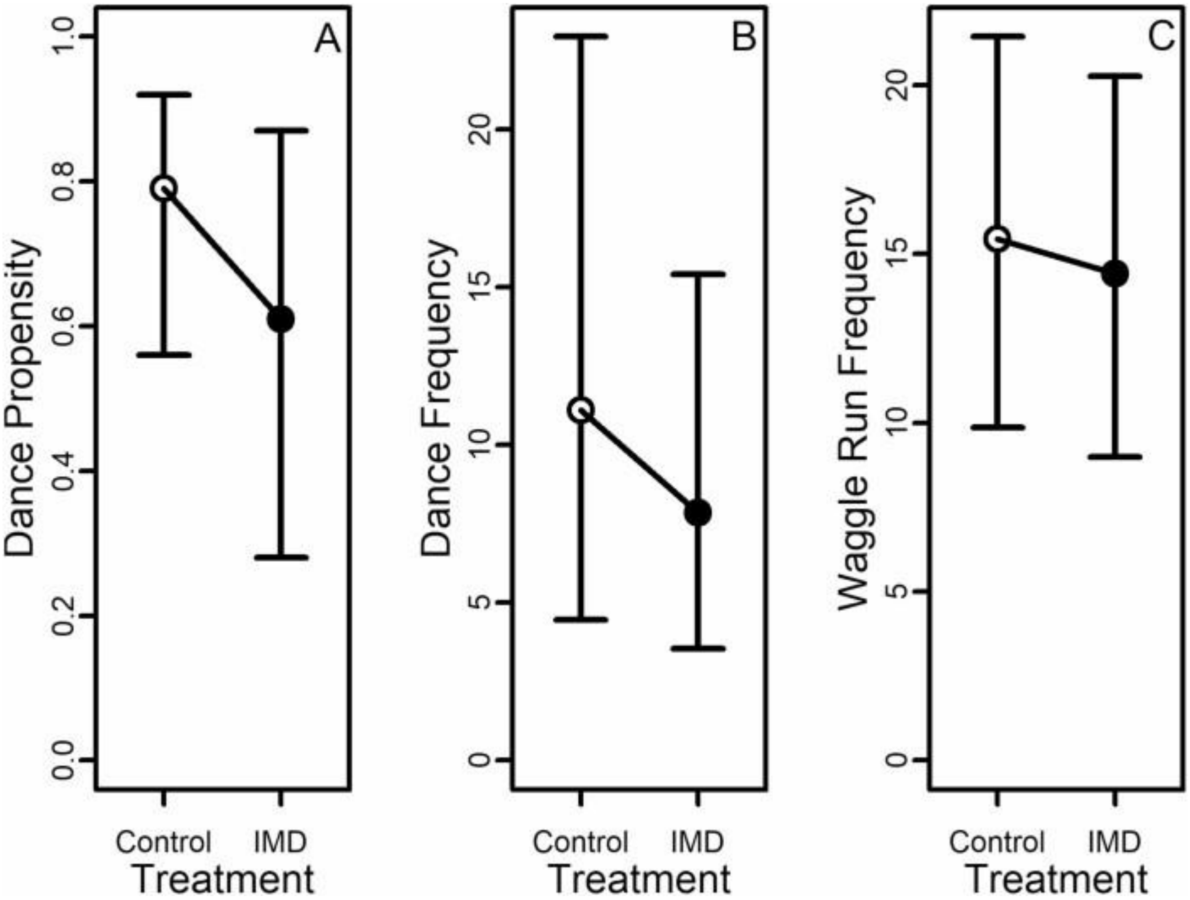

Figure 2: IMD foragers displayed numerical decreases in dance propensity (A), dance frequency (B) and waggle run frequency (C). The vertical lines represent the 95% credibility intervals sampled from the posterior for the mean behavioral responses, while the white and black points indicate the mean values from the posteriors for the control and IMD foragers, respectively. Although these results are nonsignificant, they all demonstrate a decrease in recruitment behaviors with IMD exposure.

IMD decreased foraging persistency on Day 2, but not Day 1

There was a significant, negative effect of IMD on foraging persistency on Day 2 (Control: mean = 0.34, 95% CI [0.05,1.07]; IMD: mean = 0.14, 95% CI [0.02,0.49]; proportional mean difference = 0.44, 95% CI [0.18,0.87]), with the IMD foragers displaying a 66% [-82 to -13%]

decrease in foraging persistency compared to control foragers (Figure 3). In contrast, there was no effect of IMD on foraging persistency on Day 1 (Control: mean = 3.41, 95% CI [1.46,6.13]; IMD: mean = 3.25, 95% CI [1.32,6.19]; proportional mean difference = 0.96, 95% CI: [0.63,1.41], Figure 3).

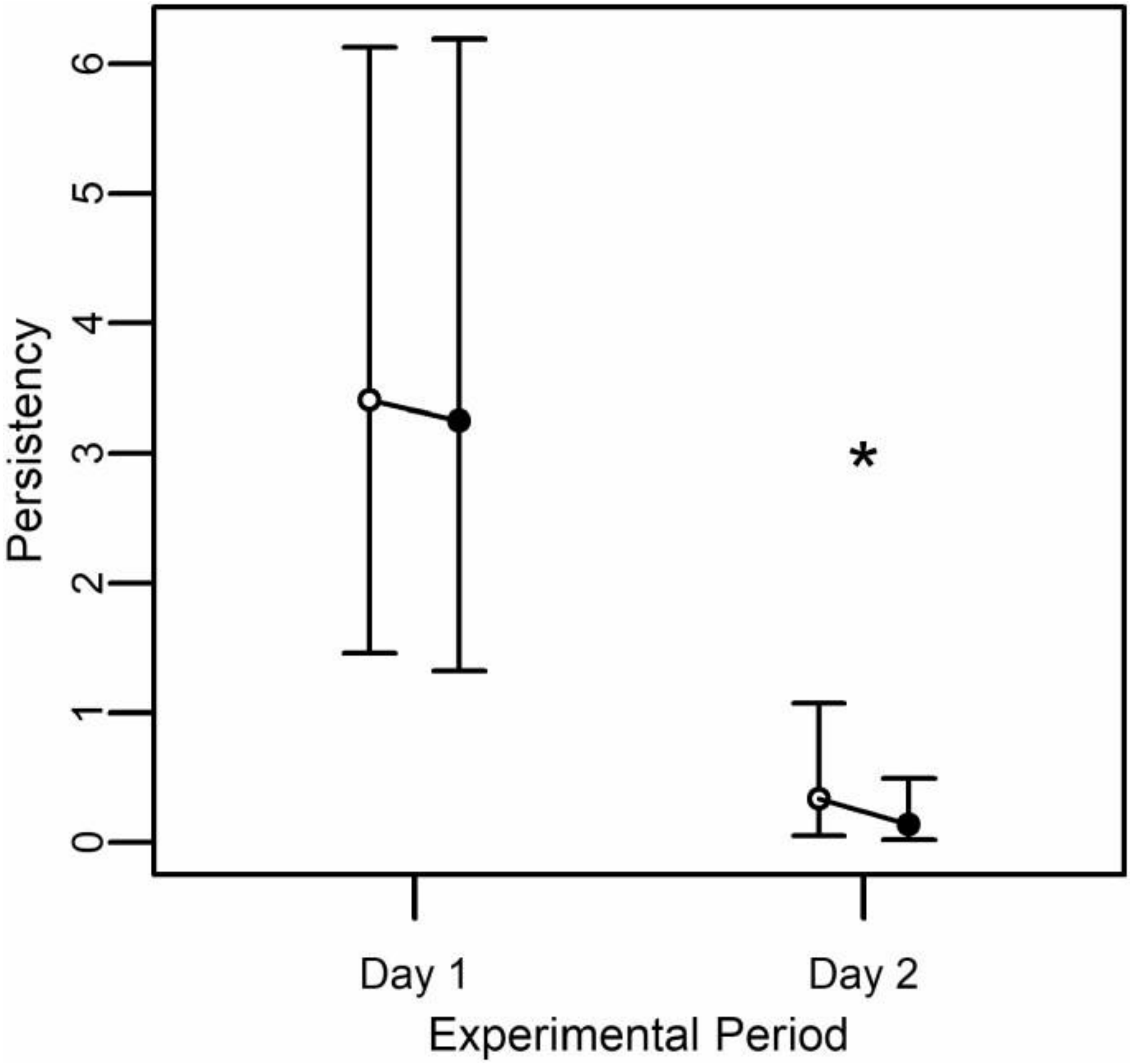

Figure 3: IMD foragers displayed a nonsignificant decrease in persistency on Day 1 and a significant decrease in persistency on Day 2 (*). The vertical lines represent the 95% credibility intervals sampled from the posterior for the mean total persistency visits, while the white and black points indicate the mean values from the posteriors for the control and IMD foragers,

respectively. IMD treated bees were c. –4% [–37 to +41%] less persistent on Day 1 and c. –66% [–82 to –13%] less persistent on Day 2 compared to control foragers.

IMD did not affect site specificity

There was no significant effect of IMD on site specificity on Day 1 (Control: mean = 0.91, 95% CI [0.79,0.96], IMD: mean = 0.90, 95% CI [0.73,0.96]; mean relative odds: 0.88, 95% CI [0.41, 1.89]), or on Day 2 (Control: mean = 0.87, 95% CI [0.73,0.96], IMD: mean = 0.93, 95% CI [0.80,0.99]; mean relative odds: 2.31, 95% CI [0.65,8.81]; Figure 4).

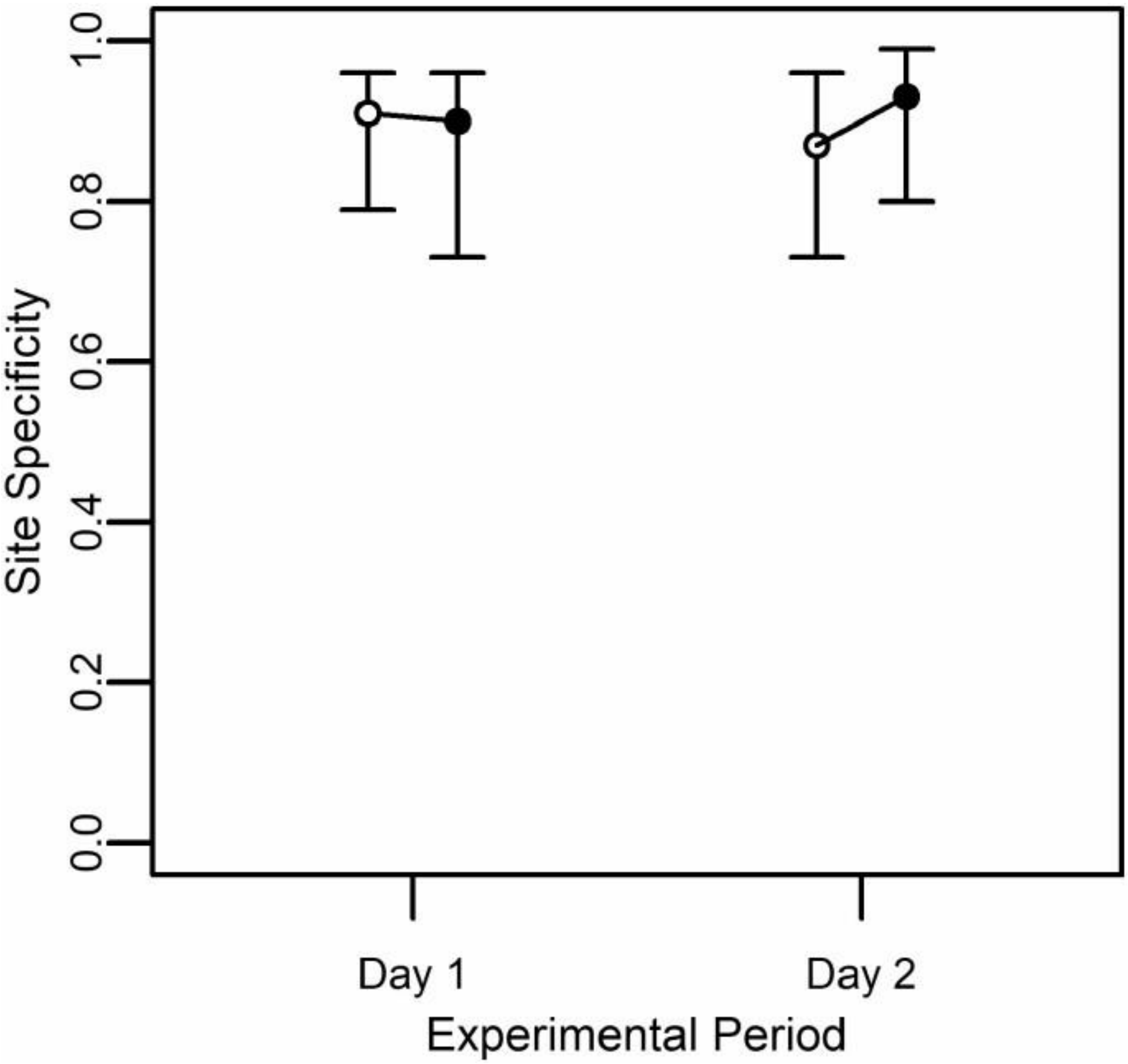

Figure 4: IMD foragers did not affect site specificity on Day 1 or Day 2. The vertical lines represent the 95% credibility intervals sampled from the posterior for the mean total persistency visits, while the white and black points indicate the mean values from the posteriors for the control and IMD foragers, respectively.

Discussion

Here we used freely flying bees in a semi-field feeder experiment to determine the effects of a sublethal, field-realistic, concentration of the neonicotinoid imidacloprid (IMD) on honey bee foraging and recruitment. We report significant effects of IMD on foraging frequency and foraging persistency, and non-significant, but considerable effects on waggle dance propensity, waggle dance frequency and waggle run frequency. In all these latter instances, the data are consistent with IMD decreasing the foraging (Yang et al. 2008, Schneider et al. 2012, Tison et al. 2016, Tison et al. 2020) and recruitment behaviors (Eiri and Nieh 2012, Tison et al. 2016, Tison et al. 2020) in bees, though we cannot completely rule out non-effects. This suggests that IMD causes the honey bee foragers to devalue the reward (Seeley 1994), even though both treatment and control solutions were equal in molarity and equidistant from the lab. These effects were limited to the honey bees that foraged exclusively on the treatment solution versus those that foraged only on the control solution. The observed effects support the hypothesis that honey bees might employ an optimal foraging strategy when exposed to sublethal doses of neonicotinoids, where an organism limits exposure to toxic substances in the field by decreasing their foraging and recruitment activity to them (Easton and Goulson 2013). Additionally, our findings must be considered in the broader context of lab, semi-field and field studies to determine whether these results represent a general decrease in motivation to forage and recruit, which we could call a general inhibition, reduced physical (Williamson et al. 2014), sensory (Andrione et al. 2016) or cognitive abilities (Aliouane et al. 2009, Wright et al. 2015), which we could call foraging impairment, or a specific aversion to neonicotinoid-laced foods, which we could call an adaptive aversion.

Previous research, both laboratory (Eiri and Nieh 2012, Kessler et al. 2015) and semi-field (Yang et al. 2008, Schneider et al. 2012, Tison et al. 2016, Tison et al. 2020) has demonstrated that neonicotinoids inhibit foraging in honey bees. However, Kessler et al. (2015) reported that caged honey bees preferred solution containing a 26 ppb concentration of IMD of control solutions in two-choice assays. Interestingly, Kessler et al. (2015) also report that caged honey bees foraged preferentially on solutions containing a 1000 nM concentration of thiamethoxam or clothianidin over control solutions in the same two-choice assays. Although these bees drank less total solution than those with access to only control solutions. The latter findings suggest that honey bees simultaneous increase their proportional intake of neonicotinoids while decreasing their total solution consumption when exposed to certain concentrations of some neonicotinoids. These seemingly contrasting results probably reflect the differences between their experimental methods and ours: Firstly, our study compares the foraging behavior of honey bees that were trained to visit a treatment or control solution only, while Kessler et al. (2015) compared the choices of honey bees with equal access to treatment and control solutions against those with access to control solutions alone. Therefore, our experimental design was only able to demonstrate the overall decrease in foraging, but neither confirm nor refute the proportional increase in foraging to neonicotinoid treatment. Secondly, we used freely flying foragers in a feeder experiment that more closely simulates the complexities of a typical foraging scenario: our experiment provided a more cognitively and energetically demanding foraging task, in which honey bees were tasked with navigating to and from a feeder positioned 145 m away from their colony. Freely flying foragers might be more susceptible than caged foragers to the reported adverse physiological (Williamson et al. 2014) and cognitive effects (Wright et al. 2015, Mustard et al. 2020), which could have caused the neonicotinoid induced decreases in foraging to occur at lower doses in our semi-field experiment than in the lab (Kessler et al. 2015).

Lab studies provide a highly controlled system that is well-suited for describing the basic behavioral responses of honey bees to pesticides. Common laboratory methods, such as proboscis extension response experiments, have demonstrated that neonicotinoids decrease sucrose responsiveness (Aliouane et al. 2009, Eiri and Nieh 2012, Démares et al. 2016, Démares et al.

2018, Jiang et al. 2018), as well as learning (Tan et al. 2015, Mustard et al. 2020) and memory capabilities (Tan et al. 2015, Wright et al. 2015) in honey bee foragers. These studies provide foundational insights into the behavioral responses of honey bees. However, honey bee foraging is an extraordinarily complex task, requiring a suite of navigational (Menzel et al. 2005), sensory (Balbuena et al. 2012), memory (Menzel and Müller 1996) and learning (Menzel and Müller 1996) capabilities to make optimal foraging decisions. In our study, we used feeder experiments to incorporate the additional variables that honey bees might encounter when freely foraging in a landscape. In doing so, we demonstrate that neonicotinoid-treated honey bee foragers decrease their foraging frequency to rewarding solutions and persistency to newly unrewarding solutions in a semi-field context. These results indicate that the behaviors observed in individual honey bees in the lab are relevant to colony-level foraging and recruitment behaviors in the semi-field.

