Waggle Dance Your Own Way: Individuality, Network Structure, and an Herbicide Stressor in Recruitment, Foraging, and Neurobiology in the Honey Bee (*Apis mellifera* L.).

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

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SCIENTIFIC ABSTRACT

The waggle dance of the honey bee (*Apis mellifera* L.) is perhaps the most celebrated animal communication behavior. With a waggle dance, a forager bee who has discovered a profitable resource on the landscape, usually floral nectar or pollen, can inform her nestmates of its location and recruit them to exploit it by communicating both a distance and a direction. Since Karl von Frisch described the waggle dance in 1942, scientific exploration of the dance has exploded into the realms of its structure, function, role in the regulation of collective foraging in the context of the hive as a super-organism, and even its utility as a study system for understanding sublethal behavioral effects of pesticide exposure. This dissertation presents three novel studies of the waggle dance. In the first, we asked whether consistent inter-bee differences (i.e., individuality) in a waggle dance distance - duration calibrations could affect communication success. In the second, we characterized the networks of recruitment arising from waggle dance communications and explored the role of the aforementioned individuality in network formation. In the third, we tested whether sublethal exposure to glyphosate (GLY), the most-applied herbicide in the world, could affect foraging, recruitment, or the levels and balance of biogenic amines in the bee brain. In each of these experiments, we housed bees in clear-walled observation colonies and trained cohorts of bees to visit artificial feeders to record both foraging and recruitment data. In our first experiment, we found that individuality in waggle dance behavior does shape communication outcomes, indicating that individual-level behavioral differences should not be discounted as factors at work in eusocial insect societies. In the second, we present the first network density and dance burstiness data from *in vivo* bee networks, revealing that recruitment networks are sparse, and waggle dancers are partitioned into bursty and non-bursty behavioral types. In the third, we show that not only can sublethal GLY exposure reduce foraging, but it can also produce significant correlations between levels of the important insect neurotransmitter octopamine and its two biosynthetic precursors, tyramine and tyrosine, where levels in control bees were unrelated. The results of this dissertation research, while distinct by experiment, together emphasize the continuing usefulness and tractability of the honey bee colony as a system in which to study the role of individuality in animal communication and to better understand the threat posed by non-insecticidal pesticide chemistries to the planet's most economically impactful pollinator.

Waggle Dance Your Own Way: Individuality, Network Structure, and an Herbicide Stressor in Recruitment, Foraging, and Neurobiology in the Honey Bee (*Apis mellifera* L.).

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GENERAL AUDIENCE ABSTRACT

One of the most famous and well-studied animal behaviors is the waggle dance of the honey bee. A honey bee's waggle dance works similarly to a Yelp review for a restaurant: a bee who has found a good food source, like a flower patch offering especially sweet nectar or high-quality pollen, can come back to the colony and recommend it to her nestmates with a dance. The waggle dance is even more specific than a Yelp review, however, in that it also gives instructions to find the food source, communicating both a distance and a direction so that dance followers can go out into the landscape and look for the food source themselves. Even though the waggle dance has been studied extensively since it was first described by Karl von Frisch in the 1940s, there are still unknowns about how it works, and how it might be impacted by certain stressors. This dissertation presents three different experiments aimed at shedding light on these unknowns. First, it has recently been shown that there are consistent differences between bees in the way they communicate distance in the dance, and we tested whether that between-bee individuality can affect the likelihood that two bees will communicate successfully. Second, we studied how information about a food source moves from bee to bee via the waggle dance to form a communication network. Specifically, we described how efficiently information moved from bee to bee, patterns of dancing behavior, and the role of that individuality in its formation. Third and lastly, we looked to see whether exposure to a weedkiller called glyphosate (GLY) could affect either honey bees' waggle

dance or food-collecting behavior, as well as levels of certain neurotransmitters in their brains that are involved in those behaviors. In all three experiments, we collected our data by housing bees in a clear-walled observation hives that let us view and film their waggle dance behavior, and then training groups of bees to collect artificial nectar from a feeder station that we provided, so we could also observe them as they collected food. We found that individuality in waggle dance communication can indeed affect the likelihood of communication success between two given bees, where the likelihood of communication success is greater when the dancer communicates a farther distance to the food source than the follower would. In the second experiment, our study of the waggle dance communication network showed that (1) information does not flow from bee to bee very efficiently, and (2) bees either dance quite regularly or sporadically. As far as we know, we are the first to describe these aspects of the waggle dance communication network, which may be useful in the field of computing algorithms inspired by living organisms. Finally, our third experiment showed that mild GLY exposure not only reduced how frequently bees collected food from our feeder, but also changed the relative amounts of certain neurotransmitters in their brains. This result emphasizes the importance of understanding how weedkillers that are not meant to target beneficial insects like honey bees are actually affecting them, so that we can make better-informed decisions to protect honey bees and other good insects.

For David, my bee groupie.

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And finally, to the anonymous reviewer of my undergraduate capstone project who wrote “she has potential as a behaviorist” – words taped on the fridge door of my heart these last twelve years –

Thank you

TABLE OF CONTENTS

PREFACE AND ATTRIBUTION

MANUSCRIPT 1: INDIVIDUALITY IMPACTS COMMUNICATION SUCCESS IN HONEY BEES

M.J.C. and R.S. conceived this experiment, and designed it with L.C.M. L.C.M. and M.J.C. conducted the feeder training and data collection with field assistance from R.S., B.D.O., and L.E.J. L.C.M. performed data analysis with the assistance of R.S. L.C.M. prepared the manuscript with M.J.C. All other co-authors provided valuable discussions and feedback throughout. At the time of dissertation submission, this manuscript was in revision with the peer-reviewed journal *Current Biology*.

MANUSCRIPT 2: DANCING ON THE EDGE: HONEY BEE WAGGLE DANCE NETWORKS ARE SPARSE AND BIMODALLY BURSTY

L.C.M. conceived and designed this analysis of feeder training data collected with M.J.C. and with field assistance from R.S., B.D.O., and L.E.J. L.C.M. conducted the data analysis, and prepared the manuscript with M.J.C. All other co-authors provided useful comments and discussions throughout.

MANUSCRIPT 3: SUBLETHAL GLYPHOSATE EXPOSURE IMPACTS HONEY BEE FORAGING AND ALTERS BALANCE OF BIOGENIC AMINES IN THE BRAIN

L.C.M. and M.J.C. conceived and designed the feeder training experiment, and conducted it with assistance from R.S. and B.D.O. L.E.J. assisted with feeder data entry and collected dance data from video recordings. L.C.M. analyzed the data with assistance from R.S.

L.C.M. conceived the molecular portion of the experiment, and A.D.G. advised on its design and provided laboratory space and assistance. L.C.M. performed brain dissections and sonication. M.C.T. conducted the UPLC and wrote the associated portion of the materials and methods. L.C.M. and M.J.C. wrote the manuscript, and all co-authors contributed valuable discussions and comments throughout. At the time of dissertation submission, this manuscript was in revision with the peer-reviewed journal *The Journal of Experimental Biology*.

CHAPTER 1 - GENERAL INTRODUCTION

The lives of animals, including their traits, behaviors, and adaptations, have been shaped through evolutionary history by the following essential question: “How will I get my next meal?” (Second only to “how will I get my next mate?”)

In answer, the animal world displays an astounding array of foraging strategies. From anglerfish entrancing prey with bioluminescent lures (Bertelsen 1951; Munk 1999) to fungus-farming leaf-cutter ants (Wilson 1986; Fang et al. 2020), animals find their food in ways that put grocery store online-ordering to shame. Even further complexity can be found in the foraging strategies of social animals, shaped as they are by the evolutionary exigencies of group living (Krause & Ruxton 2002; Trumbo 2012; Rubenstein & Abbot 2017). For social foragers, the question then becomes: “how will we get our next meal?”

A hallmark of collective foraging is that the foraging efforts of social animals often benefit the group as a whole, rather than the forager alone (Wilson 1975; Whitehouse & Lubin 2005). While it is worth noting that social feeding does not necessarily involve cooperation between individuals (Webster & Fiorito 2001), complex cooperative foraging behavioral adaptations have arisen in many animal societies (Giraldeau & Caraco 2000). For instance, groups of crab spiderlings of the genera *Australomisidia* and *Xysticus* have higher prey capture success and hunt larger prey when together than when alone (Dumke et al., 2018); group-living shrimp (*Synalpheus regalis*) work together to protect their sponge habitat, which is also their perpetual source of food (Duffy 1996; Hultgren et al. 2014); and Galápagos sea lions (*Zalophus wollebaeki*), which live in large groups but rarely hunt together, will form hunting parties to trap and kill large yellowfin tuna when food easily obtained alone is scarce (Páez-Rosas et al., 2020).

The order Hymenoptera in particular contains a treasure trove of taxa that forage cooperatively, including ants (Hölldobler & Wilson 1990; Behmer 2009; Greenwald et al. 2019) and some social wasps (Overmyer & Jeanne 1998). For instance, in the ant *Temnothorax albipennis*, a forager with knowledge of a food source can lead a naïve recruit directly to it through a cooperative recruitment behavior called tandem running (which, interestingly, has been proposed as the first example of teaching in a non-human animal (Franks & Richardson 2006)). In addition to cooperation, many social insects' foraging efforts feature yet another layer of complexity: division of labor. Division of labor involves the divvying up of different tasks among subsets of workers, either permanently or for a period of time. Compared to systems where all workers may perform any job at any time, division of labor tends to increase foraging efficiency (reviewed in Oster & Wilson (1978), Robinson (1992), Beshers & Fewell (2001), although see Dornhaus (2008)). Indeed, it is considered a crucial underpinning of social insects' ecological success (Wilson 1976, 1990; Robinson 1992).

Even in such remarkable company, perhaps the finest described example of adaptive social foraging involving these traits—communication, cooperation, and division of labor—belongs to the European honey bee, *Apis mellifera* L.

Honey bees collect food from a wide variety of flowering plants, making them generalists in their foraging preferences (Biesmeijer & Slaa, 2006). Remarkably, a honey bee forager who has discovered a high-quality resource on the landscape can communicate its location to her sisters and direct them to it with a behavior unique to honey bees, the waggle dance. The waggle dance, also called the dance language of the bees, is a ritualized communication system wherein a forager bee informs her nestmates of the location and quality of a valuable resource (von Frisch, 1946; von Frisch, 1967). What counts as a profitable resource to a bee? A dancer most frequently advertises sources of nectar, pollen, water, and resin, or even an appealing candidate nest site (von Frisch 1967; Seeley 1995; Seeley & Visscher 2004). A bee does not dance for every resource she comes across; rather, she preferentially visits higher quality resources, and only performs waggle dances for the most profitable resources at any given time (Seeley et al. 1991; Seeley 1994; Grüter & Farina 2009; Couvillon 2012). Bees who follow the dances are then recruited to venture out in search of the advertised resources, resulting in an increase in foraging efficiency and ultimately an increase in colony-level fitness (Sherman & Visscher 2002; Dornhaus & Chittka 2004; Schürch & Grüter 2014; although see Price et al. 2019).

The dance language comprises several components, each with its own meaning. A waggle dance begins when a bee waggles her body back and forth as she moves across the comb, which is called a waggle run. The waggle run is the information-rich, repeating subunit of the dance. Its duration is proportional to the distance of the resource from the colony, while its direction – the dancer's clockwise orientation from vertical on the comb – corresponds to the angle from the sun's azimuth a bee must fly to find the advertised spot (von Frisch, 1946; von Frisch, 1967; Seeley et al., 2000). At the end of each waggle run, a dancing bee returns (the return phase) to near the start point of her dance by turning away alternatingly to the left or right. She then performs another waggle run; this time, she turns away to the opposite side. The alternating run-and-return creates the classic figure-of-eight pattern (von Frisch 1946; von Frisch 1967; Couvillon 2012).

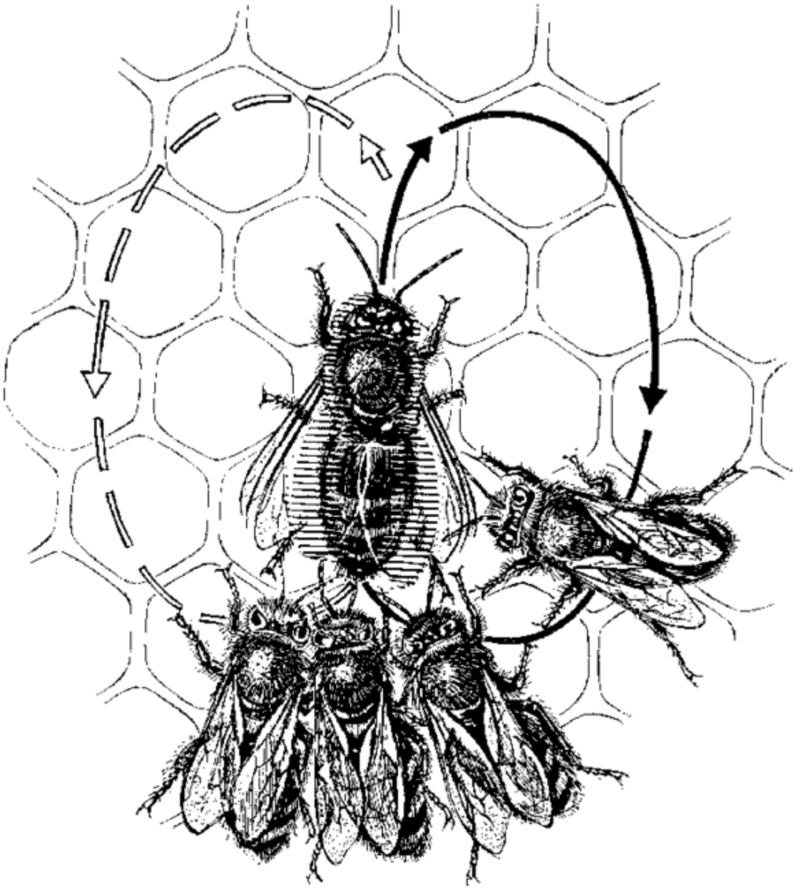

Figure 1.1 Karl von Frisch's 1965 depiction of the waggle dance, showing the waggle dancer attended by four follower bees as she performs a waggle run. (From Karl von Frisch, *Tanzsprache und Orientierung der Bienen* [Berlin, 1965], p. 57)

Few animal behaviors have been the object of so much scientific excitement, scrutiny, skepticism, and even rancor as the waggle dance of the honey bee (Gould 1975; Munz

2005; Couvillon 2012; Tautz 2023). The phenomenon in which one honey bee is observed foraging at the site of a profitable resource (usually floral nectar or pollen) soon becomes multiple honey bees, was documented as far back as Aristotle (Haldane 1955). But it was not until 1967 that Dr. Karl von Frisch won a share of the Nobel Prize in Physiology for his scientific description of the behavior at the heart of this phenomenon: the waggle dance, or the dance language of the bees. Von Frisch's discovery prompted a veritable explosion of research into the form and function of the dance in the years between then and now. Even after decades of fruitful research peppered with prolonged bouts of scientific sparring, which, in turn, prompted further research, von Frisch's words remain as accurate as ever: "The bee's life is like a magic well. The more you draw from it, the more it fills with water."

This dissertation presents three manuscripts that describe separate but related experiments – pulls from the well, so to speak – that are united in their use of honey bee foraging and waggle dance recruitment to investigate basic and applied questions along the intersecting axes of ethology, network analysis, and behavioral toxicology and neurobiology. The first explores the impact of inter-bee individuality in waggle dance behavior (Schürch et al. 2016) on communication outcomes; the second characterizes networks of recruitment arising from waggle dance communications, offering the first biologically-informed network descriptive statistics for *in vivo* recruitment networks; and the third tests the effect of sublethal exposure to the herbicide glyphosate (GLY), the most widely-applied pesticidal toxicant in the world (Benbrook et al. 2016), on foraging and recruitment, as well as on the levels and balance of biogenic amines in the bee brain.

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CHAPTER 2

INDIVIDUALITY IMPACTS COMMUNICATION SUCCESS IN HONEY BEES

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This manuscript is formatted according to *Current Biology's* Correspondence article type, which consists of (1) the primary manuscript of no more than 1,000 words and ten references, and (2) supplemental information.

Individuality Impacts Communication Success in Honey Bees

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The honey bee waggle dance is a celebrated animal communication behavior that, despite its importance to colony function, often fails. A forager's dance conveys to nestmates a vector (a distance and direction) from the hive to a valuable resource, usually nectar or pollen (von Frisch 1967). Recruitment supports colony-level foraging efficiency in dynamic environments (Dornhaus et al. 2006). Why then do only 16-25% of dance-following bees succeed in finding advertised resources (Seeley and Visscher 1988)? Intriguingly, it was demonstrated that each forager possesses an individual calibration to communicate the resource's distance (Schürch et al. 2016); however, the effect of this individuality on recruitment success is unknown. Here we tested whether calibration mismatch in dancer-follower dyads affects their ability to communicate. We created fully-marked observation colonies and trained bees to forage from artificial feeders at known locations. Concurrently, we filmed dances inside the colony to identify successful dancer-follower dyads. We then compared the distribution of calibration mismatch values among these successful dyads (n=30) to a simulated expected distribution based on a null hypothesis of

random assortment of calibration values. In support of our hypothesis, calibration mismatch was nonrandom among successful dyads: followers predicted to overshoot the resource were over-represented among successful pairs compared to the null distribution (p = 0.03). Our data demonstrate that dancer-follower calibration mismatch impacts the likelihood of recruitment success, and, more broadly, that individuality can shape communication outcomes in a eusocial insect society.

A waggle dancing forager communicates the distance and direction to the resource in the waggle run, which is the repeating subunit of the dance wherein a bee vibrates her body as she moves across the comb. The direction is encoded by the angle of the dancer's body relative to the vertical during the run, and the distance is encoded by the duration of the run. Since each forager communicates distance according to her own distance-to-duration calibration curve (hereafter simply "calibration") that increases linearly (Schürch et al. 2019), run durations may differ between individual bees even though they advertise the same location. Therefore, there exists an opportunity for miscommunication. In particular, possible calibration mismatches at a given distance may occur where: (1) dancer duration < follower duration, where the follower would interpret a shorter distance than intended by the dancer and would be predicted to undershoot the actual distance to the resource; or (2) dancer duration > follower duration, where the follower would interpret a farther distance than intended by the dancer and would be predicted to overshoot the actual distance to the resource. We hypothesized that (2) over-shooters would be more likely than under-shooters to successfully arrive at a location since they would fly over it on outbound trips and might re-encounter it, either while searching or upon their return to the colony (Menzel et al. 2005).

To investigate the impact of individual calibrations on recruitment success, we trained an initial cohort of 10-20 bees belonging to fully marked observation colonies (N = 2, each with 3,000 bees, see Supplementary online) to visit an artificial feeder at a known distance offering 2 M unscented sucrose solution to promote strong waggle dance recruitment. This initial cohort (dancers) then dance recruited a second wave of 15-20 bees (followers, but eventually dancers as well). Once both waves had danced for the feeder, we moved it to the second location, where we remained until all bees had performed second-location dances. Concurrently, we filmed both sides of the observation hive to identify successful dancer-follower pairs (n=30 dyads) and to measure bees' waggle run durations, which allowed us to determine their individual calibrations (Figures 2.1a and 2.1b; see below and Supplementary online) (Couvillon et al. 2012).

For each successful dyad, we quantified the mismatch by calculating [dancer mean waggle run duration] minus [follower mean waggle run duration]. Ideally, we would then compare a distribution of these values with a distribution obtained by calculating values from unsuccessful dyads, where the follower failed to find the feeder; however, this was impossible because unsuccessful followers never found the feeder, never danced, and were not represented in our data. We therefore compared our calculated distribution of successful dyads to a simulated expected distribution based on the null hypothesis that successful dyads would possess randomly assorted calibration values (Figure 2.1c, Supplementary online).

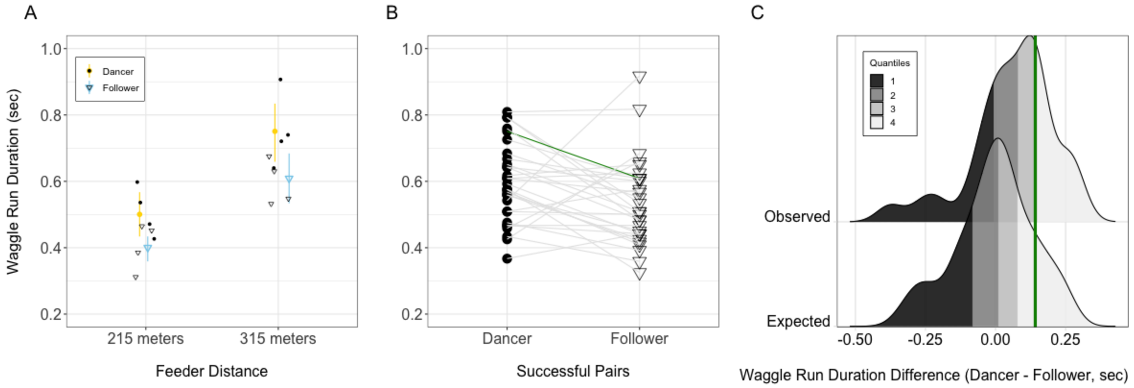

Figure 2.1. (A) The waggle run distance-durations of the actual dancer-follower dyad possessing the median mismatch value (see Supplementary Online; circles = representative dancer, triangles = representative successful follower). Individual bee waggle run durations, with multiple durations per bee, are shown in black and white, and each bee's mean waggle run duration is shown in color (dancer = yellow, follower = blue). Error bars are bootstrapped 95% CI. **(B)** Waggle run duration values for successful dancer-follower dyads (n = 30) with lines linking dyads. Green line highlights the representative dyad from A. **(C).** Observed distribution of calibration mismatch among successful dyads versus expected null distribution (one-tailed Kolmogorov-Smirnov Test, p = 0.03). Green line shows the median mismatch value for the same dyad presented in A and highlighted in green in B.

In support of our hypothesis, our observed distribution of successful dyad calibration mismatch values was statistically distinct from simulated expected null distribution and was in fact significantly right-shifted (One-tailed Kolmogorov-Smirnov Test, p = 0.03, Figure 2.1c). In other words, dyads in which the follower (ultimately) communicated a shorter waggle run duration for the feeder location than that of the dancer that recruited her, and which we would therefore assign to the predicted-overshoot condition, were disproportionately over-represented in our data of successful communication partners. Conversely, dyads assigned to the predicted-undershoot condition were underrepresented (Figure 2.1c).

Importantly, not every dance follower necessarily tried and failed to find the advertised feeder: dance-following is also associated with reactivation to and/or monitoring of the profitability status of an already-known food source (Grüter and Farina 2009). Nevertheless, our results indicate that miscommunication arising from individual calibrations can account for some recruitment failure.

If this individuality can result in miscommunication and failed recruitment, why then should it persist? We might expect stabilizing selection pressure to compress the calibration value distribution and increase signal similarity. Perhaps calibration is related to the presence of multiple patrilines in colonies (Withrow and Tarpy 2018). If the genetics underpinning calibration were linked to genotypes whose variation across patrilines confers additional, unrelated fitness benefits at the colony level (Mattila and Seeley 2007), these benefits might outweigh any benefit of calibration stabilization. The broader question remains: does this individuality exist and persist despite costs associated with miscommunication, or is there an as-yet-unknown adaptive benefit of individuality in waggle dance calibrations?

Here we have shown that individuality matters: the distinct and conserved calibration differences between forager bees (Schürch et al. 2016) can indeed modulate communication outcomes. We propose that considering the members of honey bee societies not as interchangeable subunits, but rather as individuals whose particularities may plausibly shape the emergent properties of the whole, may be a fruitful avenue to add nuance to our understanding of eusocial insect communication.

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Supplemental Information

Individuality Impacts Communication Success in Honey Bees

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Materials and Methods

Study Organism - individual marking, housing, and management

We studied two queenright experimental colonies of mixed European subspecies, primarily *Apis mellifera ligustica,* each containing approximately 3,000 workers plus brood. Since we were interested in identifying successful waggle dance communication partners (i.e., matching each newly recruited bee with the waggle dancer from whom she learned of the feeder's location), we needed to distinguish individual bees' identities for the duration of the experiment. We therefore marked each cold anesthetized worker before the start of the experiment with a unique identifier (UID) by assigning to her a combination of a colored, numbered disc tag (BetterBee, Greenwich, NY) adhered to her thorax and a stripe of color from a paint pen applied dorsally on the abdomen (POSCA, USA). We used sets of disc tags numbered 1-100 in five colors and paint pens in six colors, yielding 3,000 distinct UID combinations: a bee's UID was given as the disc color's initial, the disc number, and the abdomen stripe color's initial. For example, a bee with a blue tag with the number 8 on it and blue abdomen stripe would therefore be called “BB8.” Once marked, we placed each bee in a recovery chamber with the queen and with free access to sucrose solution until marking was complete. In all, we marked approximately 3000 workers from each colony, and we worked with two colonies sequentially.

Each observation hive was made up of three American Standard Deep frames installed in a wooden scaffold, one on top of the other such that the comb surface was in a single layer and covered by transparent Lexan to enable direct observations of activity on the comb. To support recruitment, we selected frames from the bees’ colony of origin that contained capped brood, sealed honey, and, most importantly, some empty cells, as it has been shown that a lack of empty cells leads to increased forager wait time for nectar unloading, which then decreases the likelihood of recruitment dances (Seeley 1989; Seeley 1995).

We transferred all the marked bees and the queen from the recovery chamber to the observation hive by connecting the recovery chamber to one of the observation hive’s ventilation openings with a short length of plastic tubing, gently placing the queen into the observation hive, and then allowing the marked bees to simply walk through the tube and into the hive following her scent. We housed the observation hives indoors at the Prices Fork Research Center in Blacksburg, Virginia, but allowed colonies free access to the outdoors via a plastic tube measuring approximately 5 cm x 30 cm. If any bees remained in the recovery chamber the following day, we shook the recovery chamber outside to of the observation hive entrance and allowed bees to find their own way into the colony.

We collected field data from July 11, 2022, to July 23, 2022, on days with good foraging weather. High summer tends to be a favorable time to train bees to feeders in our study area, since the relative dearth of floral resources in the landscape makes the provided sugar solution more attractive (Ohlinger et al. 2022). We provided colonies with supplemental sugar solution during periods of inclement weather, but we removed the food prior to data collection.

Feeder Training and Experimental Bee Recruitment

For each experimental colony, we first trained an initial cohort of foragers to collect 2 M sucrose solution scented with lavender (5 μL/L) from an artificial feeder station using previously established stepwise training protocols (Schürch et al. 2013; Couvillon et al. 2015; Schürch et al. 2016; Schürch et al. 2019; Ohlinger et al. 2022a; Couvillon et al. 2023). Briefly, the day before the experiment began (Day -1), we prepared a small box by placing a Petri dish inside it loaded with a few drops of the sucrose solution. We then placed the box on a tripod platform positioned so that the box covered the hive entrance, thus blocking bee access to the wider landscape but encouraging exiting foragers to explore the box and discover the food. The box had a transparent viewing window at the top that allowed us to see when at least three bees were collecting sucrose solution from the Petri dish. At that time, we gently removed the box and transferred the Petri dish directly to the tripod platform, still located just outside the hive entrance, taking care not to disturb the drinking bees. Then, once 3-5 individual bees had made at least three return visits to collect the solution, we replaced the Petri dish with the complete feeder setup, comprised of a 4-oz. Mason jar mounted on a plastic base with nectar collection wells that was on the tripod platform covered with a blue plastic tablecloth and raised 1 m off the ground.

In the stepwise training phase, we progressively moved the feeder farther from the colony across an open field in 10-12 sequential steps, with 10-15 meters between each step. To encourage the initial cohort of bees to continue foraging at the feeder as we increased the distance from colony, we kept the feeder at each step location until 5-15 individuals had visited at least three times to ensure they had learned the current feeder location and were tightly committed to it. We repeated this process until we reached the first experimental feeder location, which was 215 m in Trial 1 and 315 m in Trial 2. This typically took until late afternoon / early evening on Day -1. The accumulation of multiple visits at the final feeder location on Day -1 provided a highly rewarding experience for these foragers, thus increasing the likelihood that they would return to the feeder the next day and, more importantly, dance to recruit their sisters (Seeley 1995).