The decrease in foraging activity is consistent with various other semi-field studies showing that honey bees reduce their foraging (Yang et al. 2008, Schneider et al. 2012) and recruitment (Eiri and Nieh 2012) to even untreated solution after acute exposure to neonicotinoids. Such studies suggest that honey bees respond to neonicotinoids with a general inhibition of foraging (to both treated and untreated food sources), rather than an adaptive aversion to neonicotinoids. Neonicotinoids are often persistent in the environment (Bonmatin et al. 2005), potentially leading to chronic exposure to foraging honey bees. Therefore, the level of exposure and severity of adverse effects from exposure are determined by the behavioral responses of honey bees in the field. Our study elucidates the effects of a 3 hr exposure to sublethal concentration of neonicotinoids on honey bees. In doing so, we demonstrate that honey bees do not avoid neonicotinoid treated foods over the course of a three hr foraging period, but instead reduce their foraging activity. Only a few other studies, most containing low colony-level replication (Tison et al. 2016, Tison et al. 2020), have investigated the behavior of honey bees foraging on feeders containing sublethal doses of neonicotinoids for extended periods of time. These studies report a similar trend for decreased foraging when visiting feeders containing neonicotinoids over several weeks (Tison et al. 2016, Tison et al. 2020). Additionally, the observed decrease in foraging activity is consistent with field studies reporting decreased colony weight gain in colonies located near agricultural lands (Smart et al. 2018), as well as those reporting decreased foraging traffic (Wu-Smart and Spivak 2016), pollen stores (Wu-Smart and Spivak 2016) and decreased colony weight gain (Wood et al. 2018) in colonies fed a similar 20 ppb solution of IMD, inside the hive, over several weeks.

Feeder experiments play an important role in revealing the likely responses of honey bees to different substances, such as neonicotinoids, in the field. Furthermore, understanding these behavioral responses is important for assessing the potential risk of honey bees in urban and agricultural settings, where neonicotinoid exposure is common (Wood et al. 2019). However, it is also important to consider the mechanisms that underlay the observed behavioral effects, which could simultaneously drive the preference for neonicotinoid-laced solutions in the lab (Kessler et al. 2015) and decrease in foraging in the semi-field (Yang et al. 2008, Schneider et al. 2012, Tison et al. 2016, Tison et al. 2020). For example, honey bees could experience neurochemical effects, such as octopaminergic rewards (Barron et al. 2007) or dopaminergic punishments (Klappenbach et al. 2013), in response to a positive (or negative) sensory experience (Linn et al. 2020). Additionally, honey bees could experience pharmacological effects that increase (or decrease) their perception of reward, or affect their cognitive and motor abilities (Williamson et al. 2014). Kessler et al. (2015) used electrophysiological recordings of both sugar-sensing neurons and “bitter”-sensing neurons to determine that honey bees cannot taste neonicotinoids in nectar. Therefore, pharmacological effects likely explain the proportional increase in IMD treated solution consumption in the lab. However, freely-flying bees, like those in our experiment, might experience additional effects, such as decreased navigational (Henry et al. 2012) and flight (Tosi et al. 2017a) abilities that could further decrease their ability to forage efficiently. Taken together, pharmacological effects that decrease foraging motivation and/or capabilities are the most plausible mechanism underlying our observed decreases in honey bee foraging and recruitment activity.

Honey bee foraging and recruitment behaviors are usually highly correlated (Seeley 1995, Couvillon et al. 2015) and should respond similarly to treatment. Honey bees increase their foraging effort on a gradient from individual foraging to lower quality resources (less frequent to more frequent) to individual foraging and colony-level recruitment to higher quality resources (von Frisch 1967, Seeley 1994, Seeley et al. 2000). Feeder experiments have demonstrated that individual foraging adjusts resource exploitation linearly, while recruitment (or diminished recruitment) alters resource exploitation non-linearly (von Frisch 1967, Seeley 1995). Therefore, we expected recruitment to respond more strongly to treatment than foraging frequency. Indeed, we report significant decreases in foraging frequency and Day 2 persistency and only non-significant (but in the same direction) decreases in dance propensity, dance frequency and waggle run frequency. Why could it be that we saw a large decrease in foraging behavior (Figure 1) but only a modest decrease in recruitment behaviors (Figure 2)? Importantly, not every forager performs a waggle dance. Instead, only foragers working the best resources at any given time will dance, and it is possible for a bee to forage back and forth at a resource without recruiting her nestmates (von Frisch 1967, Seeley 1994, Seeley et al. 2000). Such a situation will occur for good but not great resources, and if that resource suddenly decreases in quality, the foraging will likewise decrease, but recruitment, which was already happening at a low level might not display as large an effect. Interestingly, Eiri and Nieh (2012) report the opposite trend for decreased recruitment, but not foraging activity after a treatment with IMD. However, in their experiment, honey bees visited a feeder positioned 1.5 meters away from the colony (Eiri and Nieh 2012), while our bees visited a feeder 145 m from the colony. Clearly, our experiment provides a more challenging task and bees might have simply struggled to navigate efficiently from the colony to the feeder.

Our non-significant (but in a consistent direction) effect on dance propensity and dance frequency might reflect the conservative statistical approach that we took, in which we used the 2018 experimental observations to produce priors for the 2019 analysis, rather than pulling the data from the two field seasons with slightly different experimental methods. Feeder training is difficult because foragers prefer to visit natural forage over artificial resources (von Frisch 1967, Seeley 1995). We selected late July-early August as our study period because we initially thought that time would constitute a nectar gap; however, despite our best efforts, we struggled to train bees in both years. We now know from a different study that we are doing in our lab that forage is actually readily available in those months, making it a non-ideal time for a feeder experiment. Additionally, we experienced the previously mentioned feeder swapping in 12.3% of visits, resulting in cross-exposure to the control and treatment solutions. We addressed this unexpected behavioral response by analyzing just the bees that exclusively visited their trained feeder. Of course, there is always a risk of introducing bias by systematically eliminating data from analyses. For example, we considered the possibility that the 100% consistent foragers represent a physiologically distinct subset within the colony, consisting of bees that are robust against the effects of IMD. However, our analysis of commitment to the training feeder (i.e. incorrect visits during the experimental phase) indicated that the treatment did not affect the odds that a forager would switch or not switch, which gave us reason to believe that the foragers were randomly allocated into the consistent forager subsample.

The most biologically relevant experimental observations are those that measure the responses of organisms under field-realistic conditions. We used a 26 ppb concentration of IMD in our experiment because it is within the range of concentrations found in at least some agricultural settings (Byrne et al. 2014) and had previously been shown to elicit behavioral effects in the lab (Kessler et al. 2015). However, it is important to note that the IMD concentrations found in the field are variable, with a range of possible concentrations being reported (Dively and Kamel 2012, Pohorecka et al. 2012, Byrne et al. 2014, Long and Krupke 2016). For example, Byrne et al. (2014) reported that the concentration of IMD found in citrus nectar depended on field site and lower concentrations were found in the crops of freely foraging honey bees. Additionally, lower concentrations have been reported in bee-collected pollen (Long and Krupke 2016) and bee-collected nectar (Dively and Kamel 2012, Pohorecka et al. 2012), with IMD being nearly undetected in bee-collected pollen in rural landscapes during the summer (Long and Krupke 2016). After examining the range of concentrations reported in different field/experimental contexts and the behavioral responses to a 26 ppb concentration of IMD reported in the lab, we decided that this concentration gave us the best opportunity to both observe behavioral effects and to gain field-relevant insights. However, our results only demonstrate the foraging and recruitment responses of Italian honey bees to a single concentration of IMD, while honey bee stocks vary in their sensitivity to pesticides (Laurino et al. 2013, Rinkevich et al. 2015) and are exposed to various neonicotinoids within a range of concentrations (Dively and Kamel 2012, Pohorecka et al. 2012, Byrne et al. 2014, Long and Krupke 2016). Future studies utilizing honey bees with different genetic backgrounds and/or other neonicotinoids across the broad range of concentrations observed in the field are needed to better assess the risk of neonicotinoids to foraging honey bees.

In summary, we report novel, biologically relevant, data describing the behavioral responses of freely-flying honey bees to a sublethel, field-realistic, concentration of IMD. Our results add to previous research demonstrating that honey bees reduce foraging to neonicotinoid-laced solutions in the lab and foraging after acute exposure in a semi-field context. Unlike several previous studies, honey bees in our experiment were given the option to continuously visit a feeder containing neonicotinoids. We found that they did not actively avoid the treatment, but instead continued to forage and recruit, but at a reduced rate. These results, along with the results of previous studies, suggest that honey bees probably do not employ optimal foraging to avoid pesticides and instead display a general inhibition of foraging to both foods containing pesticides and those not containing pesticides. Such behaviors could possibly lead to an overall decrease in food intake and poorer health outcomes.

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Chapter 3: Dance-communicated distances support nectar foraging as a supply-driven system

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Abstract

Much like human consumers, honey bees adjust their behaviors based on resources' supply and demand. For both, interactions occur in fluctuating conditions. Honey bees weigh the cost of flight against the benefit of nectar and pollen, which are nutritionally distinct resources that serve different purposes: bees collect nectar continuously to build large honey stores for overwintering, but they collect pollen intermittently to build modest stores for brood production periods. Therefore, nectar foraging can be considered a supply-driven process, whereas pollen foraging is demand-driven. Here we compared the foraging distances, communicated by waggle dances and serving as a proxy for cost, for nectar and pollen in three ecologically distinct landscapes in Virginia. We found that honey bees foraged for nectar at distances 14% further than for pollen across all three sites ($n = 6224$ dances, $p < 0.001$). Specific temporal dynamics reveal that monthly nectar foraging occurs at greater distances compared to pollen foraging 85% of the time. Our results strongly suggest that honey bee foraging cost dynamics are consistent with nectar supply-driven and pollen demand-driven processes.

Introduction

In commerce, supply and demand interact to determine the market value of goods and services. Consequently, supply-chains are managed to produce at rates and prices that profitably meet consumer demand (Whelan, Msefer & Chung 2001). Supply/demand ratios modulate the consumer cost dynamics and influence consumer decisions (Whelan, Msefer & Chung 2001). Meanwhile, these processes operate in a fluctuating market. Analogously, the foraging landscape navigated by animals also fluctuates, with dynamic shifts in both supply and demand then modulating food-collection behaviors. Unsurprisingly, many of the terms used in consumer economics have been co-opted to animal foraging ecology (Kagel *et al.* 1975; Seeley 1995).

Honey bees are highly efficient social foragers that can survey complex landscapes, identify attractive resources, and allocate their foraging efforts according to food quality and colony needs by selectively recruiting to the best resources at any given time (Seeley 1987; Seeley 1989; Seeley, Camazine & Sneyd 1991; Camazine 1993; Seeley 1994; Dreller, Page Jr & Fondrk 1999). Recruitment is accomplished via the waggle dance, where a successfully returning forager who has found a good source of food performs a stereotyped behavior that encodes the distance and direction from the hive to the forage (von Frisch 1967; Seeley 1995). Workers that follow a dance can then use the information to find the advertised food (von Frisch 1967; Gould 1975). Lastly, honey bee foragers, like human consumers, respond to supply and demand forces as they collect resources (Seeley 1995).

Honey bees' most important food resources are pollen, a source of protein and lipids that is fed to developing brood, and nectar, a source of carbohydrates that is turned into honey, which is mostly food for adult bees. Honey bees in temperate regions must also, during the foraging season (spring – autumn), create large stockpiles of honey that serve as food for the winter bees that engage in energetically costly thermoregulation (Fahrenholz, Lamprecht & Schricker 1989) and are critical to colony overwintering survival (Seeley & Visscher 1985; Döke *et al.* 2019; Abi-Akar *et al.* 2020). Honey bees therefore are strongly motivated to collect nectar and will continuously do so even if the colony already possesses honey stores (Fewell & Winston 1996). Nectar foraging, therefore, is considered supply-driven because the amount coming into the colony is only limited by its availability in the environment (Seeley 1995). In contrast, pollen foraging is considered demand-driven, where the amount of pollen coming into the hive is also strongly modulated by colony needs because pollen is required when brood is actively being reared (Fewell & Winston 1992; Camazine 1993; Eckert, Winston & Ydenberg 1994; Dreller, Page Jr & Fondrk 1999).

Decoding protocols to analyze honey bee waggle dances recover the distance and direction to the forage as discrete components (Couvillon *et al.* 2012; Schürch *et al.* 2013; Schürch *et al.* 2019). This is useful, as the encoded distance information can act as a proxy for forage availability (Couvillon, Schürch & Ratnieks 2014). Honey bees are economic foragers (Seeley 1994), and flight is costly (Schmid-Hempel, Kacelnik & Houston 1985; Wolf *et al.* 1989), so foragers will only recruit nestmates to resources as far as necessary (Couvillon, Schürch & Ratnieks 2014; Couvillon *et al.* 2015; Garbuzov, Schürch & Ratnieks 2015). In other words, increases in communicated foraging distance indicate decreases in forage (Couvillon, Schürch & Ratnieks 2014; Couvillon *et al.* 2015; Garbuzov, Schürch & Ratnieks 2015).

Although terms like supply and demand have long been applied to bee foraging ecology (Seeley 1995), the cost dynamics in supply versus demand-driven systems remain less explored. Flight distance, as a large cost associated with resource collection, is analogous to consumer prices (Wolf *et al.* 1989; Seeley 1994; Couvillon, Schürch & Ratnieks 2014) and both should respond similarly to fluctuating supply/demand ratios. Supply-driven markets, used by foragers/consumers with continuous resource demand, and demand-driven markets, used by foragers/consumers with intermittent resource demand, should produce distinct consumer/foraging responses. Foragers/consumers are expected to respond more strongly and more consistently to resource availability changes in supply-driven processes than in demand-driven processes. Therefore, one would predict that communicated honey bee foraging distances should be inversely proportional to nectar availability, as nectar collection is considered supply-driven (i.e., honey bees always need nectar). In contrast, honey bee foraging distances should only be inversely proportional to pollen availability when pollen demand is high. Additionally, the pollen dancers' communicated distances should be lower than nectar distances.

Here we investigate whether foraging distances, as communicated by the waggle dances, support supply-driven nectar foraging and demand-driven pollen foraging. We analyzed 6224 waggle dance distances, which reflect cost and are an availability proxy, from bees in three ecologically distinct landscapes in Virginia to determine overall and monthly communicated foraging distance for both nectar and pollen.

Methods

We studied nine predominately *Apis mellifera ligustica* colonies, each consisting of a queen and approximately 5000 workers, at three sites across Virginia, with three hives per site. We housed colonies in glass-walled observation hives composed of three American Standard Deep Langstroth frames. The glass provided an unimpeded view of behaviors, including dances. We maintained the hives indoors at the Prices Fork Research Center (PFRC; 37.21148, -80.48935) in Blacksburg, Virginia, the Tidewater Agricultural Research and Extension Center (TAREC; 36.66447, -76.73278) in Suffolk, Virginia and the Alson H. Smith Jr. Agricultural Research Center (WAREC;

39.11349, -78.28449) in Winchester, Virginia. Foragers were able to enter/exit colonies through a 5 cm x 30 cm PVC piping from the colony entrance to the outside. We provided the colonies with supplemental sucrose solution during times of forage dearth and to maintain consistent food stores. The landscapes surrounding the three sites provided unique ecological contexts: TAREC consisted of row croplands, WAREC of orchard croplands, and PFRC of a mix of residential, agricultural, and semi-natural lands.

We video recorded and decoded waggle dances using an updated protocol developed by Couvillon *et al.* (2012). Briefly, we decoded four waggle runs (information-rich, repeated subunits) per dance to extract run duration, which encodes the distance (von Frisch 1967). We used frame-by-frame playback for videos recorded on 177 days from 13 April – 31 October, 2018, and 10 April – 18 October, 2019. We noted whether the dancer was carrying pollen, which is highly visible in the videos. Although presumably some non-pollen dancers might be recruiting for water, this usually represents <5% of the overall foraging effort (Seeley 1995; Couvillon *et al.* 2015). In all, we decoded 622 dances, with 1931 (Nectar: 1144, Pollen: 787) at PFRC, 2282 (Nectar: 1329, Pollen: 953) at TAREC and 2011 at WAREC (Nectar: 1273, Pollen: 738).

We used the methods reported in Schürch *et al.* (2019) to convert durations into distances by using bootstrap sampling from the universal calibration dataset, consisting of run durations to known distances (Schürch *et al.* 2019) and has been shown to perform well across landscapes and contexts (Carr-Markell & Spivak 2021). The method also reflects the uncertainty inherent in the communication (Couvillon *et al.* 2012; Schürch *et al.* 2013; Schürch *et al.* 2016; Schürch *et al.* 2019). To identify temporal trends in communicated foraging distances, for each dance we simulated the distances 1000 times and then calculated the median simulated distance. Then we determined the effect of month and forage type on distance at three sites by using log transformed linear mixed models from the Lme4 package (Bates *et al.* 2015), with distance as a response variable; month, site, forage type, and the first and second order interactions as fixed effects; and hive as a random effect. We used R 4.1.1 for all analyses (R Core Team 2020) and we obtained the estimated marginal means (EMM) using the emmeans package (Lenth 2022).

Results

Across all the dances (n= 6224), we found a significant effect of the interactions among month, forage type and site (LRT = 81.05, df = 12, $p < 0.001$). We observed a significant effect of forage type on communicated foraging distance, with nectar foragers recruiting significantly further away, 13.9%, relative to pollen (Nectar: EMM = 717.2 m, 95% CI [659.4 m, 779.9 m]; Pollen: EMM = 629.3 m, 95% CI [577.2 m, 685.9 m]; Mean Difference = 87.9 m, 95% CI [58.4 m, 116.1 m], $p < 0.001$; Figure 1). There were some site-specific differences, with communicated nectar distances reflecting the overall result at PFRC (Mean Difference = 125.7 m, 95% CI [64.6 m, 181.5 m], $p < 0.001$) and TAREC (Mean Difference = 115.8 m, 95% CI (75.3 m, 153.5 m], $p < 0.001$). At WAREC, the communicated nectar distance was higher, but not significantly so (Mean Difference = 21.5 m, 95% CI [-33.3 m, 72.2 m], p = 0.422).

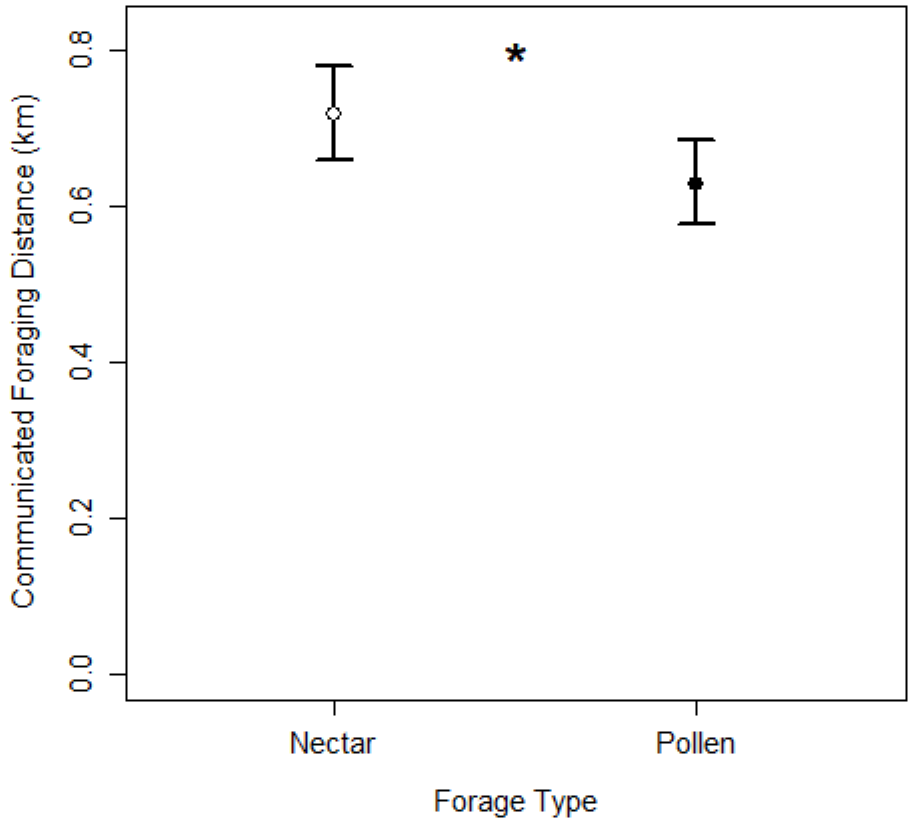

Figure 1: Nectar foraging distances, as communicated by waggle dances, were significantly (*) greater than pollen foraging distances across all three sites (n = 6224 dances). White circles are the EMM for nectar and black circles are the EMM for pollen, with the bars representing the 95% confidence intervals.

In our monthly/site specific investigations, when there were significant differences between monthly communicated foraging distance for nectar versus pollen, communicated nectar distances were higher in 11 of the 13 months, or 84.6% (Figure 2). Specifically, nectar was always higher at PFRC (Figure 2A) and TAREC (Figure 2B). At WAREC, overall nectar distances were significantly higher than pollen, as was seen in May, June, and July; pollen distances were higher in August and October (Figure 2C).

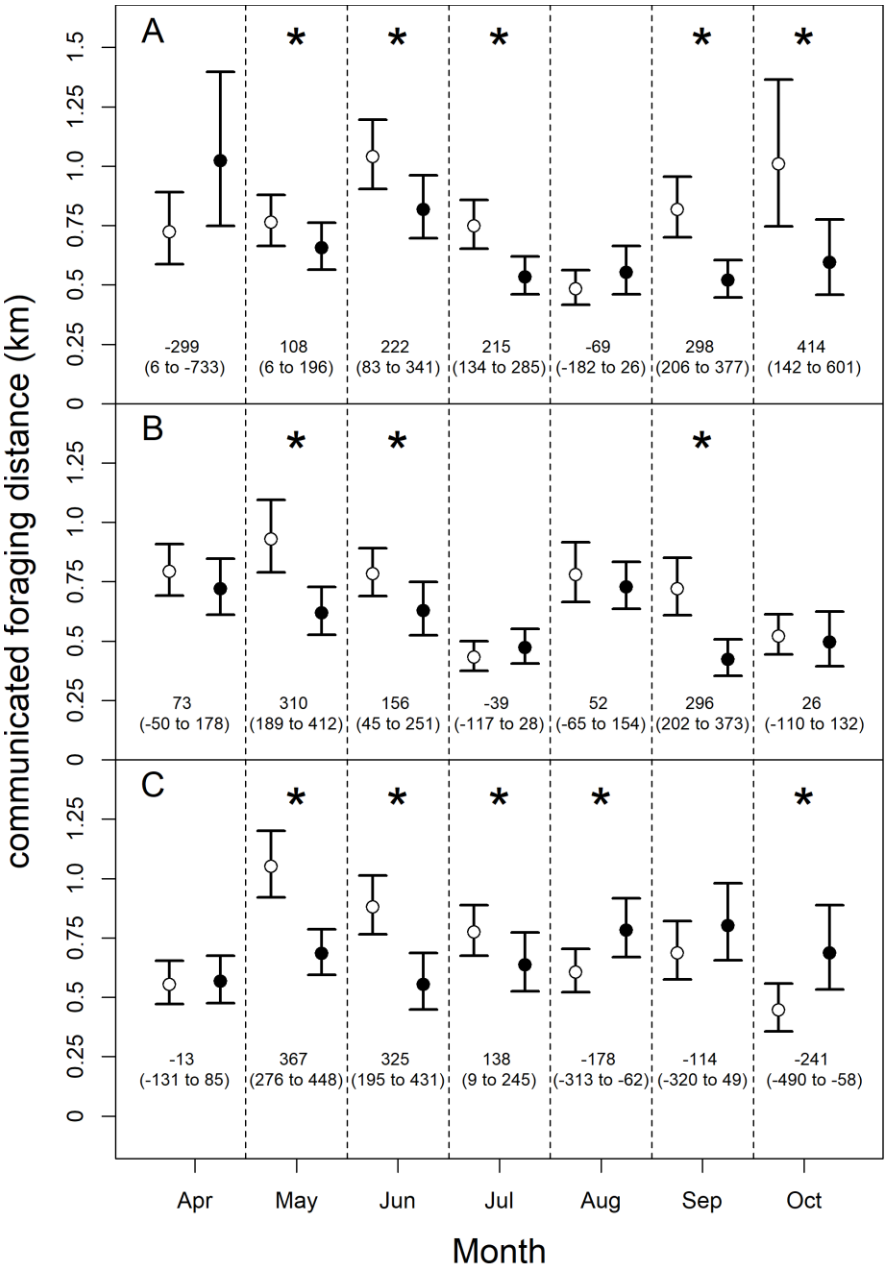

Figure 2: Month and forage type and their interactions affect communicated distance at the three sites: PFRC (A), TAREC (B), and WAREC (C). Significant differences between communicated foraging distance by resource type (nectar = white, pollen = black) is indicated by asterisk (*).

White circles are the EMM for nectar and black circles are the EMM for pollen, with the bars representing the 95% confidence intervals. When a significant difference (*) existed between resources' foraging distance, nectar was greater at PFRC, TAREC, and for three of the five months at WAREC. Mean differences (meters) with 95% CI are reported in the margins.

Discussion

Here we investigated honey bee foraging distance, as communicated by waggle dances, for nectar versus pollen across two foraging seasons in three distinct landscapes. We report that nectar versus pollen foraging distances were higher with our overall, site-specific, and monthly mean analysis. Our results suggest that the cost dynamics of nectar foragers are consistent with a supply-driven scenario, while that of pollen foragers are consistent with a demand-driven scenario.

In their decision to make a waggle dance, foragers weigh the energetic costs of flight against the energetic/nutritional content of food to efficiently meet their colony's nectar and pollen demands in dynamic environments (Seeley 1994). Therefore, honey bees adjust their foraging efforts according to the supply of resources in their environment (Couvillon, Schürch & Ratnieks 2014) and the demand for resources by their colony (Fewell & Winston 1992; Camazine 1993; Seeley 1995; Dreller, Page Jr & Fondrk 1999). The supply of both nectar and pollen varies according to biotic and abiotic factors, such as season (Couvillon *et al.* 2014; Couvillon, Schürch & Ratnieks 2014), competition (Fontaine, Collin & Dajoz 2008), weather (Benedek, Molnár & Nyéki 2000; Hassan *et al.* 2017), and time of day (Corbet 1978).

However, the demand for nectar and pollen differs: honey bees keep modest stores of pollen (Jeffree & Allen 1957; Seeley 1995) and increase pollen collection intermittently during periods of high brood production (Fewell & Winston 1992; Camazine 1993; Dreller, Page Jr & Fondrk

1999; Schmickl & Crailsheim 2004), while honey bees collect nectar continuously to meet their metabolic needs and to build large honey stores to buffer against nectar gaps and provide overwintering food (Seeley 1995; Fewell & Winston 1996). We demonstrate that nectar foragers, compared to pollen foragers, displayed overall higher communicated distances (Figure 1), a result that is likely driven by comparatively low foraging distances for pollen during periods with low pollen demand (i.e., when brood is not being reared). In other words, the colony does not need pollen during times of low demand and, consequently, is less willing to pay the "cost" of a further flight. Lastly, the overall result of higher nectar foraging distances is further supported by our site-specific analyses, which revealed significantly higher nectar distances at PFRC and TAREC and non-significantly higher nectar distances at the WAREC.

Why might WAREC be different? Incidentally, we observed a high number of colony and queen deaths at WAREC in 2019 (n = 2), even compared to 2018 (n = 7). Although the colonies were replaced as soon as possible, there was a small, unavoidable gap. Therefore, the non-significance at WAREC might be due to the high pollen demand in replacement colonies, as they experienced a break and then surge in brood rearing as new queens and/or colonies are introduced. Importantly, nectar distances were in fact significantly higher in 2018 (Mean Difference: 117.9 m, 95% CI [44.1 m, 183.2 m], p = 0.002), but not in 2019 (Mean Difference: -28.4 m, 95% CI [-125.7 m, 57.9 m], p = 0.527).

The temporal dynamics in foraging distance provide additional support for nectar as a supply-driven process: we observed significant differences in nectar and pollen distances in 13 of the 21 site/month combinations, with nectar foraging distances higher in 11 out of the 13 instances (Figure 2). This effect is consistent with Couvillon *et al.* (2015) and Balfour and Ratnieks (2017), who reported that nectar dancers communicated longer foraging distances across two years in a rural and orchard system in England, respectively. Interestingly, some previous studies report either no difference in foraging distance for pollen and nectar (Waddington *et al.* 1994) or longer pollen distances (Steffan-Dewenter & Kuhn 2003). However, these studies used calibration models that relate waggle dance circuit duration (waggle run + return phase) to foraging distances and were completed before recent advancements demonstrating that run duration alone encodes distance, while the return phase duration responds to reward quality (Seeley, Mikheyev & Pagano 2000). Therefore, these studies could be confounded by potential differences between the return phases of nectar and pollen foragers, but this possibility remains uninvestigated.

Pooled communicated foraging distances, where resource type is not distinguished, are commonly used as a proxy for resource availability (Couvillon, Schürch & Ratnieks 2014; Couvillon *et al.* 2015; Garbuzov *et al.* 2015; Garbuzov, Schürch & Ratnieks 2015). Our study therefore fills a need to compare resource-specific trends in communicated distances. Such comparisons are particularly important given the distinct foraging economics of nectar and pollen foragers. The demand-driven economics of pollen foraging suggest that foragers might decrease foraging/recruitment effort when there is low pollen demand, and semi-field experiments show that pollen foragers will switch from low quality pollen sources to high quality nectar sources (Arenas & Kohlmaier 2019). In contrast, nectar-specific trends in communicate foraging, which are likely supply-driven (Seeley 1995), might provide a better indicator of general forage availability.

Although we did not directly test that nectar foraging is a supply-driven process, our results are nonetheless consistent with cost dynamic predictions. Overall, these results suggest that forage-specific waggle dance data can more precisely assess the availability of pollen and nectar in landscapes than aggregated data.

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Author contributions

MJC and BDO conceived the ideas and designed the experiment; BDO, TNS, and MRS collected the data; BDO and RS analyzed the data; BDO and MJC led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication. We declare no conflict of interest.

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Chapter 4: Searching for greener pastures: agricultural grasslands provide forage for honey bees but only when nearby

(currently in review under this title; *Agricultural, Ecosystems, and Environment*)

Abstract

1. Knowledge of foraging currencies and costs is important for understanding honey bee food collection economics and to parameterize their foraging behaviors as indicators of habitat quality, which is important in the identification of management targets in human-altered landscapes.
2. Previous research has yielded inconsistent results regarding the relationship between honey bees and important agroecosystems, such as agricultural grasslands. Waggle dance decoding provides a method for resolving these inconsistencies by mapping and quantifying bee recruitment to agricultural grasslands using statistical methods that appropriately account for foraging distance, or cost.
3. Here we decoded 3881 dances across two foraging years to investigate when and where honey bees forage in a mixed-use landscape in Virginia, with a particular interest in honey bee use of agricultural grasslands (pastures and haylands).
4. We initially observe that bees recruited heavily to agricultural grasslands compared to croplands, developed lands and forests, where the percent foraging to that land type was at 30.9% (CI: 29.5% to 32.3%), and thus significantly higher than its representation in the landscape (c. 23%). Honey bees also recruited heavily to agricultural grasslands across months, with percent foraging ranging from 26.9% (23.5% to 30.1%) in August to 38.8% (31.3% to 46.9%) in October.

5. However, when we examined distance-corrected foraging rates, which allowed us to compare land type attractiveness when flight cost is removed, we found that the agricultural grasslands were not more attractive than the broader landscape and were significantly less attractive than, for example, croplands.
6. We identify potential forage gaps in agricultural grasslands during June and August, while also distinguishing them as a possible source of forage in October before colony overwintering.
7. We qualitatively observe a hotspot, demonstrating high foraging interest that is composed of agricultural grasslands, developed lands, and croplands and is itself a mixed-use area.
8. Together, these results demonstrate that honey bees utilize heterogeneous land areas and underscore the importance of statistical analyses that incorporate biological knowledge. Lastly, these data will be important in informing future management aimed at pollinators in agricultural grasslands.

Keywords: waggle dance, pollinators, foraging, land management, grasslands, pastures, agroecosystems

Introduction

Foraging animals must decide which resources to exploit, a decision explored in optimal foraging theory (Giraldeau 2008a; Pyke & Starr 2021). One recognized prediction arising from the theory is that animals should abandon resources when the exploitation benefits no longer outweigh the costs (Charnov 1976). Whereas solitary animals must rely on their own experience when making foraging decisions, for example how long it takes to find resources in a patch (Pyke 1980), social animals possess the added benefit of learning from others (Giraldeau 2008b; Falcón-Cortés, Boyer & Ramos-Fernández 2019; Pyke & Starr 2021). Honey bees are on the extreme end of the social spectrum: colonies utilize scouts that explore a landscape, find quality resources and then recruit to them via the waggle dance (von Frisch 1967). Each bee, as she decides whether to continue to forage at a resource, considers flight distance as a major cost against the value of the resource in her cost/benefit analysis (Seeley, Camazine & Sneyd 1991; Seeley 1994; Seeley 1995). Then successful foragers also must further decide whether to recruit nestmates through the waggle dance (Seeley 1995).

Because waggle dances are observable, we can infer the bees' assessment of the landscape because bees, by definition, only dance for good resources. Thus, waggle dance decoding provides biologically relevant data for mapping high value honey bee foraging within landscapes (Couvillon & Ratnieks 2015). Recently, dance decoding methodologies have been developed to map and to analyze waggle dances, which can describe honey bee foraging at the resolution necessary to investigate foraging patterns to specific land categories (Couvillon *et al.* 2012; Schürch *et al.* 2013; Couvillon, Schürch & Ratnieks 2014a; Garbuzov *et al.* 2015; Garbuzov, Schürch & Ratnieks 2015; Balfour & Ratnieks 2017; Schürch *et al.* 2019; Carr-Markell *et al.* 2020; Carr-Markell & Spivak 2021; Silliman *et al.* 2022; Steele *et al.* 2022). However, because bees devalue resources that are located farther away, we must consider the flight distance to understand the value of a resource to the bees (Couvillon, Schürch & Ratnieks 2014a). In other words, we need to both determine how much honey bees use specific resources and at what cost they use them before we can assess their value and determine appropriate management action.

Understanding resource values is critical because bees, along with other pollinators, are challenged by the modern landscape. Decreases in quality floral resource availability and the resultant forage deficits leave managed honey bee colonies susceptible to nutritional stress (Smart *et al.* 2016; Smart *et al.* 2017), which can interact with other stressors (Huang 2012; Tosi *et al.*

2017; Dolezal & Toth 2018; Tong, Nieh & Tosi 2019). Landscape composition is therefore an important predictor of individual and population-level health in honey bees (Lecocq *et al.* 2015; Otto *et al.* 2016; Smart *et al.* 2016; Smart *et al.* 2017; Quinlan *et al.* 2021), bumble bees (Goulson *et al.* 2010; Quinlan *et al.* 2021), and wild bees (Kennedy *et al.* 2013; Evans *et al.* 2018). In particular, honey bees display increased colony weight gain (Quinlan *et al.* 2021) and overwintering survival (Smart *et al.* 2016) when herbaceous habitat such as pastures and haylands is widely available. It is therefore surprising that honey bee dance decoding studies to grasslands reported that these were less attractive compared to urban sites (Samuelson, Schürch & Leadbeater 2022), or at least found that herbaceous habitat, both residential and pasture, was not more attractive than the remaining landscape (Richardson *et al.* 2019).