On our experimental day (Day 0), we re-erected the feeder at the first experimental feeder location, which we had reached at the end of the previous day. The Day 0 experimental phase began when the first bee from the Day -1 cohort returned to the feeder to collect sucrose solution, usually within 30 minutes of re-erecting the feeder. Importantly, we filled the Day 0 feeder with unscented 2 M sucrose solution to ensure that the new recruits would need to rely on waggle dance information, without any potential scent cues, to find the feeder (Tautz 2022). Because the solution at 2 M is highly rewarding, the Day -1 bees would very quickly begin to perform waggle dances, and soon newly recruited bees (Day 0 bees, who were of course already marked but new to the feeder) would begin to arrive. Our goal was for Day -1 bees to remember and reactivate to the feeder, and then to recruit via waggle dances a new cohort of naive experimental bees who would have been recruited with no prior knowledge of the feeder and in the absence of scent cues. Four researchers participated in Day 0 data collection: two people were stationed at the feeder to monitor and record foraging visits, and two additional people were stationed at the observation hive, one on each side, to monitor and record waggle dances on the dance floor, which we concurrently filmed (see below). We recorded each feeder visit and each waggle dance as the bee's UID and the time of the visit / dance using ODK Collect installed on Samsung electronic tablets.

Since our goal was to compare the waggle dance calibrations of Day –1 bees to those of their successful recruits (Day 0 bees), it was necessary that both Day –1 bees and the newly recruited Day 0 bees not only visit the feeder, but also perform dances so that we could quantify their waggle run durations at the two experimental distances, allowing us to calculate calibrations. To keep track of how many dances each bee had performed in real time, we used free online leaderboards (from www.keepthescore.com). When a new recruit arrived at the feeder, a researcher would add her UID as a "player" on the leaderboard with zero dances. Then, researchers monitoring the dance floor on either side of the observation hive updated each bee's tally of waggle dances by assigning her a "goal" each time she danced. These KeepTheScore dance tally data were used for decision-making in the field (see below) but are not presented here since primary dance data including timestamps were collected in ODK Collect. We allowed foraging and recruitment to continue until both the Day -1 bees and 10-20 new Day 0 recruits had all had performed at least 2-3 separate waggle dances advertising this first feeder location.

We then moved the feeder to a second experimental distance. For Trial 1, our first experimental feeder location was 215 m from the colony, and our second experimental feeder location was 315 m from the colony. For Trial 2, we reversed these locations (e.g., first experimental feeder location at 315 m) to control for any effect of shifting closer versus farther away from the colony. To avoid losing bee participants during the location shift, we used one intermediate feeder location per our stepwise training protocol, where we allowed each bee to make at least one feeder visit at this intermediate location. At the second experimental distance, we continued recording feeder visits and monitoring and filming both sides of the dance floor until each bee had performed at least 2-3 waggle dances for that second feeder location. Previous work had demonstrated that two distinct feeder distances are sufficient to calculate calibration curves, as waggle run duration accrues linearly with increasing distance from the colony in this distance range (Schürch et al. 2013; Schürch et al. 2016; Schürch et al. 2019). Importantly, we were fundamentally interested in comparing calibrations between real-life communication partners at our experimental distances, rather than projecting calibration curve regression lines out to hypothetical experimental distances.

Video recording and monitoring of observation hives and extraction and analysis of video data

We filmed the observation hives continuously during the Day 0 experimental phase. To do this, we set up two Canon Vixia G50 camcorders mounted on tripods with SanDisk Extreme 256 GB SD cards recording at 30 frames per second and the 3840x2160 resolution setting, one on either side of the observation hive. We set each camera approximately 1 meter from the comb surface, and focused the camera on the "dance floor," where most waggle dance activity occurs. We labeled and stored SD cards as original video data, but also backed up all video data to a cloud-based repository and an external storage device (Seagate One Touch with Hub, 8 TB).

Broadly, our waggle dance video analysis had two goals: (1) to match successful recruits with the dancer who recruited them, and (2) obtain calibration information (i.e., waggle run duration at the feeder distance at which the recruitment event occurred) for each bee in a successful pair. For our first goal of identifying successful dancer-follower pairs, we used the video data to match each Day 0 recruit bee who successfully found the feeder during the experimental phase with the dancer from whom she learned of its location. Specifically, for each bee whose UID first appeared on Day 0 (i.e., a Day 0 recruit with no prior knowledge of the feeder), we extracted the timestamp of her first visit to the feeder, and then examined the video data preceding that time in DaVinci Resolve 18 (Version 18.0.4, Build 5) to find the last waggle dance she followed before arriving at the feeder, which we termed the recruitment event. We defined a recruitment event as the last dance for which a follower bee followed at least 1 waggle run, or the information-rich subunit of the waggle dance (Seeley et al. 2000; Couvillon et al. 2012) from a bee advertising our experimental feeder before she then made her first recorded feeder visit (note: most bees follow more than one waggle run at recruitment events). We defined “following” as being within antennal contact distance with the dancer during a waggle run (Tanner and Visscher 2008; Grüter and Ratnieks 2011). For each recruitment event, we recorded the UID of the dancer, the number of waggle runs followed, the timestamp of the last frame at end of the last waggle run for which the follower was in antennal contact with the dancer, and the timestamp of the last frame in which any part of the experimental phase recruit’s body was visible in the video frame as she scurried hastily out of view in the direction of the hive exit and, ultimately, to the advertised feeder.

To achieve the second goal of extracting waggle dance calibration information for our experimental bees, we followed the dance decoding methods of Couvillon et al.[13] in DaVinci Resolve to obtain the waggle run duration values (measured in seconds) for each member of a successful dancer-follower pair. We decoded four non-first, non-last waggle runs with two left-lead-ins and two right-lead-ins for each dyad bee (both Day -1 dancers and Day 0 recruits), the arithmetic mean of which gives a representative estimation of that bee's waggle dance distance calibration (Couvillon et al. 2012). Whenever possible, we decoded dancer waggle runs from the same dance that the successful recruit herself followed to represent as closely as possible the information that the recruit received. Rarely were the dances from recruitment events ineligible for decoding (i.e., not enough waggle runs in the dance to get four non-first, non-last waggle runs with two left- and two right-lead-ins). In the very rare cases where this occurred, we selected waggle runs from that dancer's next eligible dance for that feeder location, as individuals dancing for the same location are precise across multiple, independent bouts of dancing (Schürch et al. 2016). For the successful followers, we selected waggle runs to decode from dances advertising the same feeder distance at which they were recruited. Specifically, whenever possible, we selected waggle runs from the first dance where our waggle dance decoding criteria could be met, and which occurred after the recruit had made at least three feeder visits at that feeder distance (Chatterjee et al. 2019). This was possible in the vast majority of cases.

Statistical Analysis and Data Visualization

A key statistical challenge in this experiment was the impossibility of comparing calibration values for successful versus unsuccessful pairs of dancers and followers: we could not obtain calibration information for both members of unsuccessful pairs, as followers who failed to find the feeder would not dance for it and would not be represented in our data. To address this problem, we instead chose to calculate from our data an observed distribution of waggle run duration differences in successful pairs and to compare this distribution to a simulated expected distribution of waggle run duration differences based on the random assignment of calibration values to hypothetical dancers and followers. To obtain the observed distribution of waggle run duration differences, we simply subtracted the waggle run duration value of the successful follower from that of the dancer that recruited her for each of our successful dancer-follower pairs. We compared this observed distribution to a simulated distribution of calibration differences constructed with randomly assigned waggle run distance values, which we obtained using the bootstrapped honey bee calibration data object representing the universal waggle dance calibration (Schürch et al. 2019a). We assigned a bootstrapped waggle run duration value to each member of 1,000 hypothetical dancer-follower pairs at random for both of our experimental feeder distances. Then we subtracted the simulated followers' duration values from those of the simulated dancers to obtain an expected duration difference distribution against which to compare our observed duration difference distribution.

To confirm that the raw waggle dance calibration values we observed for our experimental bees were well-represented by the universal waggle dance calibration data used to generate the expected distribution, we compared our raw waggle dance duration values to a bootstrapped sample of 1,000 waggle run duration values for the same distance (Schürch et al. 2019a) using a two-tailed Kolmogorov-Smirnov test using the ks.test() function in R.

Next we used a one-tailed Kolmogorov-Smirnov test in R using the ks.test() function to test whether our observed waggle run duration difference distribution was statistically distinct (and, more specifically, right-shifted) from the expected distribution. Since we predicted that follower bees who likely experienced a slight initial overshoot of the resource based on calibration difference would have a higher recruitment success rate after having just passed the resource once on the wing, we specified the "greater" alternative hypothesis in ks.test() to represent this prediction.

Additionally, it is important to note that we chose the Kolmogorov-Smirnov test as a non-parametric test to handle non-independence in our successful dyad data. This non-independence is present due to the structure of recruitment, where successful followers go on to dance and recruit more nestmates, and therefore may appear in the data as both a successful follower and later as a dancer. Additionally, bees who successfully recruited multiple nestmates to the feeder may appear in the successful dyad dataset as dancers multiple times, if those recruited nestmates also went on to dance and were eligible for analysis. As a result, our successful 30 dyads are made up of 40 individual bees. While we considered excluding dyads whose members already appeared in the data, doing so would have drastically curtailed our sample size and statistical power as bee training is difficult in the best of conditions: while 106 bees were recruited to the feeder in the experimental phase across both trials, only 30 of those danced and were eligible for analysis as successful followers. We therefore chose to accept this non-independence associated with the commutative properties of waggle dance communication, and to account for it with a non-parametric approach. The Kolmogorov-Smirnov test is non-parametric and therefore a suitable choice for our analysis.

When developing our data visualization, we chose to highlight data from a representative successful dyad to help illustrate our hypothesis and interpretation of the data. To select a representative dyad, we first calculated the mismatch value for each dyad as the raw waggle run duration difference in seconds (dancer – follower) divided by the waggle run duration of that dancer. (This can also be thought of the predicted percent overshoot, where a negative value would indicate a predicted undershoot and a positive value a predicted overshoot). We then selected the dyad possessing the median mismatch value to highlight in our data visualization.

We carried out all data cleaning, analysis, and visualization in R version 4.4.1 (R Core Team 2024) with the use of the following R packages: knitr v. 1.46 (Xie 2014; Xie 2015; Xie 2024), rmarkdown v. 2.26 (Xie et al. 2018; Xie et al. 2020; Allaire et al. 2024) and tidyverse v. 2.0.0 (Wickham et al. 2019). We used the package grateful v. 0.2.4 (Rodriguez-Sanchez and Jackson 2023) to facilitate proper citation of all R packages used.

Data Availability

All data and analysis code will be made available with dedicated static DOIs, maintained at Virginia Tech's Library, upon acceptance.

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CHAPTER 3

DANCING ON THE EDGE: HONEY BEE WAGGLE DANCE NETWORKS ARE SPARSE AND BIMODALLY BURSTY

Manuscript in Preparation

Dancing on the edge: honey bee waggle dance networks are sparse and bimodally bursty

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Abstract

Social network analysis is increasingly and fruitfully applied to study animal societies' collective structure and function across space and time. Eusocial insects such as honey bees (*Apis mellifera* L.) offer tractable model systems rich in social relationships and dynamics. Despite the vast body of literature describing the honey bee's social life and communication behaviors, including the famous waggle dance by which foragers recruit nestmates to profitable resources, more about the foraging recruitment network(s) that arise from waggle dance communication is needed. Here, we conducted a field experiment with fully marked experimental colonies (N=2 colonies, 3,000 bees each) to characterize the honey bee waggle dance recruitment network structure concerning network density, burstiness in waggle dance bouts, and the role of individuality in communication behavior. First, we found that each dancer successfully recruited only a tiny proportion of unoccupied nestmates, resulting in sparse recruitment networks. This remained true even when we corrected density values by comparing our observed networks to a simulated graph of maximal honey bee recruitment as a biologically-informed density upper bound (experimentally observed density values: 1.5-3.5% of the edges predicted). Second, we found that one group of bees performed waggle dance bouts at regular intervals (non-bursty dancing), while another group did so at irregular intervals (bursty dancing). K-means clustering analysis yielded a between-cluster ratio of 65.9% indicating good separation between the two dance burstiness groups. Finally, we found that individuality in waggle dance communication behavior did not have a detectable effect on recruitment network formation (QAP regression, t = -1.09, p = 0.28). Our results offer the first empirical and biologically-informed network descriptive statistics for honey bee waggle dance and may be informative in the parameterization of bio-inspired computing models.

Introduction

Social network analysis and its application in biological research is a burgeoning field, increasing our understanding of how information is spread in animal societies (Krause et al. 2007; Finn et al. 2019; Firth 2020). Information comes in one of two main varieties: private information, which is obtained by an individual's sampling of the environment (Dall et al. 2005), and public information, which is gleaned through interaction with other group members or their products (Danchin et al. 2004; Dall et al. 2005). In publicly available information, the signal receiver has a choice about whether to alter their behavior based on the information contained in that signal, and/or to propagate the signal

(Dall et al. 2005; Schmidt et al. 2010). The successful transfer of information also usually has a spatial component, where individuals must be within some sensory distance of one another for the signal to reach a receiver (Danchin et al. 2004; Drosopoulos and Claridge 2005).

While these basic principles operate in any communication network, the biology, physiology, and ecological context of the group or species in question profoundly influence how these principles' effects are realized (Seyfarth and Cheney 2003; Drosopoulos and Claridge 2005). For instance, some signal givers may be choosy about when and with whom to communicate (e.g., a stock broker who only shares an insider tip with a close friend). In contrast, other signal givers may be physiologically incapable of modulating their communication to reach specific targets. Therefore, they are obligate signal broadcasters (e.g. insects stridulating to attract a mate cannot target a specific prospect). Receiver behavior is likewise influenced by bio-physiology. For instance, in mammals and birds, the likelihood that a receiver will act on information obtained is shaped by social familiarity (Valsecchi et al. 1996 (gerbils); Kavaliers et al. 2005 (deer mice) and social rank relative to the signaler (Radford 2004 (hoopoes); Kavaliers et al. 2005; Evans et al. 2018 (finches)). In summary, the factors that shape communication networks are as varied as the suite of species on this planet that communicate, and analysis of social network structure and function must, therefore, be carefully informed by the biology and social learning strategy of the organism in question (Cairns and Schwager 1987; Alves et al. 2023).