Amidst the above surprising and sometimes conflicting results, it is important to remember that honey bee-relevant floral resources, nectar and pollen, vary across and temporally within landscapes (Couvillon, Schürch & Ratnieks 2014b; Baude *et al.* 2016; Simanonok, Otto & Smart 2020; Ohlinger *et al.* 2022). Therefore, individual landscape characteristics can be beneficial under some conditions, but not others, suggesting that sufficient spatial and temporal resolution is needed to identify forage gaps. Furthermore, we argue that if we properly consider the costs that honey bees use to assess landscape profitability, namely foraging distance, we can resolve some of the previously reported inconsistencies.

Here we decoded, mapped, and analyzed 3881 waggle dances, made by returning honey bee foragers, across two foraging seasons (April – October, 2018-2019) in a mixed-use landscape comprised of lands categorized as either agricultural grasslands, developed lands, forests, or croplands. Our overarching objective was to determine which of these land types, within a heterogenous area, was most attractive to bees. We studied this by 1) assessing percent foraging, as advertised by waggle dances, to agricultural grasslands as compared to developed lands, forests, and croplands; 2) establishing agricultural grassland attractiveness using distance-corrected visitation rates to account for the bees' cost; and 3) examining and describing the most attractive foraging hotspot in our mixed-use landscape.

Materials and methods

Study Organism

We studied three predominately *Apis mellifera ligustica* colonies (labeled "A-C"), each consisting of a queen and approximately 5000 workers. We housed the colonies in glass-walled observation hives, composed of three vertically arranged American Standard Deep Langstroth frames, which allowed us to observe the dances. We installed plumb lines, which were used as a vertical reference for dance decoding (Couvillon, Schürch & Ratnieks 2014a). Each consisted of fishing line weighted at the bottom for vertical alignment. The plumb lines were hung 5 cm apart horizontally and extended vertically down the hive. We maintained the hives inside a building at the Prices Fork Research Center (PFRC; 37.21148,−80.4893) in Blacksburg, Virginia (see below for site description). The building provided stable temperature and diffuse light, which is important for proper dancer orientation (von Frisch 1967). Foragers entered/exited the colony through a 5 cm x 30 cm PVC piping that extended from the entrance of the colony, through the wall, to the outside. We provided supplemental sugar solution as needed after the day's data collection to standardize nutritional status. We practiced standard beekeeping to prevent swarming and to maintain a consistent population size across colonies.

Field Site

The area surrounding our lab is characterized by its diverse landscape composition, which provided an ideal context to assess the relative attractiveness of different land types, especially agricultural grasslands, as foraging resources for honey bees. We mapped the landscape by creating a shapefile of the land composition within 3.25 km of the colony (Figure 1A), a radius chosen because 99% of the dances (n= 3881) from this study advertised within this distance. The surrounding landscape consisted of croplands (c. 4%), developed lands (c. 17%), agricultural grasslands (c. 23%), and forests (c. 55%; see below for more details; Figure 1B). These values are important to note because, if all else is equal, we would expect percent foraging to a particular land type to match its representation in the landscape. Importantly, the four land types were available across the study area (Table 1).

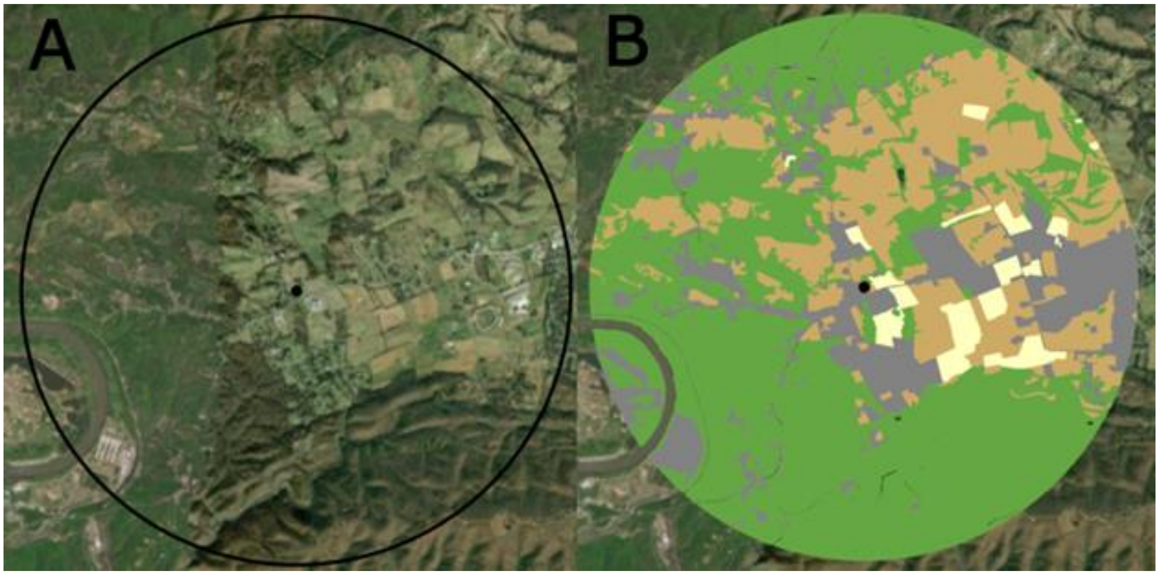
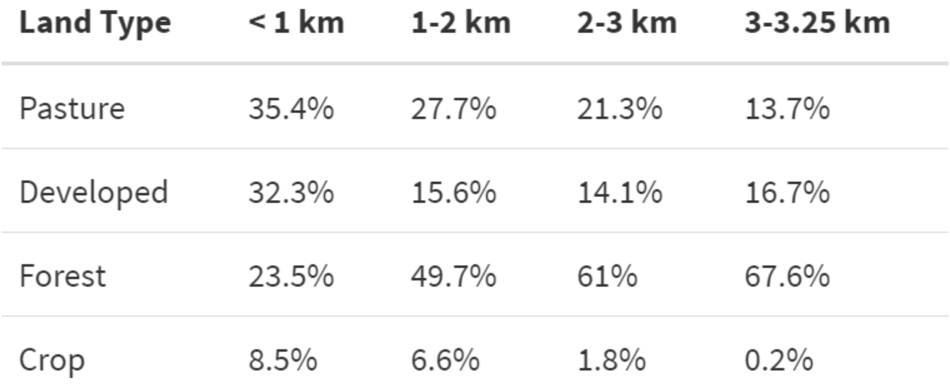


Figure 1: (A) Aerial imagery map showing the 3.25 km study area surrounding the colonies (black point). (B) Map showing the cropland (yellow, c. 3%), developed land (grey, c. 17%), agricultural grassland (c. 23%, tan) and forests (green, c. 55%) land categories within the 3.25 km study area.

Table 1: Prevalence (%) of the land categories across the study radius



Land Type	< 1 km	1-2 km	2-3 km	3-3.25 km
Pasture	35.4%	27.7%	21.3%	13.7%
Developed	32.3%	15.6%	14.1%	16.7%
Forest	23.5%	49.7%	61%	67.6%
Crop	8.5%	6.6%	1.8%	0.2%

Data Collection

We video recorded waggle dances simultaneously from each colony for one hour per day between 9:30 am and 1:30 pm, but usually 10-11am, 3-5 times a week (weather permitting) at 30 fps using a Canon Vixia HF R82. A wood partition encouraged the returning foragers to dance on the video-facing side of the frame. We focused our camera on a 25 cm by 20 cm area near the colony entrance, where most dances occurred (Seeley 1995). Metadata (date, colony ID, time, weather, temperature) were displayed on a datasheet at the video's start. The recording period spanned most of the honey bee foraging season for the study area for two years, with dances being recorded from 13 April – 15 October in 2018 and from 24 April – 4 October 2019. The videos were saved to SD cards and then uploaded to Google Team Drive (GTD).

Data Collection – waggle dance decoding

We later downloaded the waggle dance videos from the GTD, converted them to AVI files using Ubuntu (v. 2004.2021.222.0) and then imported them into ImageJ (version 1.52i) for dance decoding using the protocol developed by Couvillon *et al.* (2012) and modified for our experimental set-up (Ohlinger *et al.* 2022; Silliman *et al.* 2022; Steele *et al.* 2022). At the start of each video, we first determined the angle off-set, as honey bees use gravity to calibrate the angular component of their dances (von Frisch 1967), by measuring the angle of a vertical plumb line. Then we played the video until we saw the first dancing bee, which usually occurred within the first few minutes. We worked through the video by decoding cohorts of simultaneous dancers. To decrease the likelihood of resampling from the same dance, we skipped ahead six minutes in the video after each decoded cohort (Ohlinger *et al.* 2022; Silliman *et al.* 2022; Steele *et al.* 2022).

Dancers were identifiable because of the distinct pattern of movement consisting of consecutive waggle phases, in which successful foragers shake their abdomen from side to side as they run linearly across the comb, followed by a return phase, where the dancer circles back, usually to begin another waggle phase (von Frisch 1967). In decoding, we extracted two pieces of information: the waggle phase duration, which encodes the distance to the food, and an angle relative to vertical, which encodes the direction to the food (von Frisch 1967; Seeley 1995; Couvillon *et al.* 2012). We calculated the waggle phase duration as the difference between the start frame/time and end frame/time per phase. We measured the dance angle by drawing a line along the waggle phase path from the central portion of the thorax at its start and end. We then added this measured angle to the angle offset. Waggle dances consist of 1 – 100+ repeated waggle phases (Seeley 1995). We decoded a subset of four non-first, non-last waggle phases per dance, which has been shown to provide information consistent with averages from entire dances (Couvillon *et al.* 2012), and we then averaged these four phases to obtain a single duration and angle per dance (Schürch *et al.* 2013; Schürch *et al.* 2019). As done previously, we treat each dance as an independent sample (Couvillon *et al.* 2014a; Silliman *et al.* 2022; Steele *et al.* 2022).

Waggle dances are imprecise, consisting of both variation across successive waggle phases from a dance (Couvillon *et al.* 2012) and across dances by different foragers for the same location

(Schürch *et al.* 2016). Therefore, we plotted the foraging location advertised by dances as probability distributions that reflect our uncertainty about the communicated locations (Schürch *et al.* 2013; Schürch *et al.* 2019). We plotted these distributions using Monte Carlo sampling from the universal calibration dataset that performs well across different landscapes and experimental contexts (Schürch *et al.* 2019; Carr-Markell & Spivak 2021). We simulated the angular component of the dance by sampling from a von Mises distribution with a concentration parameter (κ) of 24.5, which is a circular analog to a normal distribution (Schürch *et al.* 2019). Each averaged dance, consisting of the four decoded waggle phases, was then simulated 1000 times.

Landscape Analysis

We used NAIP aerial imagery from 2018, with overlaid CropScape layers (USDA) from 2018 and 2019, to determine our study area landscape composition. We visually inspected the landscape in ArcGIS Pro at the 1:2500 scale and manually drew polygons over landscape patches and categorized them as either croplands (mostly corn), developed lands (low intensity residential and commercial development), forests (mostly broadleaf deciduous), or pastures and haylands, which we call agricultural grasslands. When the aerial imagery did not agree with CropScape, we used the GPS coordinates to ground truth the patches in question.

Analysis of land type use and land type preference

As has been previously done (Silliman *et al.* 2022; Steele *et al.* 2022), to determine the percent foraging to our categories of interest (agricultural grasslands, croplands, forests, and developed lands) by dancing honey bees, we first plotted the simulated dances as points over a map of polygons of the four land categories. We then calculated the proportion of dances advertising a particular category by selecting a single simulated dance location for each observed dance and extracting the land category under each simulated dance location using the over() function from the rgeos R package. Finally, we created confidence intervals for percent foraging to each land category (number of points in category / total number of points in the sample) by repeating this process 1000 times and comparing the proportion of dances advertising each category to the relative percentage of the land categories within the 3.25 km foraging area. Results are presented as percent foraging, plus and minus confidence intervals, per land category. We also used this method to calculate aggregate monthly percent foraging to agricultural grasslands for both years. We used multiple comparison tests with a Bonferroni correction (see code link below) to identify significant differences in simulated total percent foraging to the different land categories and monthly percent foraging to agricultural grasslands. Additionally, we divided the landscape into a grid of 50 m x 50 m tiles and 500 m x 500 m blocks (Figure 2). We later calculated foraging probabilities within each tile to create foraging heat maps. Foraging probability was then used as a response in statistical models (described below), while the blocks were used to correct for spatial autocorrelation among neighboring tiles (Couvillon *et al*. 2014a).

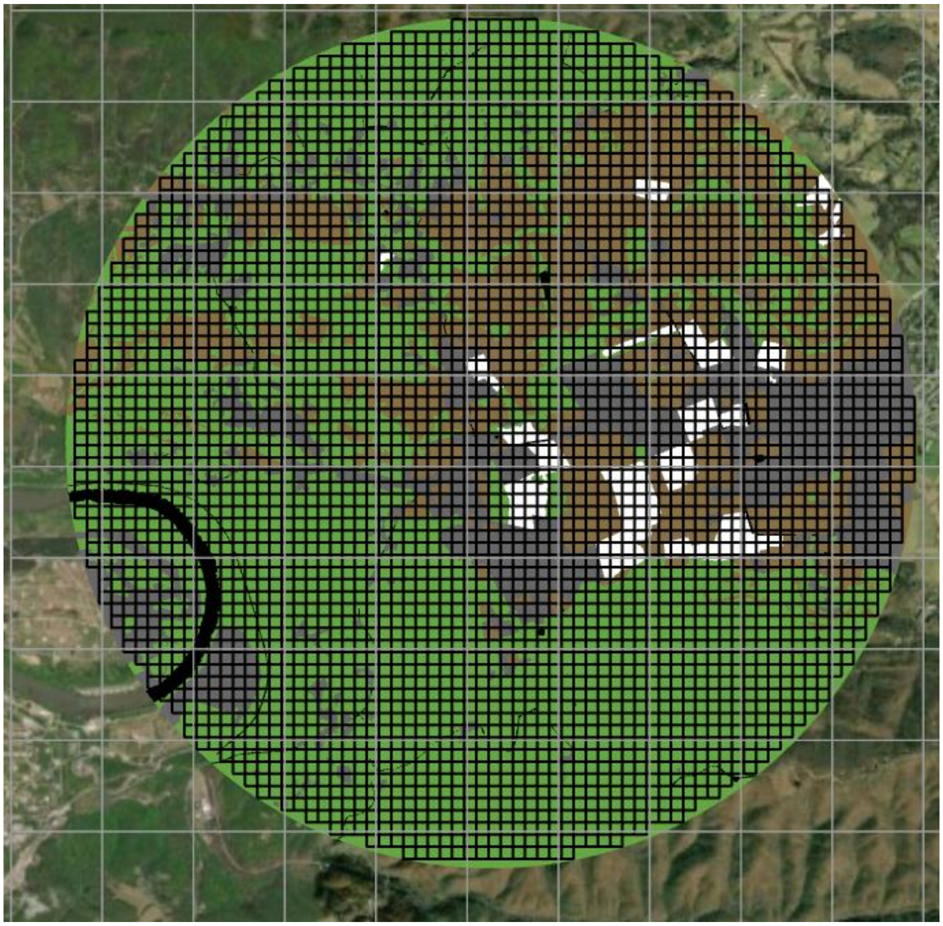


Figure 2: Study area map with overlaid grids of 50 m x 50 m tiles (black) and 500 x 500 m blocks (gray) for our distance and land area corrected models.

Statistical correction for distance in foraging preferences

The above comparison provides only a preliminary assessment of foraging preference for two reasons: firstly, land categories are unevenly distributed across the landscape, and secondly and more importantly, honey bees prefer, because flight is energetically costly, to forage near the hive (Schmid-Hempel, Kacelnik & Houston 1985; Wolf *et al.* 1989; Seeley 1994; Seeley 1995; Couvillon, Schürch & Ratnieks 2014b; Ohlinger *et al.* 2022). Therefore, to better assess honey bee foraging preference, we implement a distance correction, as previously developed by Couvillon, Schürch and Ratnieks (2014a). In this process, we collected 1000 Monte Carlo samples each consisting of a single simulated location for each dance. We then fit each Monte Carlo sample to a binomial generalized linear mixed model with visitation probability to each 50 m x 50 m tile as a response and the presence/absence (1 or 0) of the cropland, developed land, forest and agricultural grassland categories in each tile, and the distance from the colony to each tile centroid (i.e., the distance by which a response is corrected) as fixed effects and block as a random effect. We categorized land types as present if they covered at least 4% of the total area of the tile. We selected the 4% threshold within 50 m x 50 m tiles because it is consistent with the 100 m^2 area covered by individual foragers (Ribbands 1953). In other words, we modeled the effect of the presence or absence of the land categories within the tiles on foraging odds.

We tested for significance of distance-corrected foraging rates in two ways: first, we created confidence intervals of the odds ratios for the presence versus absence of each land category in our model. Confidence intervals consisting entirely of values above or below one indicated that the presence of a particular land category increased or decreased the odds of visitation, while those crossing one indicated non-significance. Second, we used multiple comparison tests with a Bonferroni correction to identify significant difference in the distance corrected-foraging odds across the different land categories. In this case, confidence intervals containing only values above or below one indicated significantly higher or lower foraging odds to the reference category, and those crossing 1 indicated non-significance.

Results

Waggle dance summary statistics

Honey bees communicated a median foraging distance of 705 m (Interquartile Range: 427 to 1027) and maximum foraging distance of 6802 m. Additionally, the median communicated foraging distance varied across months in the aggregated data from both years, ranging from 485 m in April to 1122 m in June.