A growing area of interest in social network analysis is the role of consistent, inter-individual differences between network actors (Croft et al. 2009; Pinter-Wollman et al. 2011; Firth et al. 2015; Easter et al. 2022). While some types of inter-individual variation can be explained by age, sex, position in the social hierarchy, and/or other environmental factors (Silk et al. 2006; Monclus et al. 2012; Seyfarth et al. 2014; Bouchet et al. 2017), other types may consist of behavioral traits that are particular to individuals and are conserved through time and across contexts, often referred to as behavioral types or personalities (Sih et al. 2004; Sih and Watters 2005). Our understanding of the effect of behavioral types in animal social networks is at its infancy (Hasenjager and Dugatkin 2015), but see Schürch et al. (2010) for an early example in a highly social animal.

Eusocial insects, where groups of bees, ants, some wasps, and termites live and work together in organized societies, offer particularly rich systems in which to study social networks (Wey et al. 2008; Krause et al. 2009; Grüter and Leadbeater 2014; O'Shea-Weller 2021; Hasenjager et al. 2022). Relationships between individuals in these societies are multiplex (i.e., many types of social connections may exist simultaneously and in interaction with one another) (Finn et al. 2019; Hasenjager 2020), and social networks arising from these relationships are often volitional (i.e., connections are related to signaler and/or receiver choice) rather than based on spatiotemporal proximity, such as a network formed by strangers sitting next to one another on an airplane. In these complicated systems, network analyses can yield insights into animal social life unavailable via other analytical approaches (Rosenthal et al. 2018; Finn et al. 2019; Beisner et al. 2020; Hasenjager et al. 2022).

The honey bee (*Apis mellifera* L.) is a eusocial insect that lives in large colonies comprising tens of thousands of individuals, mainly female workers, and headed by a single queen (Seeley 1985). Honey bees also possess division of labor (von Frisch 1967; Michener 1969; Seeley 1983; Beshers and Fewell 2001; Robinson 2009) and complex, well-studied signaling behaviors (reviewed in Hasenjager et al. 2022) that are experimentally-tractable, in part because of their visibility through the clear-walled observation hives where bees may be housed (Tautz and Sandeman 2003; Schürch et al. 2013; Couvillon et al. 2012a; Couvillon et al. 2015; Wario et al. 2017; Carr-Markell et al. 2020; Ohlinger et al. 2022a; Dong et al. 2023). Most importantly, honey bees possess one of the most highly-celebrated communication behaviors in the animal kingdom, the waggle dance.

In the dance, a worker bee who has discovered a profitable resource in the landscape, usually floral nectar or pollen, can inform her nestmates of both a distance and a direction to that resource (von Frisch 1967; Seeley 1995; Couvillon 2012). To do this, she repeatedly shakes her body energetically in a figure-of-eight on the vertical comb surface, with the information-rich repeating subunit of the behavior being the waggle run, which occurs in the middle of the eight. The angle of the waggle run's trajectory encodes the direction to the resource she is advertising, where the vertical corresponds to the sun's azimuth at the time of the dance. The duration of the waggle run in seconds is proportional to the resource's distance from the colony (von Frisch 1967; Couvillon 2012). Bees performing waggle dances (signal givers) move on the comb through crowds of nestmates (potential signal receivers), frequently bumping into and being likewise bumped by nestmates. The waggle dance is, therefore, a broadcast signal (Nieh and Tautz 2000), but with a relatively short spatial range, with 90% of followers attracted from within 3-6 cell diameters, or 18-27 mm distance from the dancer (Tautz and Rohrseitz 1998; Kietzman and Visscher 2019). Additionally, unlike in some Hymenopteran recruitment behaviors such as tandem running, where naïve ants are led bodily to a profitable resource by a knowledgeable nestmate (Franks and Richardson 2006; Franklin 2014; Sasaki et al. 2020), dance followers attempt to locate the resource in the field without direct aid (von Frisch 1967; Seeley 1995). Therefore, the successful transmission of waggle dance information is a multi-step process. A follower bee must first (1) opt to closely attend a dance signal occurring in her vicinity, (2) decide to act on the information she has received, and (3) successfully locate the resource outside the hive, sometimes at as far as 10-12 kilometers (von Frisch 1967).

Recent evidence further suggests that inter-individual variation may also play a part in the recruitment network. Each forager bee possesses a distinct and conserved waggle run distance-duration calibration (hereafter, calibration) that is particular unto herself: different individual bees dancing to advertise the same resource location will consistently perform waggle runs that differ in duration (Schürch et al. 2016). In other words, each bee measures the real-world distance she perceived from the resource to the colony (Srinivasan 1992; Esch et al. 2001) and scales it to a waggle run duration according to her own calibration. Given that these inter-individual differences are distinct and conserved, individuality in waggle run calibration might constitute a form of a behavioral type. Furthermore, it was recently demonstrated that calibration differences between a dancer and a follower can affect the likelihood of recruitment success (McHenry et al., in review), indicating that individuality may affect the structure and function of social networks.

Since Karl von Frisch's description of the waggle dance (von Frisch 1946; von Frisch 1967, decades of research have described the form and function of the dance (reviewed in Seeley 1995; Anderson and Ratnieks 1999; Couvillon 2012; Kietzman and Visscher 2015). However, descriptions of the network(s) of information flow arising from dancer-follower (or dyad-level) interactions are limited, mostly because of methodological challenges: while it may be relatively simple to install bees in an observation hive, the logistical difficulty of detecting and tracking interactions among uniquely-identifiable individuals in colonies consisting of thousands of indistinguishable members and then handling the volumes of resulting data poses a massive challenge and has been, up to now, largely impossible (Hasenjager et al. 2022; although see Biesmeijer and Seeley 2005, Wild et al. 2021, and Liberti et al. 2022). As a result, relatively little is known about the structure and properties of waggle dance communication networks and the factors that influence them.

Here we conducted a field experiment with fully-marked experimental colonies of honey bees housed in clear-walled hives to track the formation of real waggle dance communication networks *in vivo.* We took two analytical approaches in exploring the properties of the resulting network data: (1) basic and biologically-informed network descriptions, and (2) hypothesis-driven data analysis. Specifically, we (1a) calculated and report here representative network density using a biologically-informed simulated upper-bound, (1b) report patterns and clustering of burstiness in bees' waggle dance behavior; and (2) test whether individuality in waggle dance communication (Schürch et al. 2016), which has been shown to affect communication outcomes between a given dancer and follower (McHenry et al. in review), may also affect the structure of the network that emerges from dyad level interactions.

Materials and Methods

Study Organism, Individual Marking, and Colony Management

We studied two queenright experimental honey bee colonies of mixed European subspecies (*Apis mellifera subsp.*), each containing approximately 3,000 workers plus brood. Since we were interested in tracking the flow of information from bee to bee via waggle dance communications, bees needed to be individually identifiable from the experiment's outset. To that end, we uniquely marked each cold-anesthetized worker with a unique identifier (UID). Each UID consisted of a combination of a colored, numbered disc tag (BetterBee, Greenwich, NY) adhered to the thorax and a stripe of color from a paint pen (POSCA, USA) applied dorsally to the abdomen. We used sets of disc tags numbered 1-100 in five colors and paint pens in six colors to yield 3,000 distinct UID values, where a bee's UID was given as the abdomen stripe initial followed by the disc color's initial and number. For example, a bee with a blue abdomen stripe and a blue disc tag number eight would be referred to as "BB8." Once marked, we placed each bee in a recovery chamber with the queen and free access to sucrose solution until marking was complete. In all, we marked approximately 3,000 workers from each colony. Workers were installed together in an observation hive with the queen after marking was complete, usually by midafternoon on the second day. We created and worked sequentially with two fully-marked colonies.

Each observation hive comprised three American Standard Deep frames installed in a wooden scaffold, one on top of the other such that the comb surface was in a single layer and covered by transparent Lexan to enable direct observations of activity on the comb. To support recruitment, we selected frames from the bees' colony of origin that contained capped brood, sealed honey, and, most importantly, some empty cells, as it has been shown that a lack of empty cells leads to increased forager wait time for nectar unloading, which then decreases the likelihood of recruitment dances (Seeley 1989; Seeley 1995).

We transferred all the marked bees and the queen from the recovery chamber to the observation hive by connecting the recovery chamber to one of the observation hive's ventilation openings with a short length of plastic tubing, gently placing the queen into the observation hive, and then allowing the marked bees to simply walk through the tube and into the hive following her scent. We placed the observation hives indoors at the Prices Fork Research Center in Blacksburg, Virginia, but allowed colonies free access to the outdoors via a plastic tube measuring approximately 5 cm x 30 cm. If any bees remained in the recovery chamber the following day, we shook the recovery chamber outside of the observation hive entrance and allowed bees to find their own way into the colony.

We collected field data from July 11, 2022, to July 23, 2022, on days with good foraging weather. High summer tends to be a favorable time to train bees to feeders in our study area, since the relative dearth of floral resources in the landscape makes the training sugar solution more attractive (Seeley et al. 1991; Ohlinger et al. 2022). We provided colonies with supplemental feeding during periods of inclement weather, but we removed the food prior to data collection.

Key Network Terms and Definitions

The field of network analysis has its own vocabulary that is not commonplace even in closely related fields. Here we briefly define some key terms that we use to describe our networks and analyses:

Node: an individual actor in a network (e.g., a bee).

Edge, Tie: (synonymous) a connection between two nodes.

Dyad: Any two connected nodes.

Directed edge/tie: a connection between nodes that has a directional component, originating at one node and terminating at another (e.g., the flow of information from a waggle dance recruiter to her successful dance follower).

Network, Graph: (synonymous) a dataset comprised of nodes and the edges that connect them.

Directed network: A network with directed ties.

Complete Network / Graph: (synonymous) a network in which all possible edges exist (i.e., every node is connected to every other node). These graphs possess density values of 1 (see below)

Subgraph: a subset of a larger network.

Pendant: a node that is connected to the network by only one tie.

Isolate: a node that is part of the network but not connected to any other node

Edge list: a network data storage format with two columns, where each row represents an edge and the column values specify the nodes connected by that edge. In other words, this is a list of edges.

Adjacency Matrix: a matrix where the each *ij* cell stores dyadic information about node[*i*] and node[*j*]. Most often, a 1 denotes the presence of a tie between nodes[i, j] while a 0 indicates absence of a tie. When networks are directed, the underlying adjacency matrix is **asymmetric** (i.e. bee 1 recruited bee 2, but bee 2 did not recruit bee 1).

Density: For a given network, the proportion of observed edges to all possible edges. A graph wherein all nodes are connected to all other nodes (i.e. a complete graph, see above) has a density value of 1.

Burstiness: A measure of the temporal clustering of events, where burstiness is proportional to the variance of inter-event times.

Network Time: The duration of time between an individual's first and last network event (here: feeder visit or waggle dance), excluding down time (here: when the feeder was not set up).

Day -1 Pre-Training Phase: Feeder Training and Recruitment of Foundress Bees for Network Establishment

For each experimental colony, we trained bees to forage at an artificial feeder station in two phases: the pre-training phase and the experimental phase. In the pre-training phase, we trained an initial cohort of foragers to collect 2 M sucrose solution scented with lavender (5 µL/L) from our artificial feeder station using previously established stepwise training protocols (Schürch et al. 2013; Couvillon et al. 2015; Schürch et al. 2016; Schürch et al. 2019; Ohlinger et al. 2022; Couvillon et al. 2023), which we also describe briefly below. This initial pre-training cohort would become our "foundress" bees, so named because they would go on "found" their own waggle dance communication networks during the experimental phase.

Briefly, during pre-training on the day before the experiment began (Day -1), we prepared a small box by placing a Petri dish inside it loaded with a few drops of the sucrose solution. We then placed the box on a tripod platform positioned so that the box covered the hive entrance, thus blocking bee access to the wider landscape but encouraging exiting foragers to explore the box and discover the food. The box had a transparent viewing window at the top that allowed us to see when at least three bees were collecting sucrose solution from the Petri dish. At that time, we gently removed the box and transferred the Petri dish directly to the tripod platform, still located just outside the hive entrance, taking care not to disturb the drinking bees. Then, once 3-5 individual bees had made at least three return visits to collect the solution, we replaced the Petri dish with the complete feeder setup, comprised of an artificial feeder (a 4-oz. Mason jar mounted on a plastic base with nectar collection wells) on the tripod platform that was covered with a blue plastic tablecloth and raised 1 meter off the ground.

In a stepwise fashion, we progressively moved the feeder farther from the colony across an open field in 10-12 sequential steps, with 10-15 meters between each step. To encourage the initial cohort of bees to continue foraging at the feeder as we increased the distance from the colony, we kept the feeder at each step location until 5-15 individuals had visited at least three times to ensure they had learned the current location and were tightly committed to it. We repeated this process until we reached the first experimental feeder location, which was 215 m in Trial 1 and 315 m in Trial 2 (see below our explanation of the different distances). This typically took until late afternoon / early evening on Day -1. The accumulation of multiple visits at the final feeder location on Day -1 provided a highly rewarding experience for these foragers, thus increasing the likelihood that they would return to the feeder the next day to participate in the experiment as a foundress bee, dancing to recruit her sisters (Seeley 1995).

Day 0 Experimental Phase: Recruitment of Nestmates by Foundress Bees

During our experimental phase (Day 0), we re-erected the feeder at the first experimental feeder location, which we had reached at the end of Day -1. The Day 0 experimental phase began when the first bee from the previous day's cohort returned to the feeder to collect sucrose solution, usually within 30 minutes of re-erecting the feeder. Importantly, we filled the Day 0 feeder with unscented 2 M sucrose solution to ensure that the new recruits would need to rely on waggle dance information, without any potential scent cues, to find the feeder (Tautz 2022). Because the solution at 2 M is highly rewarding (Pamminger et al. 2019), most of the Day -1 bees would very quickly begin to perform waggle dances, and soon newly recruited bees (Day 0 bees, who were of course already marked but new to the feeder) would begin to arrive. Our goal was for Day -1 bees to remember and reactivate to the feeder, and then to recruit via waggle dances a new cohort of naive experimental bees who would have been recruited with no prior knowledge of the feeder and in the absence of scent cues. Four researchers participated in Day 0 data collection: two people were stationed at the feeder to monitor and record foraging visits, and two additional people were stationed at the observation hive, one on each side, to monitor and record waggle dances on the dance floor, which we concurrently filmed (see below). We recorded each feeder visit and each waggle dance as the bee's UID and the time of the visit / dance using ODK Collect installed on Samsung electronic tablets.

Since we were interested in studying the role of waggle dance calibration on network formation, it was necessary that both foundress bees and the newly recruited Day 0 bees not only visit the feeder, but also perform dances. To keep track of how many dances each bee had performed in real time, we used free online leaderboards (from www.keepthescore.com). When a new recruit arrived at the feeder, a researcher would add her UID as a "player" on the leaderboard with zero dances. Then, researchers monitoring the dance floor on either side of the observation hive updated each bee's tally of waggle dances by assigning her a "goal" each time she danced. These KeepTheScore dance tally data were used for decision-making in the field (see below) but are not presented here since primary dance data including timestamps were collected in ODK Collect (see above). We allowed foraging and recruitment to continue until both foundress bees and 10-20 new Day 0 recruits had all performed at least 2-3 unique waggle dances advertising the first feeder location.

Since we wanted to calculate individual calibration curves (Schürch et al. 2016) for our experimental bees, we then moved the feeder to a second experimental distance. Previous work had demonstrated that two distinct feeder distances are sufficient to calculate calibration curves, as waggle run duration accrues linearly with increasing distance from the colony (Schürch et al. 2013; Schürch et al. 2016; Schürch et al. 2019). For Trial 1, our first experimental feeder location was 215 m from the colony, and our second experimental feeder location was 315 m from the colony. For Trial 2, we reversed these locations (i.e., first experimental feeder location at 315 m) to control for any effect of shifting closer versus farther away from the colony. To avoid losing bee participants during the location shift, we used one intermediate feeder location per our stepwise training protocol, where we allowed each bee to make at least one feeder visit. As before, at the second experimental distance, we continued recording feeder visits and monitoring and filming both sides of the dance floor until each bee had performed at least 2-3 waggle dances for that second feeder location.

Obtaining adequate sample sizes in our experimental set up proved extremely challenging. In both trials, a wave of late-stage, new Day 0 recruits would arrive at the feeder at the second experimental distance towards the end of the trial. When this occurred, in an effort to increase our sample size of calibratable bees, we would continue collecting data and, after this fresh wave had performed the requisite 2-3 dances, move the feeder back to the first experimental distance with a stopover halfway between, as described above. As a result, in both trials the feeder location toggled back and forth between Distance 1 (215 meters) and Distance 2 (315 meters) over the course of the experimental phase.

Video Recording and Monitoring of Observation Hives, Extraction of Waggle Dance Data from Video

We filmed the observation hives continuously during the Day 0 experimental phase. To do this, we set up two Canon Vixia G50 camcorders mounted on tripods with SanDisk Extreme 256 GB SD cards recording at 30 frames per second and the 3840x2160 resolution setting, one on either side of the observation hive. We set each camera approximately 1 meter from the comb surface to reduce parallax and focused the camera on the “dance floor,” where most waggle dance activity occurs. We labeled and stored

SD cards as original video data, but also backed up all video data to a cloud-based repository and an external storage device (Seagate One Touch with Hub, 8 TB).

Our waggle dance video data extraction had two main goals: (1) to match each Day 0 recruit with the dancer who recruited her, and, when possible, to (2) obtain dancers' calibrations. For our first goal of identifying recruitment relationships, we used the video data to match each Day 0 recruit bee who successfully found the feeder during the experimental phase with the dancer from whom she learned of its location (recall that this dancer could have been a foundress bee or a fellow Day 0 recruit). Specifically, for each bee whose UID first appeared on Day 0 (i.e., a Day 0 recruit with no prior knowledge of the feeder), we extracted the timestamp of her first visit to the feeder, and then examined the video data preceding that time in DaVinci Resolve 18 (Version 18.0.4, Build 5) to find the last waggle dance she followed before arriving at the feeder, which we termed the recruitment event. Specifically, we defined a recruitment event as the last dance for which a follower bee followed at least 1 waggle run, or the information-rich subunit of the waggle dance (Seeley et al 2000; Couvillon et al. 2012) from a bee advertising our experimental feeder before she then made her first recorded feeder visit (note: most bees follow more than one waggle run at recruitment events). We defined "following" as being within antennal contact distance with the dancer during a waggle run (Rohrseitz and Tautz 1999; Tanner and Visscher 2008; Grüter and Ratnieks 2011). For each recruitment event, we recorded the UID of the dancer, the number of waggle runs followed, the timestamp of the last frame at end of the last waggle run for which the follower was in antennal contact with the dancer, and the timestamp of the last frame in which any part of the experimental phase recruit’s body was visible in the video frame as she scurried hastily out of view in the direction of the hive exit and, ultimately, to the advertised feeder (See Supplementary Online for these data).

To achieve the second goal of obtaining waggle dance calibrations for our experimental bees, we followed the dance decoding methods of Couvillon et al. (2012) in DaVinci Resolve to obtain the waggle run duration values, measured in seconds, for each member of a successful dancer-follower dyad (i.e., each non-isolate node) at each feeder distance. We decoded four non-first, non-last waggle runs with two left-lead-ins and two right-lead-ins for each dyad bee (both Day -1 dancers and Day 0 recruits), the arithmetic mean of which gives a representative estimation of that bee's waggle dance distance calibration (Couvillon et al. 2012).

Our original goal, as described above, had been to calculate linear individual calibration curves using waggle run duration data for each bee at each feeder distances. However, we discovered that only a fraction of the bees that comprised the successful recruitment network (i.e., bees that were members of successful dancer-follower dyads) had performed dances at both locations. This occurred because, in the field, it was impossible to tell in real-time which follower had been recruited by which dancer. Therefore, while we achieved our target sample size for bees that danced for both feeder locations, it so happened that many bees recruited to the feeder got their information from dancers who only danced for that one feeder location. This presented a challenge in terms of how to compare calibrations among bees in the communication network.

We therefore adapted our approach to calculating bees' calibrations as follows. For each successful dancer-follower dyad, we first obtained both the dancer's and follower's waggle run duration advertising the feeder distance at which the recruitment event occurred. Whenever possible, we decoded dancer waggle runs from the same dance that the successful recruit herself followed to represent as closely as possible the information that the recruit received. Rarely were the dances from recruitment events ineligible for decoding (i.e., not enough waggle runs in the dance to get four non-first, non-last waggle runs with two left- and two right-lead-ins). In the very rare cases where this occurred, we selected waggle runs from that dancer's next eligible dance for that feeder location, as individuals dancing for the same location are precise across multiple, independent bouts of dancing (Schürch et al. 2016). For the successful followers, we selected waggle runs to decode from dances advertising the same feeder distance at which they were recruited. Specifically, whenever possible, we selected waggle runs from the first dance where our waggle dance decoding criteria could be met, and which occurred after the recruit had made at least three consecutive feeder visits at that feeder distance (Chatterjee et al. 2019), which had the added benefit of allowing bees to update their distance information if the first eligible decodeable dance occurred after we had toggled between Distance 1 and Distance 2. We were able to meet these criteria in the vast majority of cases.

Finally, we scaled the data in order to control for having two experimental feeder locations represented therein. We first separated our observed waggle run duration data by distance category, and then joined them to a data frame containing a set of 1000 randomly selected, simulated waggle run duration values from the universal waggle dance calibration data object (Schürch et al. 2019) for the appropriate experimental distance. We then conducted scaling on each distance category's full dataset including simulated and observed values before re-extracting our now-scaled observed values. For our analysis, we used these scaled waggle run duration values as calibration values for experimental bees.

Network Construction

To construct the waggle dance communication network, we used the dancer-follower relationship datasets obtained from video analysis (Goal 1, see above) to create a **network edge** list using the igraph R package (Csárdi and Nepusz 2006; Csárdi et al. 2024). We represented recruitment relationships as directed edges, with the edge pointing from dancer to follower along with the flow of information. We also added **isolate nodes** to the network, which we considered to be foundress bees that continued visiting the feeder during the experimental phase but did not recruit any nestmates to it, whether from lack of dancing or lack of success. We excluded from the network any bee that participated in the pre-training phase but did not continue visiting the feeder after the start of the experimental phase.

While we could have begun filming from the very beginning at the start of the pre-training phase to capture recruitment events from the initial resource discovery, we opted instead to analyze the network components that emerged after the start of the experimental phase, treating each bee that had participated in the pre-training phase as a possible foundress of her own communication network (hence the term “foundress” bee). A key advantage of this approach was the ability to treat each foundress bee as an ego under the ego-centric network analysis framework, thereby allowing us to consider each network component initialized by a given foundress bee as a pseudo-replicate of a waggle dance communication network. This is a common and accepted way of achieving replicates in network data (Fisher 2005), and was an appealing option given the extreme labor demands of marking each member of a 3,000-bee experimental colony for each true replicate.

Statistical Analysis and Data Visualization

We carried out all data cleaning, variable calculation, analysis, and visualization in R version 4.4.2 (R Core Team 2024) and the following R packages: igraph v. 2.0.3 (Csárdi and Nepusz 2006; Csárdi et al. 2024), knitr v. 1.46 (Xie 2014, 2015, 2024), rmarkdown v. 2.26 (Xie et al. 2018; Xie et al. 2020; Allaire et al. 2024), tools v. 4.4.1 (R Core Team 2024b). We used the package grateful v. 0.2.4 (Rodriguez-Sanchez and Jackson 2023) to facilitate proper citation of all R packages used.

Network Density and Simulation of a Complete Graph as a Biologically Relevant Upper Bound By Reimagining an SI (Susceptible-Infected) Discrete Compartment Model (DCM)

Network density is defined as the proportion of observed ties relative to the maximum number of ties possible for that network, which is also referred to as a **complete graph** (i.e. if every node were connected to every other node) (Bang-Jensen and Gutin 2018).

Density is a bread-and-butter network descriptive statistic, as it gives an at-a-glance sense of the level of connectivity or sparseness of a network. However, we considered that the case of a complete graph as a baseline comparison to our observed number of ties would be biologically meaningless, as there is no real-world scenario where every bee recruits every other bee to the same food source. Rather, a **biologically-informed complete graph** (hereafter referred to as BICG) of honey bee foraging would be one where every dancing bee had a perfect record of successfully recruiting every nestmate she encountered.

We therefore set out to simulate a BICG as an empirical estimate of an upper bound on network edges for a honey bee recruitment network using a deterministic compartmental model (DCM) of the susceptible-infected (SI) model family (Anderson & May 1992; Gernat et al. 2018). Traditionally formulated deterministic SI models feature individuals that are in one of two states: either "susceptible" or "infected," and an infected individual may infect susceptible individuals when they come in contact based on a given probability value that is specified in the model. We reimagined an SI model in the context of honey bee waggle dance communication, wherein "infected" bees are those with knowledge of a food resource, "susceptible" bees are nestmates that follow dance communications, in part because they are currently unemployed foraging elsewhere, and an "infection" would represent a successful recruitment to the resource of a susceptible bee by an infected bee. To simulate BICG of honey bee foraging, we set the "infection" probability to 1. The resulting network simulation therefore represents what the recruitment network might look like if bees were 100% effective at recruiting every nestmate they contacted while dancing – a more biologically relevant and informative upper bound that we then used in place of a complete graph in calculating network density.

Since density is calculated with respect to the complete graph for the set of nodes and edges belonging to the observed network, we parameterized and simulated a BICG for both trials. To do this, we used the dcm() function from the R package EpiModel. We specified the number of initially-infected individuals for each trial as the number of foundress bees that made at least one feeder visit during the experimental phase, since these were bees that had knowledge of the feeder location and thus had the opportunity to advertise it. We set total population as 1,000 individuals, since colonies were created with 3,000 marked bees and roughly one-third of the workers within a colony may be involved in foraging activities at any given time (Seeley 1995).

Here we calculated classical density for each trial using both conventional complete graphs, and corrected density using our BICG as upper bounds. For the sake of comparability between classical and BICG (corrected) density values, we first added dummy isolate nodes to our observed networks to get the node total to 1,000. We then supplied that graph object to the edge_density() function from the igraph package to obtain classical density for each trial. Then we calculated a corrected density for each trial by dividing the number of edges in the observed graph by the number of edges in the BICG.

Burstiness of Waggle Dancing

Burstiness is a concept used to describe the temporal clustering of events in a time series. In the context of network analysis, it refers to the phenomenon where nodes exhibit alternating periods of high and low activity (Barabási 2005). This pattern indicates that events or interactions occur in clusters, and it deviates from a uniform or random distribution of actions over time. While literature agrees that burstiness in behavior can significantly impact the dynamics and structure of a network and influence how information or influence spreads, the nature of its effect on the dynamic spread of information across networks varies widely based on the topology and function of the network in question (Lambiotte et al. 2013; Bakhouya and Gaber 2015; Doi et al. 2023). While burstiness is associated with slowed spread in some human communication networks (Karsai et al. 2011), burstiness in honey bee trophallaxis networks has been shown to be associated with a speedier spread of a simulated pathogenic infection (Gernat et al. 2017). To our knowledge, no work has yet explored either the existence or role of burstiness in the context of honey bee waggle dance behavior.

To explore burstiness in waggle dance behavior, we first created a timeline of waggle dance bouts for each bee. A bee was eligible for burstiness analysis if she performed at least two waggle dances during the experimental phase, since the burstiness coefficient (B) is calculated based on inter-event times. We therefore extracted the timelines of only bees who met these criteria, and then we calculated (B) for each. Preliminary density plot visualization of (B) indicated the possible presence of a bimodal distribution, and we therefore tested for clustering in (B) values using both a Gaussian Mixture Modeling (GMM) approach and a K-Means Clustering Analysis.