Honey bees foraged in agricultural grasslands across years and months before distance correction

Before we implemented a distance correction, percent foraging to agricultural grasslands was significantly higher than its representation in the landscape (c. 23%) in 2018 (30.5%, [28.8% to 32.4%]) and 2019 (31.4%, 95% [29.3% to 33.3%]). In aggregate, foraging to agricultural grasslands was maintained in all seven months (Figure 3), suggesting that in our location, this land type provided forage for honey bees across the year. No month stands out as receiving particularly high or low foraging, although there were some fluctuations: percent foraging to agricultural grasslands was highest in October (38.8% [31.3% to 46.9%]) and May (33.6%, [30.9% to 36.1%]) and lowest during August (26.9% [23.5% to 30.1%] and June (27.9%, [24.8% to 31.1%]; Figure 3).

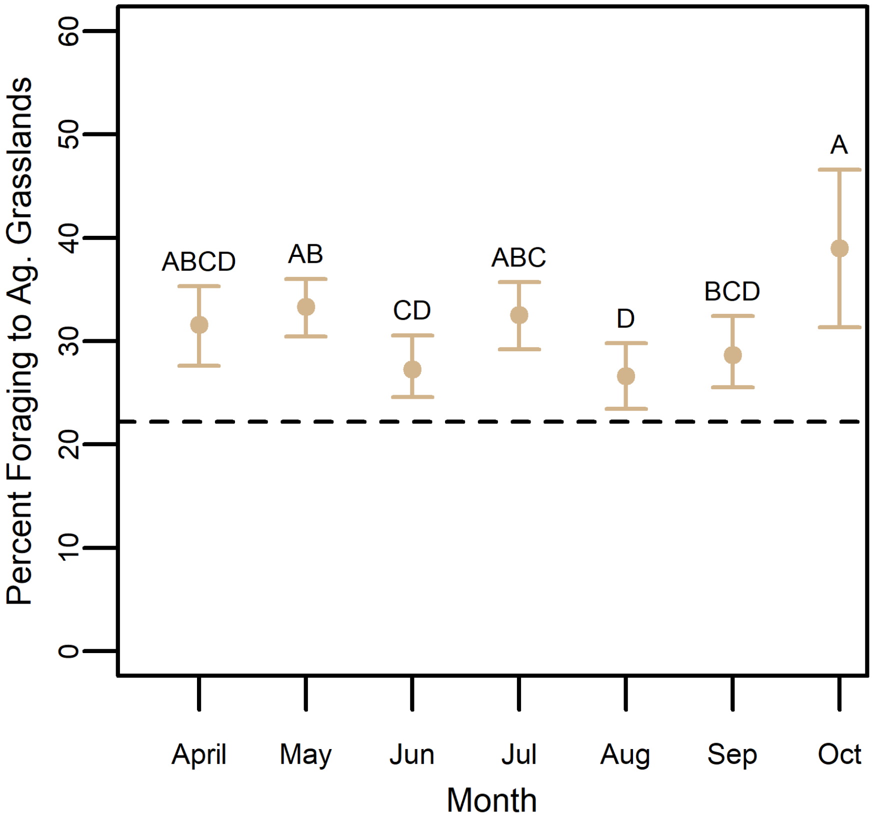


Figure 3: Percent foraging to the agricultural grasslands (tan) was significantly higher than its percentage in the landscape (23.2%, horizontal dashed line) in all seven months spanning the honey bee foraging season from the aggregated, non-distance corrected data. Points symbolize the median simulated percent foraging and the arrows symbolize the 95% confidence intervals. The letters display Bonferroni corrected multiple comparisons of median monthly simulated percent foraging: differences between categories sharing a letter are non-significant, while differences between categories that do not share a letter are significant.

Honey bees recruit to agricultural grasslands more than to developed lands and croplands, but not forests, before distance correction

Before we implemented a distance correction, we found that the proportion of honey bee foraging across two years, as determined by dance decoding, at our study site was 30.9% (29.5% to 32.3%) to agricultural grasslands, 28.2% (26.9% to 29.5%) to forests, 28.2% (27.0% to 29.3%) to developed lands, and 12.1% (11.2% to 12.9%) to croplands (Figure 4). Specifically, foraging to agricultural grasslands was significantly higher compared to both the developed land (Odds Ratio: 1.10, [1.02 to 1.17]) and cropland categories (Odds Ratio: 2.58, [2.36 to 2.86]), but not to forests (Odds Ratio: 1.06, [0.99 to 1.13]). Additionally, there was no significant difference in percent foraging to agricultural grasslands across the two years (Odds Ratio: 0.98, [0.90 to 1.06], Figure 4).

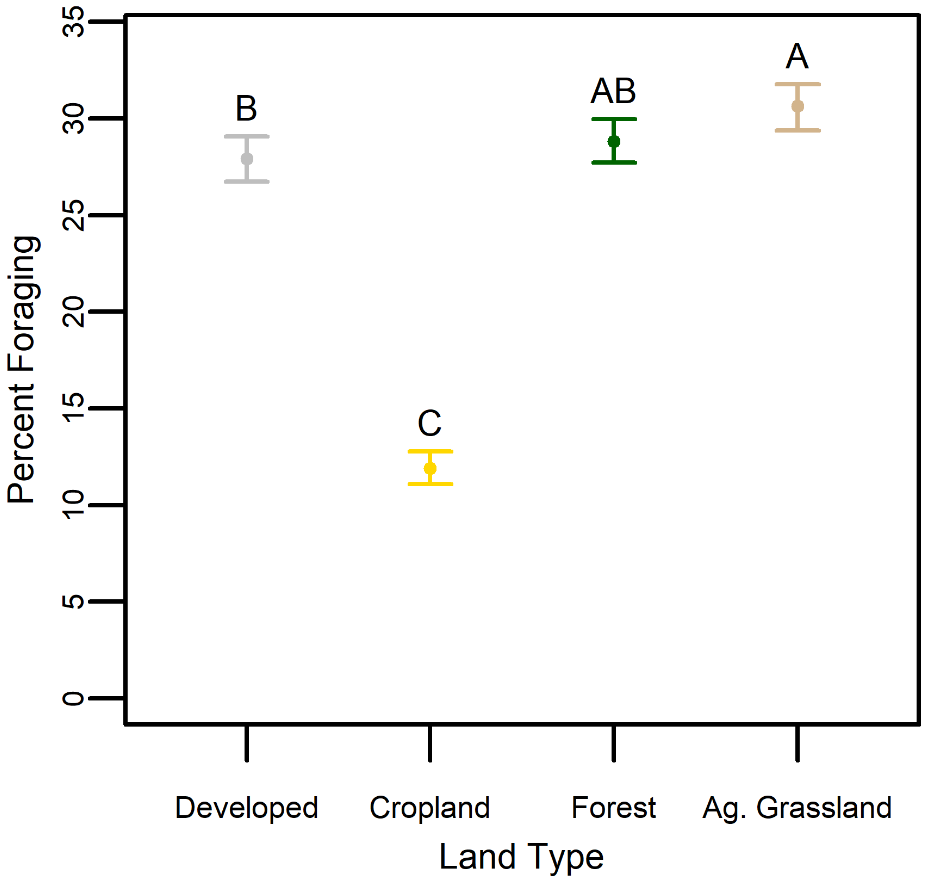


Figure 4. Percent foraging to the agricultural grasslands (tan) was significantly higher than to developed lands (gray) and croplands (yellow), but not forests (green) from the aggregated, non-distance corrected data. Points symbolize the median simulated percent foraging and the arrows display the 95% confidence intervals for simulated percent foraging. Letters display the results of Bonferroni corrected multiple comparisons: differences between categories sharing a letter are non-significant, while differences between categories that do not share a letter are significant.

After a distance correction, the presence of a land category did not affect foraging, and agricultural grasslands received significantly lower foraging than croplands

After correcting for distance, the presence of the land categories did not affect foraging to the tiles (Odds-Ratios: cropland = 1.08, [0.98 to 1.18]; developed = 0.98, [0.91 to 1.07]; forests = 0.98, [0.90 to 1.06]; agricultural grasslands = Odds-Ratio: 0.95, [0.88 to 1.03]; Figure 4). In other words, the presence or absence of a particular land category, including agricultural grasslands, did not significantly decrease or increase the likelihood that a honey bee would recruit to that area, given the distance, as indicated by all categories' confidence intervals crossing the "1" line (Figure 5). In fact, when we compare across land categories, we found that agricultural grasslands were in fact associated with a decreased honey bee recruitment trend, albeit non-significant, and tiles containing croplands received significantly higher distance-corrected foraging compared to those containing agricultural grasslands (Odds Ratio: 1.07, [1.01 to 1.12], Figure 5, see post hoc letters A versus B). In general, distance significantly decreased foraging rates to the tiles.

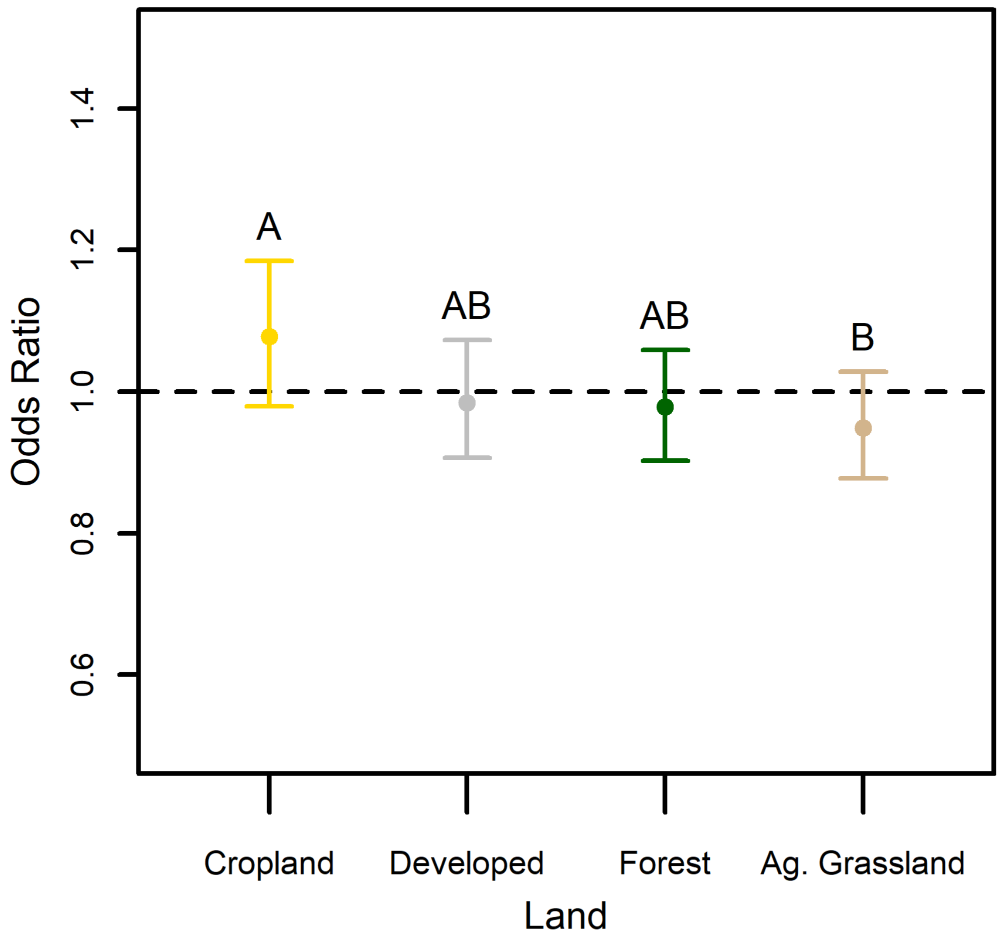


Figure 5: The presence versus absence of each land category did not affect foraging to the tiles, but agricultural grasslands (tan) significantly decreased the odds of foraging within tiles compared to croplands. Points symbolize the median odds ratio and the arrows indicate the 95% confidence interval odds ratios for the cropland (yellow), developed land (gray), forest (green) and agricultural grassland (tan) categories. The horizontal dashed line indicates even odds of visiting or not visiting, with confidence intervals entirely above or below one indicating significance (*). Letters display the results of Bonferroni corrected multiple comparisons: differences between categories sharing

a letter are non-significant, while differences between categories that do not share a letter are significant.

Honey bees preferentially foraged in a heterogenous section of the landscape

Dancing bees advertised the area directly northeast of the colonies more than expected given its distance from the colony, constituting the primary hotspot in our map of distance-corrected residual foraging probabilities (Figure 6). This hotspot is centered around a patch of early successional habitat consisting of shrubs interspersed by grasslands, and covers an area containing a stand of forest, pastures/haylands, corn fields, and an elementary school. In this way, this one, relatively small hotspot is itself a microcosm of our larger, mixed-use landscape. Dancers additionally advertised secondary hotspots with medium distance-corrected residual foraging probabilities, covering a broad area to the east of the hotspot, which consists primarily of pastures and sparse residential development, and in the western portion of the study area covering an area of low-elevation forests adjacent to a river. Finally, honey bees displayed low residual foraging probabilities, comprising cold spots, that correspond with the higher-elevation forested areas in the northwestern, northern, and southern sections of the study area.

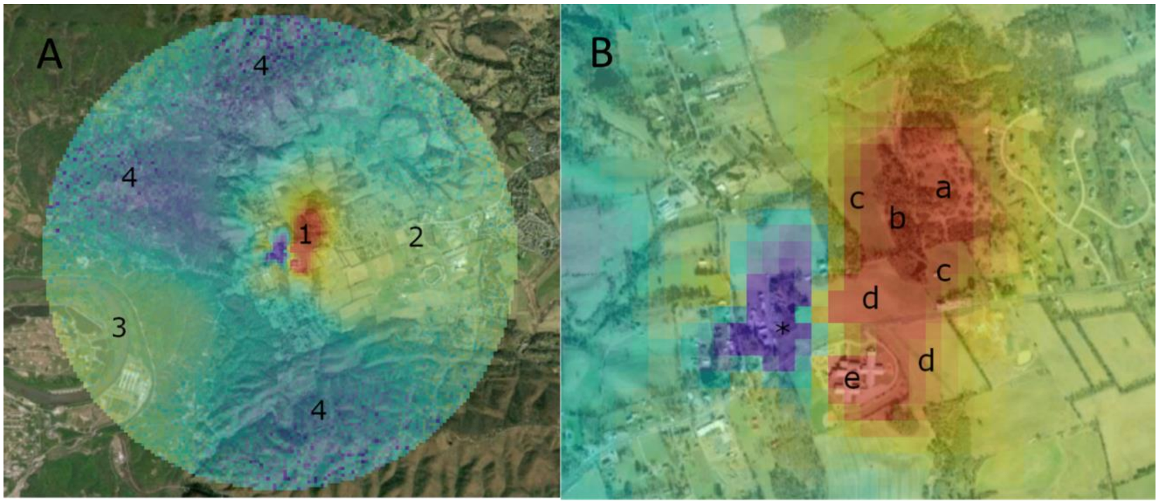


Figure 6: (A) Heat map showing distance-corrected residual foraging probabilities in the 3.25 km study area; 1) the primary hot spot in a heterogeneous section of the landscape, 2) a secondary hot spot covering an area consisting primarily of pastures and sparse residential development, 3) another secondary hotspot in low-elevation forests adjacent to a river, and 4) cold spots corresponding with higher-elevation forested areas. (B) Close-up of the primary hot spot; a) early successional habitat, b) forested area, c) pasture/haylands, d) corn fields, and e) an elementary school.

Discussion:

Here we report on honey bee foraging within a mixed-use landscape, with a particular focus on agricultural grasslands. Our initial assessments indicated that honey bees utilize agricultural grasslands across both years and all foraging months (Figure 3) at a higher level than both its representation (c. 23%) in the landscape and compared to other land categories, such as croplands and developed lands (Figure 4). However, once we correct for land type availability and distance, which is a biologically relevant foraging cost for honey bees, agricultural grasslands were not more likely to be advertised by bees and were actually less attractive than croplands (Figure 5). This demonstrates the importance of accounting for biologically relevant factors in analyses of animal behaviors. When we plot all dances simultaneously, the most advertised location is a highly heterogeneous hotspot, a microcosm of the mixed-use landscape itself, with portions of agricultural grassland, forest, developed land, and cropland (Figure 6). Overall, these data show that agricultural grasslands are attractive, but only when the cost of visiting them is low. Lastly, the most visited area in our landscape provides a small oasis of diverse land cover, with all our land categories, and, presumably, floral resources with good temporal coverage throughout the long foraging season.

The median communicated foraging distance was 699 m (n = 3881), with a minimum median distance of 485 m in April and a maximum median distance of 1122 m in June. These communicated distances are a proxy for forage availability because of the economics of honey bee foraging and recruitment decisions and, importantly, the relevance of flight distance as a cost (Couvillon, Schürch & Ratnieks 2014a; Couvillon, Schürch & Ratnieks 2014b; Couvillon *et al.* 2015; Balfour & Ratnieks 2017; Ohlinger *et al.* 2022). Interestingly, these distances are lower than those reported by previous dance decoding studies in Germany (Danner *et al.* 2016) and England (Couvillon, Schürch & Ratnieks 2014b; Couvillon *et al.* 2015; Garbuzov, Schürch & Ratnieks 2015), although comparable to another study in England (Samuelson, Schürch & Leadbeater 2022). Moreover, the maximum communicated distance in our mixed-use landscape study (6802 m) is quite lower than those reported in other Virginia landscapes, including in ones dominated by apple orchards (11.2 km; Steele et al. 2022; Ohlinger et al. 2022) and row crops (8.3 km; Silliman et al. 2022) and substantially below what is generally considered the maximum foraging range of 12 km (von Frisch 1967). These relatively low distances may indicate stable forage availability within our study area, or high landscape quality, which could potentially be attributed to the landscape's diverse land cover composition. Anecdotally, we also observed that our colonies displayed generally better health outcomes (i.e., fewer colony deaths) compared to the colonies located in apple orchards or row crops (Ohlinger *et al.* 2022; Silliman *et al.* 2022; Steele *et al.* 2022).