Having found strong support for two non-spherical clusters in our burstiness data with our GMM and K-Means analysis, we then applied a binomial mixed effects modeling approach using glmer() from the lme4 R package to explore whether we might be able to uncover node attributes related to burstiness cluster membership. Usually a bee will make several visits to a food source before beginning to advertise it with waggle dances, and thereafter her dance frequency tends to increase as she accrues experience with that food source until she is in a regular pattern of alternating feeder visits and waggle dances (Seeley 1995). We therefore modeled GMM cluster membership with respect to bees' time spent in the network (**network time**, which we defined and calculated as the cumulative hours between a bee's first and last network event while the feeder was set up) as a continuous numeric predictor, reasoning that bees who spent longer in the network would have reached a regular rhythm of feeder visits and dances and would therefore have lower burstiness than bees that had less time in the network. We also included a bee's status as foundress or Day 0 recruit as a binary predictor variable to account for the fact that no pre-training dances were recorded for these bees, and their dance timelines were therefore truncated at the front end. Lastly, we specified Trial as a random effect to control for any possible inter-colony differences. After fitting the full model, we then used backwards-elimination of non-significant terms and comparison of the resulting models' Akaike's Information Criterion (AIC) to select the model that yielded the best balance of complexity with explanatory power.

In recognition of the uncertainty introduced by foundress bees' missing pre-training dance events, we also fit a second set of models using only experimental phase recruits (n = 28), excluding all foundress bees.

Effect of Individual Calibrations on Network Structure

We set out to test whether the phenomenon of individual waggle dance calibrations and the relationship between the calibration values between a dancer and a follower might influence the formation and structure of larger waggle dance communication networks. Specifically, since we had shown previously that recruitment is more likely to succeed when a follower bee's communicated waggle run duration for a given distance is shorter than that of the dancer that recruited her (McHenry et al., in review), we wanted to test whether such a larger-smaller relationship between calibration values of hypothetical dancer-follower pairs could predict network structure. To do this, we implemented a Quadratic Assignment Procedure (QAP) regression, which tests whether there is a significant relationship between an observed network structure and specified node attributes by regressing that observed network structure against a predictor matrix containing information about node relationships. To prepare this analysis, we first extracted the subgraph of each trial's communication network containing nodes for which we could obtain scaled calibration values (described above) as an **asymmetric adjacency matrix**. This constituted the observed network structure, with directed ties showing the flow of information from dancer to recruit. Then we created a node similarity matrix for this set of bees according to bees' relative calibration values, where nodes were coded as similar ("1") if the dancer's (node[*i*]) calibration value was larger than that of a hypothetical follower (node[*j*]). Finally, we used these scaled calibration values in constructing the similarity matrix so that relative values would be comparable across all bees. This similarity matrix constituted the predictor matrix.

QAP regression handles the non-independence of network data via a permutation approach, wherein it shuffles the network data over many iterations to obtain a distribution of the test statistic under a null hypothesis, against which the coefficient of the observed network can be compared (Krackhardt 1988). To that end, we implemented a QAP regression with our observed calibrated bees' network using the netlm() function from the R package sna with 1000 permutations.

Data Availability

All data and analysis code are maintained as static datasets with Virginia Tech's library and are available upon request.

Results

Honey Bee Recruitment Networks Are Sparse, but Biologically-Informed Density Corrects Sparseness by Three Orders of Magnitude

We calculated network density values for both trials in two ways: first, we calculated density with respect to the classically defined complete graph (see above), and second, we calculated using our biologically-informed complete graph (BICG). For Trial 1, the classical network density for the observed network (Fig. 3.1, Trial 1a) was 3.60×10^{-5}, and the corrected network density calculated with our simulated BICG (Fig. 3.1, Trial 1b) was 3.68×10^{-2}. For Trial 2, the classical network density for the observed network (Fig. 3.1, Trial 2a) was 1.50 x 10-5, and the corrected density using the simulated complete graph (Fig. 3.1, Trial 2b) was 1.55×10^{-2}.

In this way, both trials reveal that honey bee waggle dance recruitment networks are sparsely connected, and that the biologically-informed density values differed from the classical density values by a factor of 1000 in both trials.

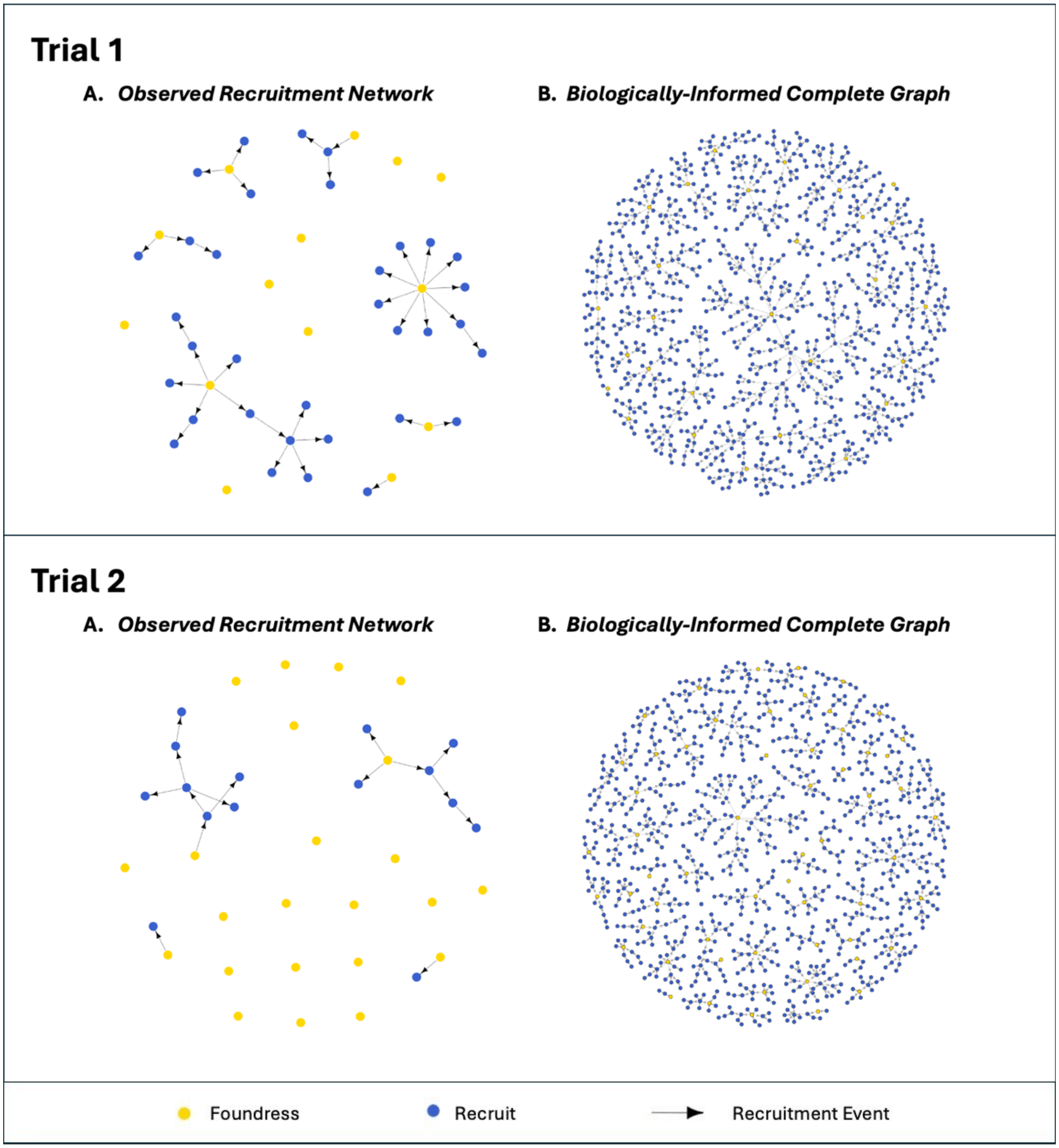

Figure 3.1. Observed waggle dance recruitment networks **(Trial 1A, Trial 2A)** and biologically-informed complete graphs **(BICG; Trial 1B, Trial 2B)** for Trial 1 (top) and Trial 2 (bottom). For observed network structures, foundress bees (yellow) participated in the pre-training phase; recruit bees (blue) were recruited to the feeder during the experimental phase. BICGs show the simulated complete graph for the corresponding trial's observed network, constructed based on DCM model output using the same number of foundress bees and a probability of recruitment success set to 1. Not shown: dummy isolate nodes added to observed networks to ensure comparability between observed and simulated networks (total nodes = 1000). Biologically-informed network density for each trial is calculated as (# Edges in Observed Network (A) / # Edges in BICG (B)).

Dancers Showed a Bimodal Burstiness Distribution That Could Not Be Explained by Network Time

Of the 63 bees that performed waggle dances during the experimental phase to advertise our feeders, 53 bees performed more than one dance and were thus eligible for burstiness analysis.

As previously mentioned, exploratory visualization of burstiness in our experimental bees indicated the possible presence of a bimodal distribution of burstiness coefficients (Fig. 2A). To investigate this, we tested for clustering in the burstiness data with two methods: Gaussian Mixture Modeling (GMM) and K-Means Clustering (KM). The results of both methods support the presence of two clusters (**method**: cluster 1 mean (95% CI), cluster 2 mean (95% CI)): **GMM**: -0.04 (-0.13 to 0.06) with 41 bees, 0.56 (0.53 to 0.59) with 12 bees; **KM**: -0.14 (-0.23 to 0.06) with 33 bees, 0.47 (0.41 to 0.59) with 20 bees (Figure 2B). The between-cluster variance ratio of 65.9% yielded by our K-Means analysis indicates that the clusters are well-separated and distinct.

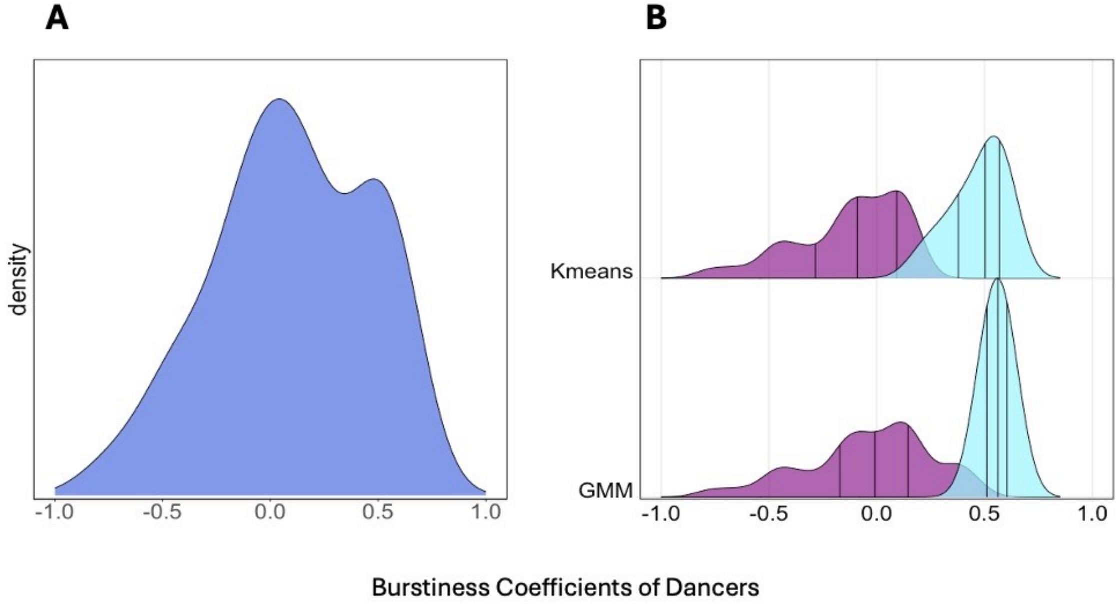

Figure 3.2. Density plots of burstiness coefficients in waggle dance recruitment among **(A):** all bees across two trials that performed more than one dance to advertise the feeder (n = 53); **(B)** those same bees separated by burstiness cluster as assigned by K-Means clustering analysis (top) and Gaussian Mixture Modeling (bottom). Bees performing waggle dances for our feeder performed dance bouts either non-burstily, dancing at relatively regular time intervals (**B**., left clusters shown in purple) or burstily, with high heterogeneity in the time intervals between dance bouts (**B.**, right clusters shown in pale blue). The between-cluster variance ratio of 65.9% indicates that the clusters are well-separated and distinct.

The next step in this analysis was to model bees' cluster membership via generalized binomial mixed-effects modeling. We chose to use GMM cluster membership assignment for this analysis rather than K-Means, since density plots of burstiness coefficients indicate that the clusters are likely neither spherical nor of equal size (Fig. 3.2B), and GMM is an appropriate method for modeling clusters of different shapes, sizes, and orientations (Patel and Kushwaha 2020).

We found that the most explanatory models for both our full (foundress + recruits) and recruit-only burstiness datasets included only network time as a predictor variable;

however, the effect of network time on cluster membership was not significant in either one. Results are given as: **model**: emmean (95% CI), p-value. **Full model**: 7.89 (0.07 to 0.51) p = 0.125; **recruits-only model**: 6.71 (0.04 to 0.50), p = 0.103.

Calibration Relationships Did Not Predict Tie Formation in the Observed Network

The relationship between bees' scaled waggle run durations among the 30 pairs of eligible dancers and followers (Fig. 3.3) was not predictive of tie formation in our observed networks. The calibration relationship coefficient for our observed network was -7.6×10^{-3} (Fig. 3.4, green dashed line) and was not statistically distinct from the permuted distribution of coefficients (QAP regression, permutations = 1000, t = -1.09, p = 0.28).

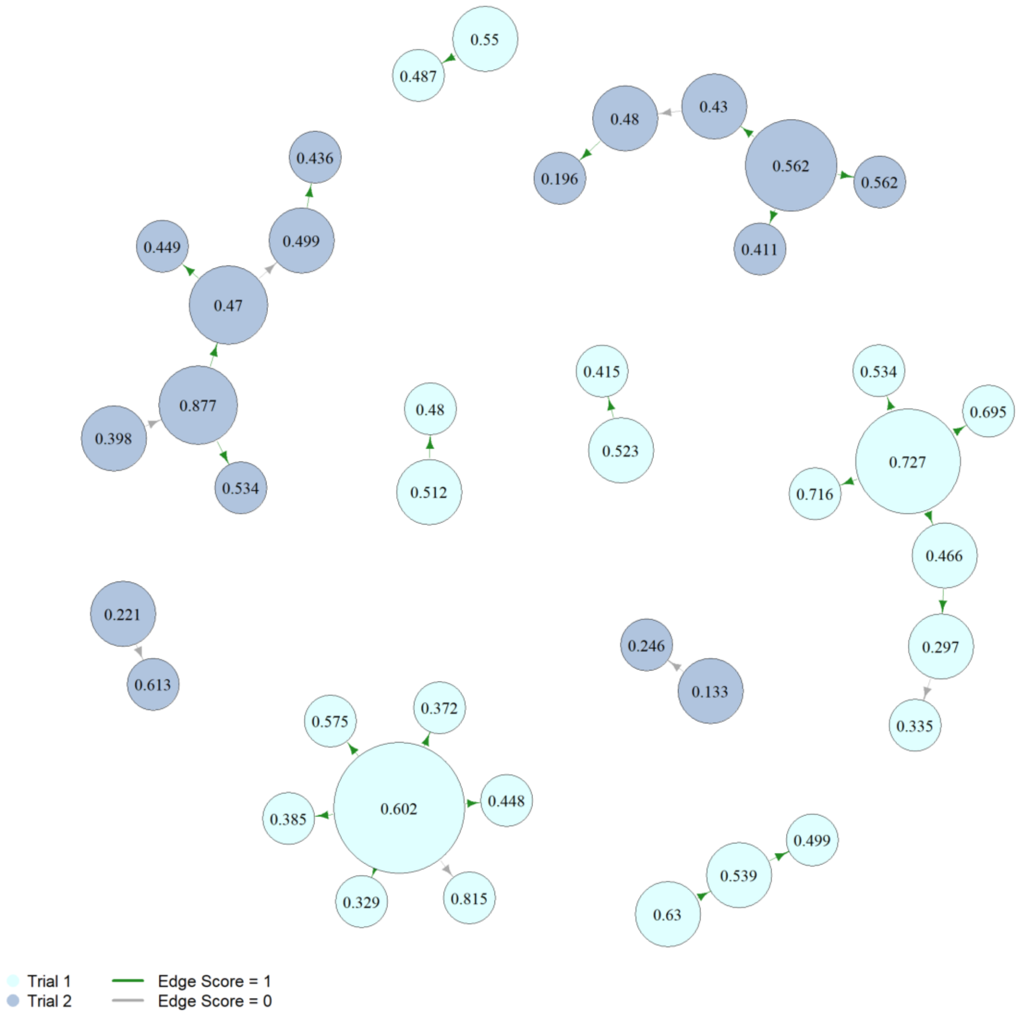

Figure 3.3. Network of waggle dance recruitment among calibratable bees. Node labels show that bee's scaled waggle run duration value in seconds for a given distance. Node color denotes which the trial to which a network component belongs. Edge color denotes similarity matrix score, where green, representing an edge score of 1, demonstrates where the dancer's scaled waggle run duration was longer than that of her successful follower. Gray denotes an edge score of 0 and represents where the dancer's scaled waggle run duration was shorter than that of her successful follower. Node size is proportional to the number of followers recruited to the feeder, or outdegree. Scaled waggle run duration differences between dancers and followers did not significantly affect the formation of network ties (t = -1.09, p = 0.28).

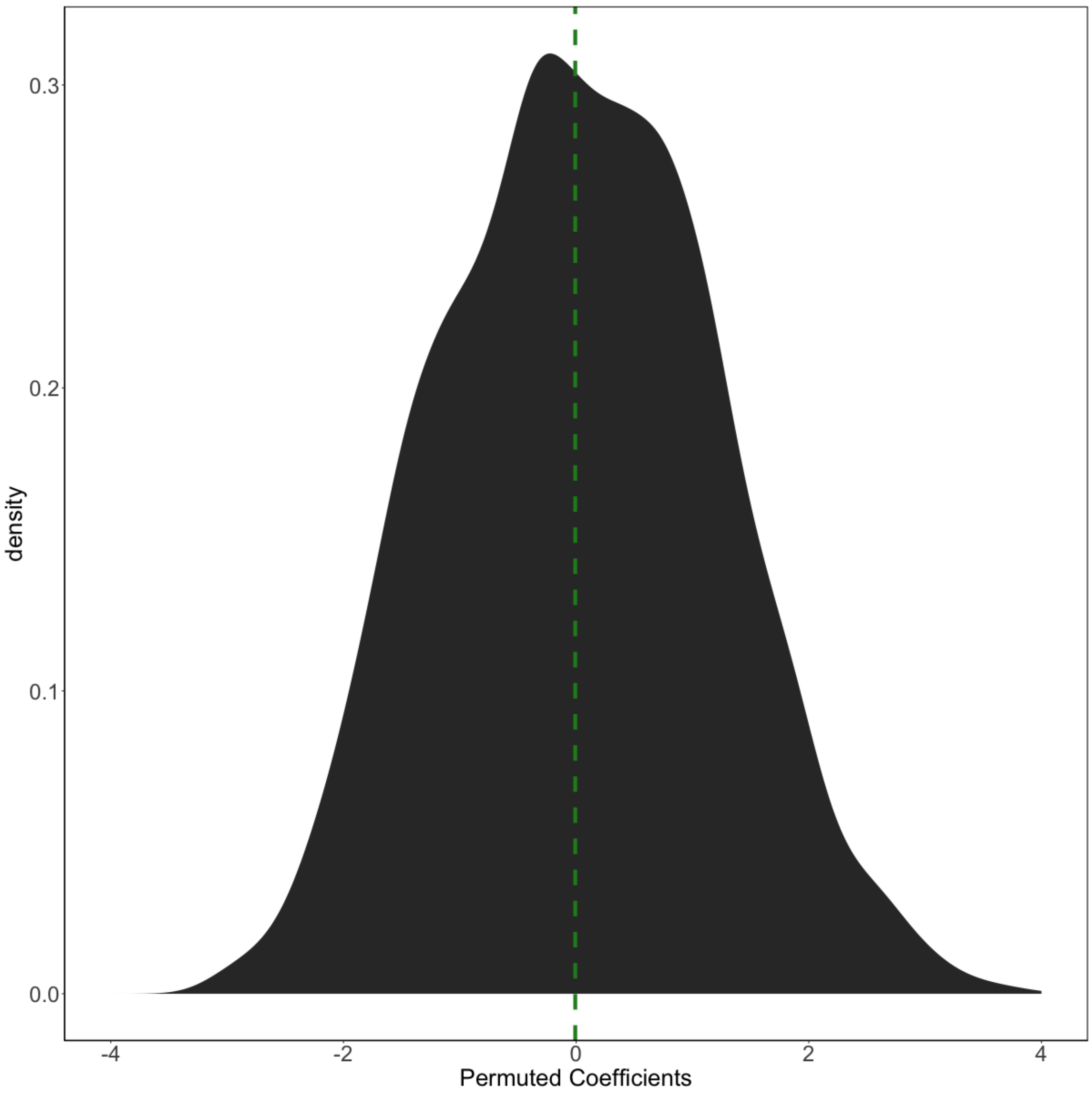

Figure 3.4. Density distribution plot of permutation coefficients (n = 1000) generated via Quadratic Assignment Procedure (QAP) regression with our observed network and a node similarity matrix representing the relationship between scaled waggle run duration values between any possible dancer-follower pair. Green dashed line shows the coefficient of our observed network (-0.0076). Scaled waggle run duration differences between dancers and followers did not significantly affect the formation of network ties (t = -1.09, p = 0.28).

Discussion

This study investigated the structure and properties of the social network of waggle dance recruitment in honey bees. While the importance of a network analysis approach in gaining insights into the life of eusocial insects is widely understood, *in vivo* studies of such networks are rare due to the steep logistical difficulty of obtaining and handling these data. This study provides an empirical description of network density, burstiness, and the role of individuality in the honey bee waggle dance recruitment network.

Recruitment Networks Are Sparse

We found that, while both the classical density and biologically-informed density of observed recruitment networks were sparse, biologically-informed density calculated using our simulated biologically complete network yielded network density values that were higher than classical density by three orders of magnitude (Fig. 3.1). This result highlights the importance of calculating and interpreting network metrics in the context of the behavior and biology of the organism under study (Alves et al. 2023). The default baseline for calculating network density, a complete graph where every node is connected to every other node (Bang-Jensen and Gutin 2018), could not occur in the context of honey bee behavior: a forager bee, once committed to a food source, could not then be recruited to it again by every other forager bee. Using a simulated upper-bound (our BICG) based on the biology of the system under study is therefore a useful method for gleaning more relevant insight into the properties of social networks inspired by biological systems. Deterministic SI models have been used previously to study the social network of trophallaxis in honey bees (Gernat et al. 2018; Sun et al. 2020, with the same dataset) to model the spread of infection through the sharing of crop content. As we did, those authors also used a deterministic SI model to simulate a graph representing the upper-bound of infection rates within a trophallaxis network, parameterized with a likelihood of infection set to 1. Here, we further customized the parameterization of the model according to the biology of waggle dance communication and recruitment: we (1) specified directed edges, since dance information flows from dancer to follower, and (2) supplied a total number of individuals (1,000) based on what is known about the proportions of workers that may be engaged in foraging at any given time (approx. 1/3) (Seeley 1995), with our total observation colony size being 3,000. To our knowledge, this is the first application of deterministic SI models to understand honey bee waggle dance recruitment networks.

Interestingly, even when using our biologically-informed, simulated upper bound for waggle dance recruitment, the resulting network density values indicate an extremely sparse network structure, with relatively few edges – between 1.5 and 3.5% of the edges predicted by our BICGs (Fig. 3.1). In other words, we estimate that each trial's pool of foundress bees recruited only 1.5 – 3.5% of the bees that they would have if they had a perfect recruitment record. The lowness of this value agrees with what is known, if not fully understood, about honey bee foraging and recruitment: the waggle dance is a famously inefficient system, where the effort put into signal production by dancers seems to translate to a relatively small amount of payoff with few successful recruits (Seeley 1995), with relative success depending upon target distance, spatial distribution of the target (feeder or patch), presence or absence of floral scent cues, the number of dances a follower was allowed to observe (Esch and Bastian 1970; Mautz 1971; Gould 1976; Seeley and Visscher 1988; Tautz and Sandeman 2003), and the relative relationship between the dancer and recruit bee's calibration (McHenry et al in review). Here, we have additionally shown that sparse density values are characteristic of waggle dance recruitment networks, further demonstrating that the waggle dance is an inefficient semiotic system.

From a network analysis perspective, we suggest that the sparseness of the recruitment network may be related to the fact that the transmission of information and successful recruitment relies upon not one but several receiver characteristics and choices. For successful recruitment to occur, a receiver bee finding herself within perceptive range of a waggle dance (Rohrseitz and Tautz 1998) must (1) decide and be able to closely attend that waggle dance for a sufficient number of waggle runs; (2) weigh this public information against possible private information that she may possess; (3) decide to act on the public dance information received; and only then (4) successfully locate the resource in the landscape. Each of these is a leaky step in the pipeline to recruitment. First, not all bees within the perceptive range of a waggle dance will follow it (Tautz and Rohrseitz 1998; Okada et al. 2008); second, approximately 75 – 88% of dance-following interactions result in reactivation to a known foraging site on the part of experienced foragers (Biesmeijer and Seeley 2005) rather than recruitment to the advertised food source (i.e., their dance-following does prompt a change in volitional state, but towards the use of private information rather than public (Grüter et al. 2008; Grüter and Farina

2009; Ratnieks 2011; Hasenjager et al. 2020)); and third, only approximately 16-25% of the bees who do set out to act on the transmitted information will actually find the resource (Seeley and Visscher 1988). In light of this leaky sequence of receiver choices associated with recruitment, perhaps such sparse network densities are unsurprising.

Bees Were Either Bursty or Non-Bursty Dancers

Our description of burstiness in waggle dance bouts revealed an unexpected partitioning of bees into low- and high-burstiness groups (Fig. 3.2). The majority of bees, approximately 75%, showed very low burstiness in their waggle dance bouts, with the 95% CI including zero (cluster mean burstiness coefficient of -0.04 (-0.13 to 0.06)). Burstiness values close to zero indicate high inter-event homogeneity, indicating that most of our experimental bees show a regular pattern of waggle dance bouts with low variance in inter-bout intervals. In contrast, a minority of bees (approximately 25%) belonged to a well-separated, high-burstiness group (0.56 (0.53 to 0.59)). This unexpected result prompted us to expand our exploratory burstiness analysis beyond network description into the realm of hypothesis testing, as we attempted to uncover any factor that might account for low- or high-burstiness cluster membership.

We first explored the impact of network time on cluster membership since we know that the longer a bee visits a feeder (i.e., increased network time), the more regularly she tends to forage and subsequently dance for that feeder. To account for unknown differences in waggle dance history between foundresses and recruits (explained above), we also tested for a relationship between foundress/recruit status and burstiness. Contrary to our predictions, however, neither network time nor foundress/recruit status was related to being a high- or low-burstiness bee. A bee's burstiness therefore may be intrinsic to that bee. It is important to note that, unlike in a real-world foraging landscape, the feeder provides a single, *ad libitum* source of higher quality food, which generates tight commitment to a feeder and, consequently, very regular foraging visits (Couvillon et al. 2015; Schurch et al. 2016, 2019; Ohlinger et al. 2022; Couvillon et al. 2023). In a natural scenario, bees must visit, probe, and load from many (tens, hundreds, or even thousands) flowers on each foraging trip, and the time required to fill a crop might vary on each foraging trip. This is in stark contrast to the flow of a feeder training experiment, where bees can get into a tight rhythm, coming back and forth from the feeder every 3-4 minutes without the need to probe patchily dispersed resources (Ohlinger et al. 2022).