Agricultural grasslands, which can be either pastures or haylands, comprise nearly two-thirds of United States' agricultural land area and are an important target for management (Sanderson, Jolley & Dobrowolski 2012). However, despite their high representation and range of possible effects on honey bee health (Sponsler & Johnson 2015; Smart *et al.* 2016; Quinlan *et al.* 2021), there is a dearth of basic information necessary to develop targeted solutions that complement existing foraging resources. We quantified overall and monthly foraging in agricultural grasslands to assess food availability and identify forage gaps: use of the agricultural grasslands was lowest during June and especially August (Figure 3). Interestingly, June is also when the honey bees traveled the farthest, indicating that nearby agricultural grasslands provide insufficient forage during this time, and bees prefer instead to visit other land categories. These months, therefore, may constitute forage gaps in local agricultural grasslands and provide targets for supplemental plantings with complementary flowering phenology. In contrast, foraging to agricultural grasslands was highest in October, suggesting that local pastures and haylands might provide late season forage before colony overwintering (Döke *et al.* 2019).

How were agricultural grasslands simultaneously highly visited (Figure 4) throughout the foraging season (Figure 3) and yet not attractive for honey bees (Figure 5)? Honey bees are efficient foragers that first weigh the flight cost against energetic/nutritional gain (Schmid-Hempel, Kacelnik & Houston 1985; Wolf *et al.* 1989; Seeley 1995) and then forage and recruit at a level approximately proportional to food quality (Seeley, Camazine & Sneyd 1991; Seeley 1994; Seeley

1995; Seeley, Mikheyev & Pagano 2000). Additionally, the proportional abundance of forage might impact the probability that it will be discovered by a scout. In our study, the land categories differed in both their proportional abundance and the distance at which foragers visited them. We accounted for these factors by modeling distance-corrected foraging probabilities to equally sized patches. After doing this, our initial assessment of high agricultural grassland use changed: instead, it became clear that agricultural grasslands are utilized by honey bees, as indicated by the higher percent foraging to that land category, but only when the cost (distance) is low.

The decreased foraging to agricultural grassland after our distance-correction may reflect the fact that they were disproportionately located near the hives compared to, for example, forests. However, this seems unlikely because both categories were present across the foraging area. Alternatively, it may be that forage quality in agricultural grasslands either did not warrant long distance foraging compared to forests or did not align with the colonies' temporal demands (Ohlinger *et al.* 2022). The seemingly conflicting percent foraging and distance-corrected foraging odds are consistent with previous research reporting that honey bees increase their foraging to canola fields after crop rotations brought them closer to the colony (Garbuzov *et al.* 2015) and that honey bees foraged proportionally more in apple orchards, and preferentially in canola fields, which was only evident after correcting for distance and land area (Balfour & Ratnieks 2017).

Statistical corrections, such as ours with distance and foraging probabilities, are not unusual in behavioral ecology, where investigators seek to understand how an organism responds to ecological pressures. The process usually involves observations and measurements of traits/behaviors, measurements that may then be corrected as part of the analysis. Usually, these adjustments are based on knowledge of a study system, with the assumption that the corrections are biologically relevant and bring better understanding of the evolved traits/behaviors. For example, in comparative analyses across multiple species, where shared ancestry creates a non-independence, an algorithm may be used to account for phylogenetic closeness, and the data are transformed into independent values (Felsenstein 1985): testes are larger in bird taxa that do not participate in feeding offspring, a relationship that is only evident after controlling for phylogeny (Pitcher, Dunn & Whittingham 2005). Sometimes statistical adjustments are standardizations, such as correcting for varying body sizes in investigations on the adaptive benefit of a characteristic: tail shape significantly affects tadpole swimming performance, which is only revealed once the data are corrected by body size (Van Buskirk & McCollum 2000). Lastly, it may be necessary, as we have seen here, to consider the currency that an animal evaluates in its choices. These considerations may involve a direct manipulation of either the cost, the benefit, or both, and a measurement of the animal's resultant behavior: parental damselfish alter their cannibalistic behavior when investigators manipulated cost-to-benefit parental care ratios (Manica 2004). Alternatively, these currency considerations may also occur *ex post facto*, where a response variable is mathematically adjusted by a currency value, which is what was done here and previously (Couvillon, Schürch & Ratnieks 2014a; Balfour & Ratnieks 2017; Samuelson, Schürch & Leadbeater 2022).

By correcting for distance, we were able to identify a primary foraging hotspot covering an area that comprised all four land categories. This region was qualitatively one of the most heterogenous sections of the landscape and a microcosm of the broader landscape. Importantly, heterogeneous landscapes are generally associated with diverse and phenologically complementary flowering plant communities that provide sustained and nutritionally superior forage for insects pollinators (Andersson *et al.* 2013; Hass *et al.* 2018; Lázaro & Alomar 2019; Jachuła, Denisow & Wrzesień 2021). These benefits are present for managed pollinators as well:

honey bee colonies gain more weight and collect more pollen in heterogeneous compared to homogenous landscapes (Nürnberger, Steffan-Dewenter & Härtel 2017).

Overall, our results establish agricultural grasslands as a potential management target for improving bee foraging habitat, especially in June and August, while also recognizing them as a potential source of late season forage. Interestingly, we only began to see the relative and perhaps limited importance of agricultural grasslands after implementing our distance correction. In this way, our work demonstrates the impact of currency considerations. This currency correction helps to disentangle the factors driving behavioral and ecological phenomena, while producing insights for pollinator-focused land management efforts. Lastly, by mapping honey bee foraging, we identified a primary hotspot covering a highly heterogenous section of the landscape, which supports previous research showing the importance of landscape heterogeneity for pollinators (Andersson *et al.* 2013; Hass *et al.* 2018; Lázaro & Alomar 2019). Future research that investigates foraging hotspots and communicated foraging distances across landscape heterogeneity gradients is needed to further inform management efforts.

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Chapter 5: Good fences make good neighbors – adjacent honey bee colonies establish colony-specific foraging aggregations across landscapes

Abstract

Optimal foraging theory (OFT) predicts that animals employ foraging strategies that maximize a particular foraging currency, such as net energetic efficiency, to meet their nutritional demands. Two non-exclusive foraging patterns that arise from OFT are convergence on high-quality resources and resource partitioning. As social foragers, honey bees make collective foraging decisions by integrating their individual foraging with social, namely recruitment, behaviors. Honey bee foragers famously use the waggle dance to communicate the approximate vector of high-quality resources. Importantly, waggle dance decoding provides a valuable tool for testing optimal foraging theory predictions by allowing us to map how honey bees are using the landscape. In this study, we decoded 8049 waggle dances from groups of co-localized colonies across three distinct landscapes to investigate whether neighboring colonies forage optimally by converging their foraging on the same patches, or by resource partitioning, under which neighboring colonies establish colony-specific foraging territories. We observed no difference between the inter-dance distances, nor k-nearest neighbor clustering, among dances from the same colony versus those from different colonies. However, k-means cluster analysis demonstrated that dance locations advertised by the same colony aggregated non-randomly into clusters on 195/230 foraging days across the three sites. Overall, 45% of k-means clusters were distinct foraging territories, consisting entirely of dances from a single colony. Together, these results demonstrate that honey bees do converge, but infrequently, and instead mostly partition: neighboring colonies display similar large-scale patterns in the spatial distribution of their foraging, while establishing local, colony-specific foraging territories.

Introduction:

Foraging is necessary for animals to acquire the energy and nutrition needed for development, survival, and reproduction. Animals collect food in complex environments, comprised of multiple foraging options that may vary in their nutritional content and quality (Chalcoff, Aizen & Galetto 2006; Razeng & Watson 2015; Pamminger *et al.* 2019; Quinlan *et al.* 2021; Venjakob *et al.* 2022), quantity (Bagchi, Goyal & Sankar 2003; Lucas *et al.* 2017), distribution (Holmes & Schultz 1988), and appearance (Chittka & Raine 2006). As a result, foragers must reliably and efficiently decide not only how much time and effort to invest in foraging versus other tasks (Martindale 1982), but also which foraging resources to collect and how to distribute their efforts across foraging options (Seeley 1986; Seeley, Camazine & Sneyd 1991). To accomplish this task, animals integrate a suite of sensory (Chittka & Raine 2006; Farina, Grüter & Arenas 2012; Rusch *et al.* 2016), cognitive (Gumbert 2000; Abramson *et al.* 2013; Howard *et al.* 2018), and motor capabilities (Laverty 1994; Abramson, Dinges & Wells 2016; Diquelou, Griffin & Sol 2016) to identify resources in the environment, assess their nutritional value against the cost of collection (Ohlinger *et al.* 2022), and then perform the necessary behaviors to successfully gather and transport the resources (Thorp 1979; Raine & Chittka 2007; Diquelou, Griffin & Sol 2016).

Optimal foraging theory (OFT) proposes that organisms forage to maximize a particular nutritional/energetic currency, such as the net rate of energetic uptake, as seen in starlings (Bautista, Tinbergen & Kacelnik 2001), or net energetic efficiency, as seen in honey bees (Schmid-Hempel, Kacelnik & Houston 1985; Seeley 1994). This prediction has been tested by decades of research investigating foraging behavior through the lens of OFT across various taxa with distinct diets, social nesting strategies, and habitats (Ding *et al.* 2020; Roeder *et al.* 2020; Keesing 2021).

Additionally, OFT research suggests that we can reliably predict foraging patterns if we understand the currencies of fitness used and the characteristics of the foraging landscape (Pyke, Pulliam & Charnov 1977; Pyke 1984; Pyke & Starr 2021).

Honey bees are highly efficient social foragers that employ both individual- and colony-level adaptations to meet their nutritional demands maximally (Seeley, Camazine & Sneyd 1991; Seeley 1994; Seeley 1995). At one level, individual foragers' nervous systems integrate various relevant factors during foraging, such as the distance traveled to find the food (Seeley, Camazine & Sneyd 1991; Seeley 1994), the food's nutritional content (Arenas & Kohlmaier 2019), and current weather conditions (Riessberger & Crailsheim 1997; Clarke & Robert 2018), with cues inside the colony that inform about its nutritional status (Seeley 1989; Camazine 1993; Seeley 1995; Dreller, Page Jr & Fondrk 1999; Dreller & Tarpy 2000). Individual foragers then respond to these stimuli with the appropriate foraging and recruitment responses, which, together, allow the colony to allocate its foragers across the available food sources in proportion to their relative qualities (Seeley 1986; Seeley, Camazine & Sneyd 1991; Seeley 1994) and weighted against the colony's nutritional demands (Seeley 1989; Camazine 1993; Dreller, Page Jr & Fondrk 1999; Dreller & Tarpy 2000). Interestingly, the foraging process often begins with random searching for available food sources via scouting (Biesmeijer & Seeley 2005), and then ends with selective exploitation of a few, high quality resources via individual foraging and recruitment (Seeley, Camazine & Sneyd 1991; Seeley 1994; Seeley 1995). As a result, honey bees are famous for their ability to identify small patches of high-quality resources reliably within a large foraging radius (Seeley 1987; Couvillon, Schürch & Ratnieks 2014a).

The selective exploitation occurs in this way: after finding a high-quality resource, a foraging bee communicates its approximate location to her nestmates using the waggle dance (von

Frisch 1967; Seeley 1995). The dancing bee will perform a repeated figure-eight movement consisting of two discrete components: a waggle phase, during which she shakes her body and runs linearly across the comb, and a return phase, during which she turns back around to perform another waggle phase. The waggle phase's direction relative to the vertical comb communicates the solar azimuth from the colony to the food and the duration of the waggle phase communicates the distance to the food (von Frisch 1967; Couvillon 2012). By following waggle dances, individual foragers can find the advertised food in the landscape (von Frisch 1967; Gould 1975) and then focus their foraging on resources that meet their colony's current nutritional needs (Seeley 1989; Camazine 1993; Seeley 1995; Dreller, Page Jr & Fondrk 1999; Dreller & Tarpy 2000). Importantly, waggle dance decoding has been developed for extracting the information from waggle dances to map honey bee foraging dynamics (Couvillon *et al.* 2012; Schürch *et al.* 2013; Schürch *et al.* 2019). This emerging method has been used to investigate honey bee foraging in both agricultural (Garbuzov *et al.* 2015; Balfour & Ratnieks 2017; Lin *et al.* 2022; Silliman *et al.* 2022; Steele *et al.* 2022) and urban landscapes (Garbuzov, Schürch & Ratnieks 2015; Sponsler *et al.* 2017) and is now considered an effective method for investigating the spatial patterns of honey bee foraging (Couvillon & Ratnieks 2015).

Our current, OFT inspired, theoretical understanding of the honey bee foraging process predicts that co-localized, or neighboring, colonies should converge on the same, high quality patches within their foraging range; additionally, OFT also predicts that limited resources may be partitioned, and we do indeed know that competition between conspecifics often leads to resource partitioning in both solitary animals, such the common raven (Marzlufi & Heinrich 1991) and the Iberian lynx (López-Bao *et al.* 2011), and highly social animals, like the red imported fire ant (Wilson, Dillier & Markin 1971) and termites (Haverty, Nutting & Lafage 1975; Grace, Abdallay

& Farr 1989). Interestingly, previous research has suggested that honey bee foragers from neighboring (i.e., different) colonies may in fact partition the landscape (Waddington *et al.* 1994; Beekman *et al.* 2004); however, these data are limited in their scope and experimental design, and more research is needed to determine whether neighboring, and perhaps competing, honey bee colonies forage optimally by converging on the same high-quality patches, or, instead, by partitioning the landscape into distinct foraging territories, or some hybrid of the two.

In this study, we decoded 8049 waggle dances from nine colonies across three landscapes, with three co-localized colonies per landscape, to determine foraging dynamics in respect to those of neighboring colonies. In particular, we used nearest neighbor and k-means cluster analysis to 1) compare the spatial distribution of foraging among neighboring colonies, 2) to identify waggle dance communicated foraging clusters, 3) to quantify foraging overlap among neighboring colonies within those clusters, and 4) determine whether and how often neighboring colonies establish colony-specific foraging territories (defined here as foraging clusters consisting entire of dances from a single colony). In doing so, we explore whether spatial honey bee foraging patterns are deterministic within landscapes, producing consistent foraging patterns across neighboring colonies, or if neighboring colonies partition the landscape by establishing distinct foraging territories.

Materials and Methods

Study Organism

We studied nine predominately *Apis mellifera ligustica* colonies, consisting of a queen and approximately 5000 workers across three distinct landscapes in Virginia. Colonies were randomly allocated into groups of three adjacent colonies, spaced approximately two meters apart, and maintained within research buildings at the Prices Fork Research Center (PFRC; 37.21148,

−80.48935) in Blacksburg, VA, the Tidewater Agricultural Research and Extension Center (TAREC; 36.66447, −76.73278) in Suffolk, Virginia, and the Alson H. Smith Jr. Agricultural Research Center (WAREC; 39.11349, −78.28449) in Winchester, Virginia. Each colony was housed in a glass-walled observation hive comprised of three vertically arranged, American Standard Deep Langstroth frames, which provided a clear view of the colonies' waggle dances. We installed plumb lines on each hive, which served as a vertical reference for dance decoding. The plumb lines consisted of several weighted fishing lines, spaced 5 cm apart, and hung across the top of the observation hives. Bees were able to forage freely via a 5 cm x 30 cm PVC piping that extended from the colony to the outside. Importantly, the three landscapes provided distinct ecological conditions for the foraging bees: TAREC consisted primarily of row croplands, WAREC of orchard croplands, and PFRC of a diverse mix of developed, agricultural, and semi-natural lands.

Data Collection

We video recorded waggle dances for one hour per day 3-5 times per week (weather permitting), at 30 fps using a Canon Vixia HF R82. Waggle dances were typically recorded from 10-11 am, but they were always recorded between 9:30 am - 1:30 pm. A wood partition in the observation hive design encouraged the returning foragers to dance on the camera facing side of the hive. We focused the video cameras on a 25 x 20 cm area of high dance activity on each colony. The date, colony ID, time, temperature, and weather were displayed on a datasheet at the beginning of each video. The study period spanned from 18 April – 31 October in 2018 and 10 April – 15 October in 2019, comprising most of the honey bee foraging season. We saved the video recordings to SD cards in the field and later uploaded them to a Google Team Drive (GTD) for analysis in the lab.

Data Collection – waggle dance decoding

We later downloaded the video recordings from the GTD and converted them into AVI files using Ubuntu (v. 2004.2021.222.0) and then imported them into ImageJ (version 147 1.52i) for dance decoding using the protocol developed by Couvillon *et al.* (2012). It was important to calculate the angle offset using the plumb lines because honey bees use gravity to orient the angular component of their dances, and the cameras were not always precisely level during recording (von Frisch 1967). We looked for the first waggle dance and then proceeded through the video by decoding batches of simultaneously-dancing bees. To decrease to the probability of resampling the same waggle dances, we skipped ahead six minutes after each decoded dance batch before decoding another. We were able to easily identify dancing bees because of their distinct pattern of movement (described above). To decode the dances, we extracted the waggle phase duration, which communicates the distance to the advertised food (von Frisch 1967; Schürch *et al.* 2016), and the waggle phase angle, which communicates the solar azimuth from colony to the food (von Frisch 1967). We calculated the waggle phase duration as the difference between its start/end. Additionally, we calculated the waggle phase angle by drawing a line along the path from the center of the dancing bees' thorax at its start and end. We then added this angle to the angle offset to correct for the camera's position. Dancing bees perform 1 – 100 + waggle phases in a single dance (Seeley, Mikheyev & Pagano 2000); however, we averaged the decoded waggle phase duration and angles from a subset of four non-first, non-last, waggle phases. By doing so, we were able to efficiently extract average duration and direction values that correlate with entire dance averages (Couvillon *et al.* 2012).

Waggle dance communications are imprecise: the angular and durational components vary across successive waggle phases in a single dance (Couvillon *et al.* 2012), and across dances for the same location by different bees (Schürch *et al.* 2016). Therefore, we plotted waggle dance locations as probability distributions with dispersion parameters that accurately reflect the imprecision of the waggle dance. To do this, we used repeated Monte Carlo sampling from a universal calibration model to simulate the distance component of the dance, and from a von Mises distribution with an appropriate concentration parameter (k = 24.5) to simulate the angular component of the dance (Schürch *et al.* 2019).

Statistical analysis

Calculating within- versus among-colony inter-dance distances

After plotting the dances, we compared the spatial distribution of foraging locations advertised by waggle dances on individual foraging days, which was appropriate because honey bee foraging patches change continuously throughout the foraging season (Visscher & Seeley 1982; Waddington *et al.* 1994; Beekman & Ratnieks 2000). We first calculated the mean distance among dance locations from the same colony (within colony inter-dance distance) and also among the dance locations advertised by different colonies (among colony inter-dance distance) using the pointDistance() function from the "raster" R package. Specifically, we analyzed 1000 Monte Carlo samples per foraging day at each site consisting of a single dance location per dance. By using this approach, we were able to calculate point estimates and 95% confidence intervals for the mean within colony inter-dance distance and mean among colony inter-dance distance to explore spatial clustering of dances from single colonies. We additionally created 95% confidence intervals for the mean difference between the mean within colony inter-dance distances and the mean among colony inter-dance distances.

Investigating foraging overlap among co-localized colonies using k-nearest neighbor analysis

We further investigated the spatial clustering of the dances using k-nearest neighbor analysis. For each foraging day, we randomly selected a dance location (focal dance location) from each colony (focal colony). We then calculated inter-dance distance values, as described above, to identify the k-nearest dance locations ("k-nearest neighbors") around the focal dance locations, with k being the number of dances from the focal colony on the foraging day. At each site, we analyzed 1000 Monte Carlo samples, each consisting of a single, randomly selected, simulated foraging location per dance for each foraging day. We used this process to construct point estimates and 95% confidence intervals for the proportion of the k-nearest dances that were from the same colony (neighboring values). For this analysis, we viewed perfect clustering as instances in which 100% of the k-nearest neighbor dance locations were from focal colony (neighboring value = 1). The neighboring values were additionally converted to a neighboring ratio, which was the quotient of the neighboring value and the proportional abundance of dances from the focal colony in the days dance location sample. Foraging days during which the 95% confidence intervals for neighboring ratio values were distributed entirely above 1 indicated that dance locations (foraging) from individual colonies were non-randomly clustered.

Investigating foraging overlap among co-localized colonies using k-means cluster analysis

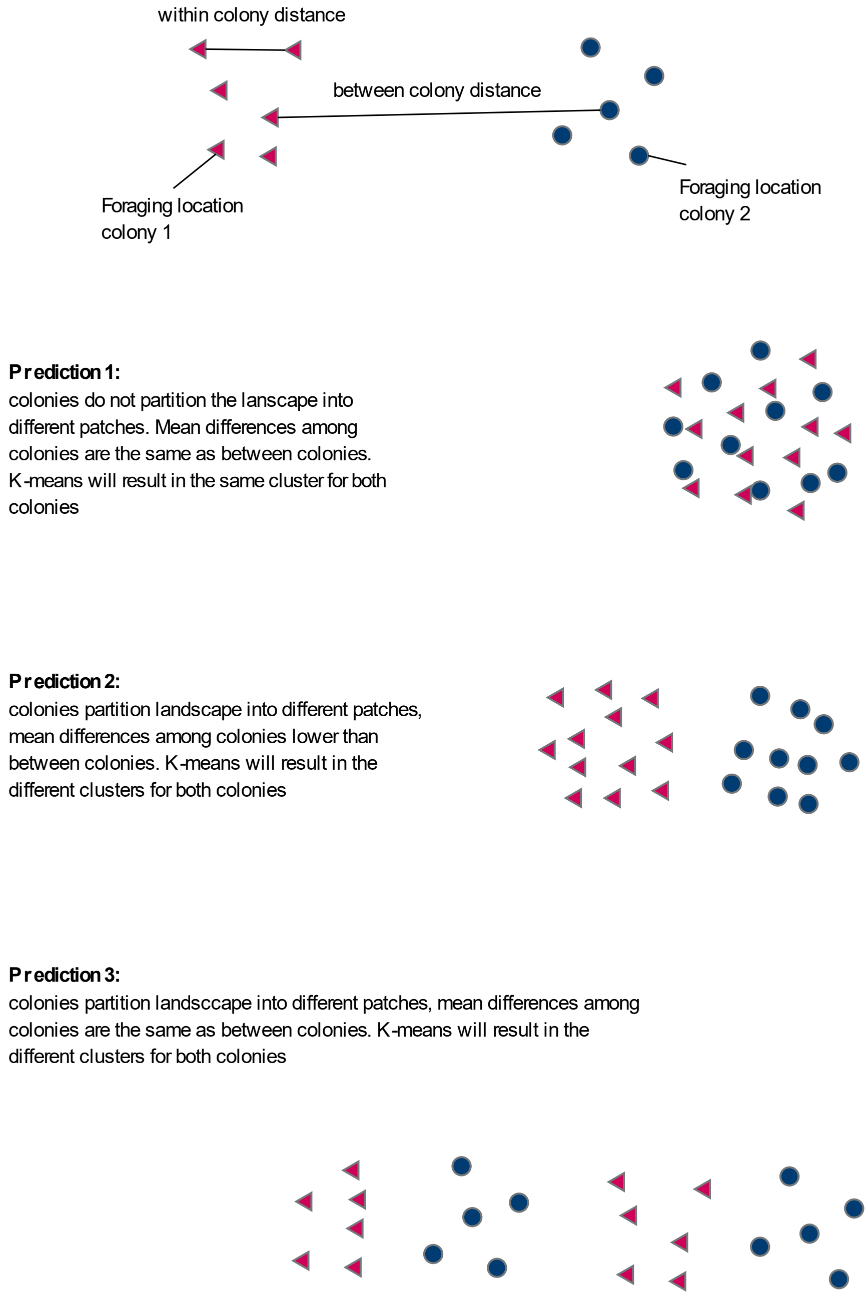
Finally, we performed k-means cluster analysis to assign the simulated dance locations into groups based on their spatial position in the landscape. Specifically, we used the kmeans() function from the "stats" R package, which applied the algorithm developed by Hartigan and Wong (1979). The optimal number of clusters for each kmeans() run was selected using the elbow method, in which clusters are added until the change in within-cluster sum of squares error decreased below a threshold. We selected 10% of the maximum decrease in the within-cluster sum of square error as our threshold value, which occurs when the first cluster is added (k =2). In other words, we selected the highest k value that decreased the within-cluster sum of squares by a value greater than our threshold. As described above, we analyzed 1000 Monte Carlo samples, each consisting of a single, randomly selected, simulated foraging location per dance for each foraging day across the three sites. In each sample, we calculated the proportional abundance of dances from each colony in the clusters and identified the most abundant colony within each cluster (dominant colony). We then created confidence intervals for the proportional abundance of dances from the dominant colony within clusters for the full dataset, for each site, and for each separate foraging day (clustering values). For this analysis, perfect clustering occurred where all dance locations in a cluster were from the same colony (clustering value = 1). We define such clusters as distinct foraging “territories”. Additionally, we converted the clustering values into clustering ratios by taking the quotient of the clustering values for each cluster and the proportional abundance of dances from the dominant colony on the foraging day. Foraging days with 95% CI for neighboring ratio values that were distributed entirely above 1 indicated that dance locations (foraging) from individual colonies was non-randomly clustered.

Optimal foraging pattern predictions

We identified three different optimal foraging predictions for the spatial distribution of dance locations communicated by the neighboring colonies, which were compared against the results of the inter-dance distance, k-nearest neighbor, and k-means clustering analyses:

- Prediction 1 – Colonies do not partition the landscape. This pattern is characterized by similar spacing of within and between colonies (difference between the mean within- and between-colony inter-dance distances spanning across 0), even representation of dances from the focal colony as nearest neighbors compared to those from the neighboring colonies (neighboring ratios span across 1), and even representation of dances from different colonies in the same k-means foraging clusters (clustering ratios span across 1).

- Prediction 2 - Colonies partition the landscape. This pattern is characterized by closer proximity of dance locations advertised within a colony compared to those between colonies (mean difference between the mean within- and between-colony inter-dance distances entirely below zero), overrepresentation of dances from the focal colony as nearest neighbors (neighboring ratio confidence intervals entirely above 1), and k-means clusters consisting predominately, or entirely, of dances from the same colony (clustering ratio confidence intervals entirely above 1).
- Prediction 3 - Colonies distribute foraging widely, while partitioning into localized foraging aggregations. This pattern is characterized by similar dance spacing within and between colonies (mean difference between the mean within- and between-colony inter-dance distances spanning across 0), even representation of dance locations from the focal colony as nearest neighbors compared to those from the neighboring colonies (neighboring ratio confidence intervals span above and below 1), and k-means clusters consisting predominately, or entirely, of dance locations from the same colony (clustering ratio confidence intervals entirely above 1).



Prediction 1:
colonies do not partition the lanscape into different patches. Mean differences among colonies are the same as between colonies. K-means will result in the same cluster for both colonies

Prediction 2:
colonies partition landscape into different patches, mean differences among colonies lower than between colonies. K-means will result in the different clusters for both colonies

Prediction 3:
colonies partition landsccape into different patches, mean differences among colonies are the same as between colonies. K-means will result in the different clusters for both colonies

Figure 1: Diagram showing the three predictions for the spatial distribution of dance locations advertised by co-localized colonies.

Results

Distances between dance locations communicated by the same and by different colonies did not differ

Dance locations advertised within a colony (mean daily inter-dance distance: 1232.5 m, 95% CI [668.2 m, 2759.4 m] were not located in closer proximity to each other than those from different colonies (mean daily inter-dance distance: 1399.1 m, 95% CI [1083.7 m, 2311.4 m]; daily mean difference: - 123.8 m, 95% CI [-767.1 m, 533.3 m]). In other words, the relative spatial distribution of dances within or between colonies was not itself different. These results support prediction 1 and 3 but fail to support prediction 2 (Figure 1).

Additionally, when looking within a site (PFRC, TAREC, WAREC), dances locations within or between colonies were similarly distributed at PFRC (mean colony inter-dance distance: within colony = 1176.6 m, 95% CI [729.5 m, 2364 m]; between colony = 1315.5 m, 95% CI [1051.2 m, 2043.1 m]), TAREC (mean colony inter-dance distance: within colony = 1152.1 m , 95% CI [553.5 m, 2909.6 m]; between colony = 1346.7 m , 95% CI [1001.8 m, 2406.8 m]) and WAREC (mean colony inter-dance distance: within colony = 1375.5 m, 95% CI [741.6 m, 2954.7 m]; between colony = 1535.9 m , 95% CI [1206.4 m, 2451.4 m]; Figure 2). In fact, dance locations from the same colony were as close to each other as they were to dance locations from the neighbor colonies on all 230 foraging days (site x date) across the two foraging seasons at the three sites.

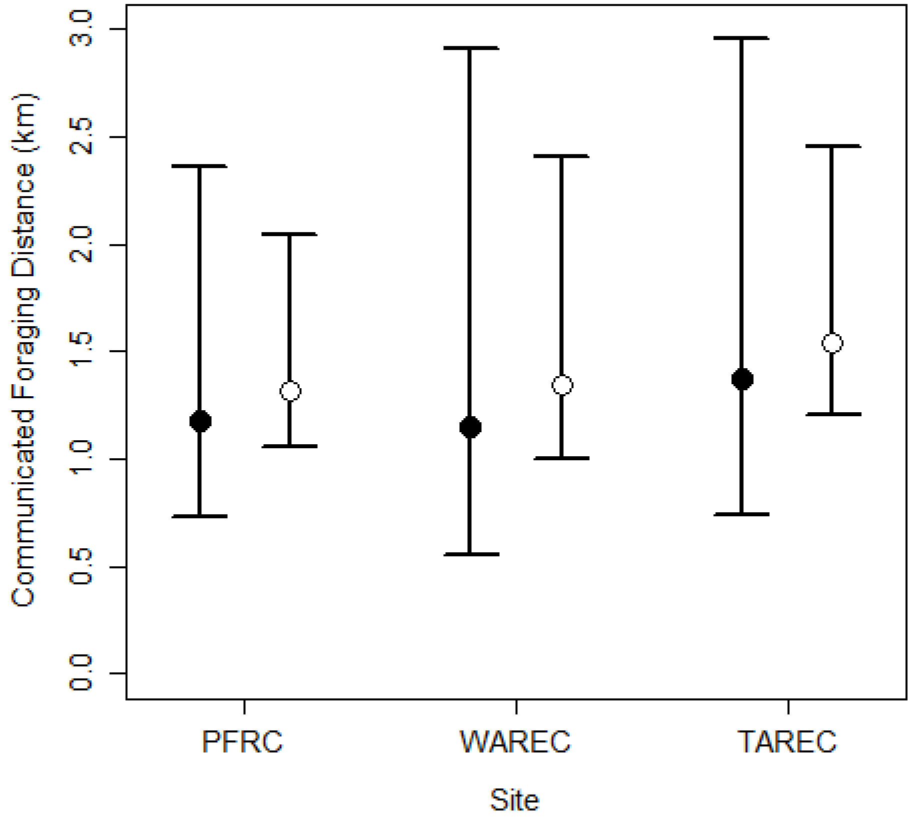


Figure 2: Dances locations advertised by the same colony were not in closer proximity to one another than dance locations advertised by different colonies. Black points symbolize the point estimates for the within colony inter-dance distances. White points symbolize the point estimates for the among colony inter-dance distances. Arrows represent the 95% confidence interval for the neighboring ratio values.

Neighboring dance locations were not disproportionately from the same colony

Dance locations from the focal colony did not make up a significant majority of its k neighboring dance locations (mean daily neighboring value: 0.50, 95% CI [0.13, 0.74]). We similarly observed low representation of the focal colony in its neighboring dance locations at

PFRC (mean daily neighboring value: 0.50, 95% CI [0.16, 0.71]), TAREC (mean daily neighboring value: 0.49 , 95% [0.08, 0.76]), and WAREC (0.51, 95% CI [0.16, 0.73]; Figure 3). The neighboring ratios, which corrected for the proportional abundance of the focal colony, further suggest random distribution of dance locations from the same and different colonies (mean daily neighboring ratio: 1.07, 95% CI [0.31, 2.11]. In fact, the 95% confidence intervals for neighboring ratios spanned across “1” on all 230 foraging days, which suggests that dance locations from the same colony did not aggregate together. These results also support prediction 1 and 3 but fail to support prediction 2 (Figure 1).

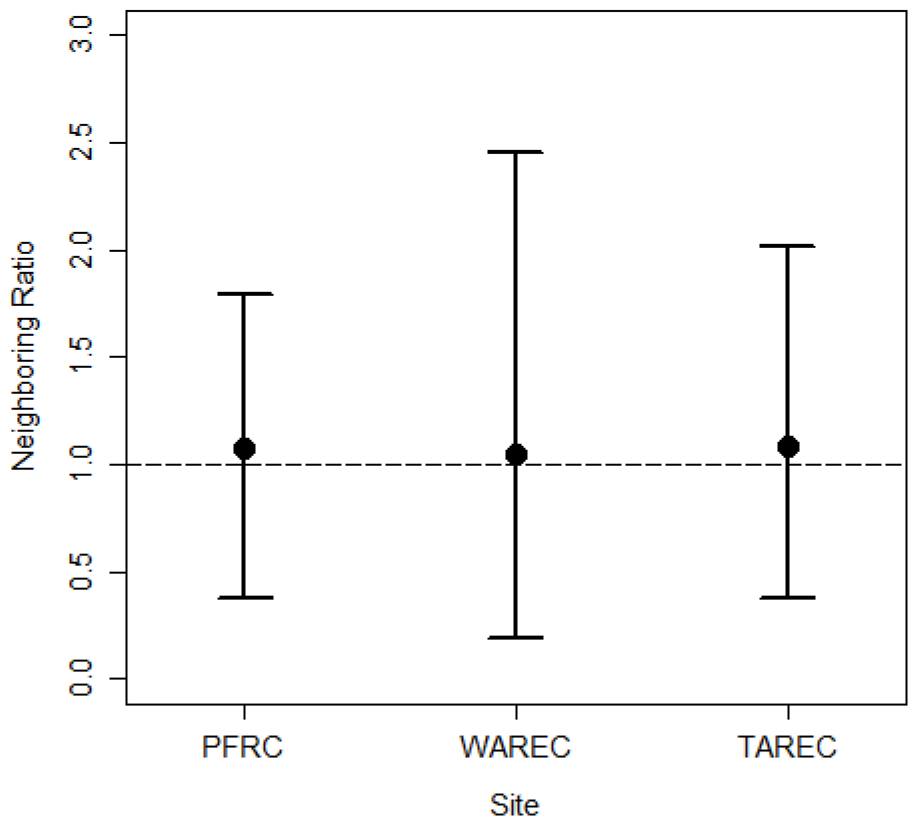


Figure 3: Neighboring dance locations were disproportionately advertised by the same colony at all three sites. Black points symbolize the point estimates for the neighboring ratio values from the k-nearest neighbor analysis. Arrows represent the 95% confidence interval for the neighboring ratio values. The dashed line reflects our baseline expectation for clustering ratios under random foraging.

Dance locations advertised by same colony aggregate into localized clusters and colony-specific foraging territories

Dance locations from the same colony comprised the majority of locations within clusters 0.79, 95% CI [0.47, 1]. Additionally, the mean clustering ratio, which corrected for the proportional abundance of the colonies suggests that colonies non-randomly aggregated into the clusters (mean clustering ratio: 1.60, 95% CI [1.09, 2.99]. In fact, the 95% confidence intervals for clustering values were significantly greater than 1 on 195 of 230 foraging days, which further suggests colonies non-randomly concentrated their foraging. We similarly observed non-random aggregation of dance locations from the same colony at PFRC (mean clustering ratio: 1.60, 95% CI [1.06, 2.99]), TAREC (mean clustering ratio: 1.64, 95% CI [1.15, 4.30], and WAREC (mean clustering ratio: 1.58, 95% CI [1.07, 3.40]; Figure 4). Over the entire foraging season, 45% of the k-means clusters of the simulated dances constituted distinct foraging territories, consisting entirely of dances from a single colony. These results support prediction 2 and 3 but fail to support prediction 1 (Figure 1).

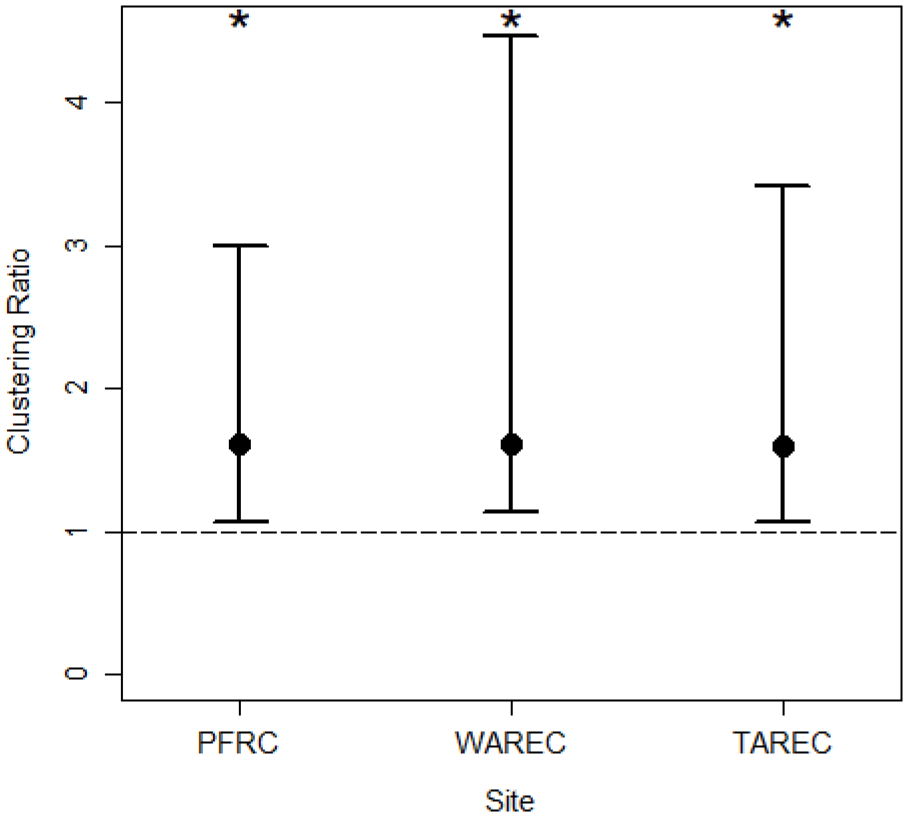


Figure 4: Dance locations advertised by the same colonies aggregated into localized clusters (*). Black points symbolize the point estimates for the mean daily clustering ratio. Arrows represent the 95% confidence interval for the mean daily clustering ratio. The dashed line reflects our baseline expectation for clustering ratios under random foraging.

Discussion

Here we used k-nearest neighbor and k-means cluster analyses to investigate spatial patterns in waggle dance communicated foraging in groups co-localized colonies distributed across three, ecologically distinct, landscapes. We report that adjacent, possibly competing, honey bee colonies display similar large-scale spatial foraging patterns, while simultaneously establishing localized, colony-specific, foraging aggregations. In other words, although we did see some convergence from honey bee foragers from adjacent colonies upon the same resources, we mostly saw resource partitioning of the landscape. Our results therefore suggest that the honey bee foraging process produces an emergent pattern that decreases competition both among nestmates and between competing colonies.

The honey bee foraging process has adapted to flexibly exploit resources according to both the availability of quality resources in their environment (Seeley 1986; Seeley, Camazine & Sneyd 1991; Seeley 1994) and colony nutritional demands (Seeley 1989; Camazine 1993; Dreller, Page Jr & Fondrk 1999; Dreller & Tarpy 2000). Importantly, this process involves both scouting, which is a random search behavior for finding new resources (Biesmeijer & Seeley 2005), and recruitment, which concentrates foraging on valuable resources (von Frisch 1967; Seeley 1995). Together, these processes allow honey bees to identify and exploit even small patches of quality resources consistently over a large area (Seeley 1987). As a result, the honey bee foraging system is thought to be an optimal foraging strategy that maximizes the net energetic efficiency of resource collection (Schmid-Hempel, Kacelnik & Houston 1985; Seeley 1994). Therefore, we expect that honey bee colonies located in proximity to each other will similarly assess and exploit the same resource patches. Alternatively, within-patch competition might deplete the quality of resources, which could influence foraging decisions and potentially lead to resource partitioning.

Previous waggle dance decoding studies suggest that neighboring colonies often exploit different foraging patches, suggesting that co-localized colonies might partition their foraging (Waddington *et al.* 1994; Beekman *et al.* 2004). However, these studies investigated foraging of paired colonies, positioned in single landscapes, over short time periods. Additionally, they used analytic approaches that do not reflect the imprecision inherent in the dance communication

(Couvillon *et al.* 2012; Schürch *et al.* 2013; Schürch *et al.* 2016; Schürch *et al.* 2019). Here we report that dance locations advertised by the same colonies were not more closely distributed than dances advertised by different colonies (Figures 1 + 2). At first glance, these results indicate broad coverage of the landscape by individual colonies, and potentially convergence on the same patches (Prediction 1; Figure 1). Interestingly, we observed disproportionate aggregation of dances form the same colony in k-means cluster analysis, which is suggestive of resource partitioning as an optimal strategy (Prediction 2; Figure 1). Together, these two, seemingly contradictory findings, suggest that colonies establish numerous foraging aggregations (mean: 5.76/day) that are widely distributed across the landscape. Put another way, dances from the same colony were non-randomly aggregated in foraging clusters, at one level, but, at another level, the relative position of those clusters was randomly distributed in the broader landscape (Prediction 3; Figure 1).

How might this spatial pattern fit into an optimal foraging strategy? Importantly, optimal foraging experiments have demonstrated that competition influences foraging by depleting resource values (exploitative competition; Balfour, Garbuzov & Ratnieks 2013; Balfour, Gandy & Ratnieks 2015) and by increasing the costs of extraction through direct interactions among competitors (interference competition; Nagamitsu & Inoue 1997). For example, honey bee foragers are deterred from lavender via bumble bee-imposed exploitative forces (Balfour, Garbuzov & Ratnieks 2013), and various social bees' aggressive foraging produces a foraging dominance hierarchy among social bees through interference competition (Nagamitsu & Inoue 1997). Therefore, the foraging activity of competitors is a relevant factor, both indirectly and directly, in foraging currency calculations (Milinski 1982; Balfour, Garbuzov & Ratnieks 2013; Balfour, Gandy & Ratnieks 2015). In our case, the simultaneous, random, landscape-scale pattern and aggregated patch-scale pattern is consistent with a foraging strategy that decreases within- and among-colony competitive forces.

How might the honey bee foraging and recruitment process produce this spatial pattern? Critically, the opposing stochastic scouting and the deterministic foraging and recruitment processes may be key features allowing for the hierarchical spatial pattern with large-scale random dispersion of dances among neighboring colonies and small-scale aggregation of dances within colonies. For example, scouts from different colonies may find different patches of the same resources, or different resources of comparable quality. In this case, each of the colonies then concentrates their foraging on the best among their identified options through selective individual foraging and recruitment (Seeley 1986; Seeley, Camazine & Sneyd 1991; Seeley 1994). Scouts might occasionally converge on the same patches; however, each colony is likely to find these patches at different points in time. As a result, foragers from the different colonies may experience different levels of competition and resource quality/availability in the patches. Therefore, honey bee colonies might only converge on the largest and most nutritionally valuable patches under good foraging conditions, or on the few available patches during extremely poor conditions. Interestingly, this possibility could be tested by investigating the relationships among mean daily waggle dance communicated foraging distance, which is a proxy for forage availability (Couvillon, Schürch & Ratnieks 2014b; Couvillon & Ratnieks 2015; Couvillon *et al.* 2015; Ohlinger *et al.* 2022), the area of patches exploited, and the foraging overlap among adjacent colonies.

While our results are strongly suggestive, additional analysis is needed to confirm the foraging patterns described above. In particular, the k values in our k-nearest neighbor analysis assume that all dances from the same colony should form a single cluster. Therefore, we can verify the local clustering of dances from the same colony by performing k-nearest neighbor with smaller k values. Additionally, we do not know whether the localized k-means clustering occurs at a biologically relevant spatial scale, or if the clusters are "overfit" for our purposes. Future research could calculate the inter-cluster distances to describe the relationship among clusters dominated by the same and different colonies. Finally, another limitation of our study is that we derived the confidence intervals for the full dataset and for full site-specific datasets by averaging the single day confidence intervals for inter-dance distances, neighboring values/ratios, and clustering values/ratios. Such a method reduced the precision of our confidence intervals and limited our statistical power. Future analysis should resample parameter values at all levels of our analysis to describe the foraging patterns more precisely.

Although we did not directly test the influences of competition on the spatial foraging patterns communicated by co-localized colonies, the patterns that we did observe are consistent with optimal foraging theory predictions. In particular, our results strongly suggest that the honey bee foraging system produces an emergent foraging pattern that reduces both within and between colony competition by clusters and territories that partition the landscape. In doing so, these results provide relevant insights into the potential complimentary roles of stochastic scouting and deterministic foraging and recruitment processes for efficient social foraging.

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Chapter 6: Does the magic well hold water? Research conclusions and future directions in honey bee behavioral ecology

von Frisch's magic well – historical and current trends in honey bee behavior ecology

"The bee's life is like a magic well: the more you draw from it, the more it fills with water". – Karl von Frisch

Every honey bee biologist knows this quote. We repeat it, *ad nauseam,* in our casual speech and our scientific communications. It is a source of inspiration, a justification for our hard work, and a means of sharing our enthusiasm with our families, friends, and fellow scientists. We relate to it – strongly. And we do so because the words encapsulate the awe and mystery that drive us to spend long, hot, sting-filled, days trying to quench our thirst with a drink from the well. But, above all, it is a call to action!

However, while this quote is so ubiquitous among honey bee researchers, I can't help but wonder if it is true. Words can be beautiful – and false. It poetically states that the more we learn about the honey bee, the more we have yet to know. In other words, honey bee research discoveries produce more questions than answers. If true, the discovery rate regarding the honey bees should be increasing non-linearly through time. There are certainly examples of scientific advances that have triggered proliferations of new discoveries and resulted in entirely new areas of honey bee research: for example, the sequencing of the honey bee genome (Sequencing Consortium 2006) has opened research avenues into the molecular underpinnings of sociality (Robinson, Grozinger & Whitfield 2005; Yan *et al.* 2014; Kohno & Kubo 2019), while advances in sequencing and culture-based technologies have been a catalyst for research into the honey bee gut microbiome and its role in their health and behavior (Jones *et al.* 2018; Wang *et al.* 2018; Zheng *et al.* 2018;

Castelli *et al.* 2020). However, one could argue that these are simply examples of the broader merging of fields across the sciences (Wilson 1997). In contrast, Karl von Frisch was talking purely about behavior.

While it is not my intention here to formally investigate historical trends in the productivity/innovation of honey bee behavioral ecologists, it is undeniable that the field is experiencing an explosion of waggle dance decoding studies (Couvillon, Schürch & Ratnieks 2014; Couvillon *et al.* 2015; Garbuzov *et al.* 2015; Garbuzov, Schürch & Ratnieks 2015; Balfour & Ratnieks 2017; Sponsler *et al.* 2017; Carr-Markell *et al.* 2020; Lin *et al.* 2022; Ohlinger *et al.* 2022; Samuelson, Schürch & Leadbeater 2022; Silliman *et al.* 2022; Steele *et al.* 2022; Shackleton *et al.* 2023). In fact, the google scholar search phrase, "waggle dance" AND "decoding", reveals a rapidly growing body of literature: 9.3 per year (1980 - 89), 30.9 per year (1990-99), 84.3 per year (2000 - 09), 181 per year (2010-2019), and 207.2 per year (2020 - April 2023). Such a trend coincides with the emergence of an increasing pollinator health crisis and a heightened scientific and public attention to the problem (Potts *et al.* 2010; Goulson *et al.* 2015). Scientists have concurrently produced emerging tools that allow us to solve these problems in new ways and from fresh perspectives (Couvillon *et al.* 2012; Schürch *et al.* 2013; Couvillon & Ratnieks 2015; Schürch *et al.* 2019; Markell & Spivak 2021).

Clearly, I have embarked on my Ph.D. during an exciting time, or dare I even say, a time when the well is refilling with every draw. In the broadest sense, my dissertation demonstrates the versatility and utility of waggle dance analysis for honey bee research. Each chapter applies waggle dance analyses differently, but they are bound together by a common goal of investigating broad trends in honey bee foraging, and stress responses, across landscapes. In short – I drew from the well. Now in this concluding chapter, I will first describe what made it into my pail (discoveries), and then hypothesize perhaps what might be waiting to be drawn (future directions):

Summary of Chapter 2

In this chapter, we investigated the effects of a sublethal dose of imidacloprid on honey bee foraging and recruitment using a semi-field feeder experiment. We report that honey bees decreased their foraging, but not recruitment, to an artificial feeder containing a sublethal dose of IMD, compared to a control solution (discovery). Together, these effects could challenge honey bees by simultaneously increasing their exposure to pesticides and decreasing their food intake. However, we still do not know the underlying mechanisms driving the observed effects. For example: 1) Do honey bees decrease their overall food intake after exposure to imidacloprid, or just their intake of foods containing IMD or other pesticides (future direction)? 2) Do sublethal doses of pesticides decrease honey bee foraging by inhibiting their motivation to forage, or their physical, sensory, or cognitive abilities (future direction)? 3) Do honey bees reduce their foraging to pesticides when collecting natural forage (future direction)?

Summary of Chapter 3

In this chapter, we compared waggle dance communicated nectar and pollen foraging distances, which served as a proxy for cost, across three distinct landscapes. In doing so, we found that honey bees foraged at 14% farther distances for nectar compared to pollen across all three landscapes and across months within sites (discovery). These results suggest that nectar foraging is a supply-driven system, while pollen foraging is a demand-driven system (discovery). While our results increase our understanding of the systems, less is known about the potential drivers of our observations. For example: 1) Do honey bees foraging costs respond to changes in brood production (future direction)? 2) Do honey bee foraging costs respond to varying levels of brood pheromone (future direction)? 3) Does forage availability influence how honey bees respond to these factors (future direction)?.

Summary of Chapter 4

In this chapter, we used waggle dance decoding to map and quantify honey bee foraging to agricultural grasslands, a common and underexplored land type, in a mixed-use landscape in Virginia. In doing so, we found that honey bees recruited disproportionately to agricultural grasslands compared to croplands, developed lands, and forests (discovery). However, after correcting for foraging distance, which is a biological relevant cost, we found that agricultural grasslands were not more attractive than broader landscape, and, instead, were less attractive than croplands (discovery). Finally, we qualitatively observed a foraging hot spot covering a heterogenous land patch, which consisted of all four land classes and comprised a microcosm of the entire landscape (discovery). Together, these results identify agricultural grasslands as a potential management target, while further supporting the importance of landscape heterogeneity to pollinators (discovery). Additional research investigating foraging across landscape heterogeneity gradients and agricultural grassland foraging across landscapes is needed to further guide management efforts. For example: 1) Is landscape heterogeneity directly attractive to honey bees, or do heterogenous patches simply provide temporally stable forage (future direction)? 2) Are heterogenous patches highly visited across landscape contexts (future direction)? 3) Does foraging to agricultural grassland differ across landscapes (future direction)? 4) Which plants do honey bees visit in agricultural grasslands (future direction)? 5) Are there specific management practices that improve agricultural grassland attractiveness (future direction)? 6) Do honey bees predominately visit the agricultural grassland fields, or their field margins (future direction)?

Summary of Chapter 5

In my final research chapter, we used waggle dance decoding to investigate spatial patterns in honey bee foraging across landscapes through the lens of optimal foraging theory. In particular, we explored whether co-localized, or neighboring, honey bee colonies forage optimally by converging on the same patches, or by partitioning the landscape into distinct foraging territories. Our analysis revealed that neighboring colonies display similar large-scale patterns in the spatial distribution of their foraging, while partitioning the landscapes into colony-specific foraging territories at the patch-scale (discovery). While we did not directly test within- and between colony foraging competition, our results suggest that the honey bee foraging process produces an emergent foraging pattern that limits both within- and among colony foraging competition (discovery). Future research that explores foraging under different levels of forage availability and among-colony competition and across different spatial arrangements of colonies and differently structured landscapes is needed to further understand how among-colony competition influences honey bee foraging patterns. For example: 1) Under what circumstances do co-localized colonies converge on the same patches versus partition the landscape (future direction)? 2) What is the relationship between among-colony spatial foraging overlap (convergence versus partitioning) and communicated foraging distance, which is a proxy for food availability (future direction)? 3) What is the relationship between among-colony spatial foraging overlap (convergence versus partitioning) and colony nutritional status (future direction)? 4) During which times of the foraging season do colonies converge/partition to landscape (future direction)? 5) Do temporal trends in convergence versus partitioning differ across landscapes (future direction)? 6) Do honey bees forage over a larger area (cluster size) when forage is scarce, as demonstrated by increased communicated foraging distance (future direction)? 7) Do colonies cover a larger foraging area when converging on foraging patches, or when partitioning (future direction)? 8) Do chemical foraging signals, such as flower-marking (Free & Williams 1983; Giurfa & Núñez 1992; Giurfa 1993; Stout & Goulson 2001), facilitate foraging convergence and/or partitioning among colonies (future direction)?

When I sit back and think of these questions and envision where the next generation of new scientists, myself included, might focus, I see boundless opportunities in a frontier more vast and mysterious than von Frisch could have imagined. And I am indeed able to conclude that the well is full!

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