It is therefore possible that our result showing a majority of low-burstiness bees with a highly regular timeline of waggle dances (Fig. 3.2B, purple distributions) might be overstated compared to a natural foraging scenario. Importantly, however, given that network time could not explain burstiness, our data do not support the idea that high-burstiness bees (Figure 3.2B, pale blue distributions) were simply those who hadn't yet had enough time or feeder experience to settle into a tight commitment and regular dance routine. We therefore suggest that waggle dance burstiness may be an intrinsic trait particular to a bee, possibly constituting a behavioral type (Sih et al. 2004).

Additionally, it may be that a bimodal distribution of waggle dance burstiness, with some low-burstiness waggle dancers performing their dances at regular intervals while other bursty waggle dancers performing their dances in clustered bouts, may be an adaptive feature in the regulation of collective foraging. Indeed, networks and their features constitute group-level behavioral phenotypes that may be shaped by selection (Hasenjager et al. 2022). Collective foraging by a honey bee colony is known to be governed by a complex system of interlinked positive and negative feedback loops (Seeley 1989; Seeley et al. 1991; Seeley 1994; Seeley and Tovey 1994; Nieh 1998; Biesmeijer 2003; Nieh 2010; Kietzman and Visscher 2015; Borofsky et al. 2020). Even as each bee functions as a sensory unit of the colony (Seeley 1994), variation in individual foraging strategy has been shown to enable a colony to flexibly deploy foragers to make efficient use of resources in dynamic landscapes (Schürch and Grüter 2014; Alves et al. 2023). For instance, each honey bee forager tends toward either behaving as a scout bee, searching the environment independently for a profitable resource (gathering private information) and then recruiting nestmates to it, or behaving as a recruit by waiting for public waggle dance information to become available on the dance floor (von Frisch 1967; Seeley 1983; Biesmeijer and Seeley 2005; Grüter et al. 2008; Grüter and Ratnieks 2011). This partitioning of strategies within a foraging workforce helps the colony balance the energy expense of bees actively exploiting resources versus that of bees sampling the environment to find new ones (Wilson 1971; Mosqueiro et al. 2017). Furthermore, evidence suggests that this phenomenon is related to individual learning behavior, with scout bees showing higher latent inhibition (propensity to ignore familiar information) compared to recruit bees (Cook et al. 2018). In other words: in the case of the scout/recruit behavioral phenotype, the foraging workforce is adaptively partitioned into two strategy groups, or behavioral types. Might foragers also be adaptively partitioned into two burstiness behavioral types?

It is known that burstiness as a feature of node behavior can either increase or decrease information flow, depending on the context and the social learning strategy governing the network in question (Karsai et al. 2011; Evans et al. 2020). How then might honey bees' learning strategy interface with dance burstiness? In a scenario where the transmission of social information depends upon a strategy of repeat exposures, that is, where repeated exposures to the same information in rapid succession can push receivers above their "information acceptance threshold" (Karsai et al. 2011; Min et al. 2013; Rocha and Blondel 2013; Evans et al. 2020), it seems possible that having a combination of the non-bursty, regularly-dancing bees (Fig. 3.2, left side of the bimodal distributions) and burstily-dancing bees (Fig. 3.2, right side of the bimodal distributions) might be adaptive. Specifically, the partitioning of dance burstiness into two behavioral types could conceivably play a role in the regulation of collective foraging by periodically modulating the "volume" of the waggle dance. If non-bursty bees maintain a steady level of dance activity, and then a smaller but active group of bursty bees intermittently heighten the signal volume with saltatory periods of dance bouts, such temporary amplification of the signal being broadcast inside the hive might push a new wave of recruits over an information acceptance threshold. We recommend that future research focus on elucidating the functional role of this bimodal burstiness distribution among forager bees.

Individual Calibration Does Not Affect Network Structure in a QAP Framework

Finally, we looked to see whether individuality in waggle dance duration calibration affected the network structure that arises from these interactions. Previously we examined the effect of waggle run duration calibration on the likelihood of recruitment success in dancer-follower pairs, demonstrating that successful recruits tended to communicate shorter waggle run durations for the same feeder distance than the dancer that recruited them (McHenry et al. in review). In other words, a follower bee was more likely to succeed at locating an advertised resource when it is closer to the colony than she expected it to be based on her interpretation of the dancer's waggle run duration versus when the resource is further away to the colony than she expected. At the dyad level, then, it is thus the relationship between calibrations in given dancer-follower pairs that matters, rather than any individual attribute of one of the communication partners that impacts the likelihood of communication success. In contrast to this dyad-level effect and contrary to our predictions, however, calibration difference between dancers and followers did not produce a detectable effect on the formation of our observed recruitment networks in a QAP regression framework (Fig. 3.4).

A major benefit of QAP is that it controls for network structure, leaving intact the edges while permuting the nodes through different arrangements in that network structure to obtain a distribution of similarity coefficients against which to compare those of our observed networks. However, it is important to note that QAP regresses the observed network against a node similarity matrix in place of a predictor variable, and we therefore had to interpret dancer-follower calibration difference into this format, specifying a "1" (similar, where the dancer's scaled waggle run duration was larger than the follower's) or a "0" (not similar, where the dancer's scaled waggle run duration was smaller). A visual inspection of the data (Fig. 3.3) indicates that the transformation to a binary similarity matrix may have stripped the data of some important nuance. Specifically, this approach lumps any pair of bees for whom the scaled waggle run duration value of the dancer was shorter than that of the follower into the "0" category, regardless of the magnitude of the difference. Some pairs of bees therefore, whose calibration values were in reality so similar that they would likely be statistically identical if associated with an error term, were nevertheless assigned to the "0" similarity group, when their biological reality might be better represented by a "1" (Irwin & McClelland 2003; Royston et al. 2006; Fedorov et al. 2009). Overall, we remain unconvinced that individuality in calibration has no role to play in the recruitment network structure and recommend that future work continue to examine the effect of individuality in network nodes on the formation of communication networks (O'Shea-Wheller et al. 2021).

Future Directions

Managing complexity is one of the greatest problems facing modern-day human organization (Beer 1972; Espejo and Reyes 2011). The honey bee colony has already inspired a wide variety of models and algorithms seeking to refine strategies to accomplish this goal in fields ranging from computing networks (Wedde et al. 2004; Wedde et al. 2005; Chamoli et al. 2015; Sun et al. 2020; Doi et al. 2023) and informatics (Beynon-Davies 2010) to the management of "big data" with artificial intelligence (Foss and Espejo 2018). However, lack of empirical knowledge about honey bee network structures and their real-world parameters has mostly restricted the bio-inspiration available to these algorithms and models to general roles associated with division of labor in a colony (Farooq et al. 2008).

A critical concern in stream-based computing is achieving low latency (temporal lag in data transmission) coupled with high throughput (volume of data transmission). Recent attention has focused on the testing of parameterization conditions of bee-inspired algorithms and models, including arbitrarily-chosen network density conditions, in order to achieve these goals (Bakhouya and Gaber 2015). As far as we are aware, our data are the first to offer density and burstiness parameterization guidance directly from *in vivo* bee networks.

We therefore hope and anticipate that these results may be of interest to researchers wishing to test bio-inspired algorithm parameterizations. In particular, it would be interesting to test the behavior of stream-based computing models when parameterized according to the density and burstiness properties observed in real bee networks. Would bee-inspired parameterizations offer good latency and throughput, or would they result in inefficient networks, as we might predict based on the famous inefficiency of the waggle dance? Such studies could provide mutually valuable insights into both bee biology and bio-inspired computing.

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CHAPTER 4

SUBLETHAL GLYPHOSATE EXPOSURE IMPACTS HONEY BEE FORAGING AND ALTERS BALANCE OF BIOGENIC AMINES IN THE BRAIN

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Sublethal Glyphosate Exposure Impacts Honey Bee Foraging and Alters Balance of Biogenic Amines in the Brain

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Abstract

Glyphosate (GLY) is a broad-spectrum herbicide that inhibits the shikimate pathway, which non-target beneficial organisms such as pollinating insects do not possess. Nonetheless, sublethal GLY exposure can impair gustation, learning, memory, and navigation in the honey bee (*Apis mellifera* L.), the most economically important pollinator. While these impacted physiologies underpin honey bee foraging and recruitment, the effects of sublethal GLY exposure on these important behaviors themselves remain unclear, and any proximate mechanism of action in the honey bee is poorly understood. We trained cohorts of honey bees from the same hives to forage at one of two artificial feeders offering 1 M sucrose solution, either unaltered (N = 40), or containing GLY at 5 mg/L (N = 46) and compared key foraging and, on a smaller subset of bees, recruitment behaviors. Next, we quantified levels of octopamine, tyramine, and dopamine, and tyrosine in the brains of experimental bees collected three days after the exposure. We found that treatment bees reduced their foraging by 13.4% ($p = 0.022$), and the brain supply of tyramine was modulated by a crossover interaction between GLY treatment and number of feeder visits ($p = 0.004$). Levels of octopamine were significantly correlated with both its precursors tyramine ($p = 0.011$) and tyrosine ($p = 0.018$) in treatment bees, but not in control bees. Our findings emphasize the critical need to investigate non-target impacts of the world's most-applied herbicide and to elucidate its non-target mechanism of action in insects to create better-informed pollinator protection strategies.

Introduction

Pesticides, including insecticides, herbicides, and fungicides, are globally ubiquitous, and their use is projected to increase alongside agricultural intensification (Zhang et al. 2011; Benbrook 2016; Brovini et al. 2021). Therefore, it is critical that we balance the benefits of pesticide use with the cost of impacts to non-target organisms, and particularly beneficial insects like pollinating bees (Pimentel 1997, 2005). The presence and degree of such impacts depend on the pesticide encountered (Decourtye et al. 2004; Charreton et al. 2015), the susceptibility of the organism to that pesticide (Wu et al. 2017; Goñalons and Farina 2018), the route of exposure (Krupke et al. 2012), the dosage experienced (Almasri et al. 2021), and the timing (chronic vs. acute) of that exposure (Tosi et al. 2017). While such non-target effects of insecticides on bee health have received increased attention in recent years (Abati et al. 2021; Bernardes et al. 2022; Gonçalves et al. 2022), those of fungicides and herbicides are less-studied (Desneux et al. 2007; Schmidt-Jeffris 2023), partly because regulatory requirements for toxicity testing on insects are generally less stringent for non-insecticidal chemistries.

The herbicide glyphosate (GLY) is a weedkiller in the [*N*-(phosphonomethyl) glycine] chemical group that targets the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) within the shikimate pathway (Steinrücken and Amrhein 1980; Sikorski and Gruys 1997; Duke and Powles 2008). Although GLY was initially approved in 1974 for the limited removal of all vegetation, the introduction of crops genetically modified to resist GLY, such as RoundUp Ready® (RR) maize, soybean, and cotton varieties in 1996, followed by alfalfa in 2005 and sugar beets in 2008, launched a dramatic increase in its use as a broadcast herbicide: between 1974 and 2014, GLY use increased more than 15-fold in the United States (Duke and Powles 2008; Duke 2018), with a corresponding global increase as well (Zhang et al. 2011, China; Brovini et al. 2021, Brazil). Today, GLY is the most widely used pesticide in the world (Benbrook 2016).

The widespread use of GLY is due in part to its relatively low acute oral and contact toxicity to honey bees. With an LD50 value over 100 µg/bee (Frasier and Jenkins 1972; Chen et al. 2023), the herbicide has been touted as being pollinator-friendly (Frasier and Jenkins 1972; Duke and Powles 2008). However, a growing body of literature suggests that GLY may nevertheless cause sublethal, non-target effects on both the behavior and physiology in a variety of bee taxa, including the European honey bee (*Apis mellifera* L.). Recent work has documented reduced honey bee survivability after exposure to GLY in formulation (Abraham et al. 2018), as well as sublethal effects of non-formulated GLY, including altered physiological homeostasis (Almasri et al. 2022), immune dysregulation (Motta et al. 2022), sleep behavior (Vázquez et al. 2020), reduced navigational capabilities (Balbuena et al. 2015), decreased gustatory responsiveness (Farina et al. 2019), impaired associative learning (Herbert et al. 2014; Goñalons and Farina 2018), and reduced olfactory memory retention (Hernández et al. 2021). As the honey bee is the most economically impactful pollinator in agricultural systems, contributing to the pollination of 44% of commodity and food crops (Klein et al. 2007) and providing billions of dollars annually in pollination services (Southwick and Southwick 1992), understanding these non-target impacts to honey bees is critically important.

Physiological disruptions to navigation, gustatory responsiveness, associative learning, and memory would hold particular importance for forager honey bees, who rely on these functions to find food successfully in a dynamically changing landscape. Foragers evaluate the profitability of a floral resource as its net energetic efficiency, which balances its sugar reward versus energetic cost (primarily flight distance) associated with working that resource (Schmid-Hempel 1987; Garbuzov et al. 2015; Seeley 1995). These foragers may also recruit nestmates to a profitable resource by performing a waggle dance, which communicates a distance and direction to that resource (von Frisch 1967), thereby increasing colony-level foraging success and ultimately fitness (Seeley 1995; Sherman and Visscher 2002; Grüter and Farina 2009; Grüter and Ratnieks 2011; Schürch and Grüter 2014; Nuernberger et al. 2019; although see Price et al. 2019). Successful foragers must therefore not only learn the location of a resource in the landscape, but also assess its profitability and adjust visitation frequency and waggle dance recruitment accordingly (Grüter and Farina 2009; Couvillon 2012). Despite the evidence that GLY may disrupt the physiological processes that underpin and modulate foraging and recruitment, the effects of sublethal exposure on these behaviors themselves remain unclear. Furthermore, the mechanism of non-target toxicity in bee physiology is poorly understood (Battisti et al. 2021; Battisti et al. 2023; Schmidt-Jeffris 2023).

Here we conducted a feeder training experiment to study the impact of GLY on behavior and brain neurochemistry in the honey bee. In particular, we investigated the effects of sublethal, field-realistic GLY exposure on key honey bee foraging and recruitment behaviors via quantification of foraging frequency (our primary behavioral outcome), foraging persistency, waggle dance propensity, waggle dance frequency, and waggle run repetitions per dance (our secondary behavioral outcomes, studied with subset of bees). Since these behavioral metrics tend to scale positively alongside a bee's valuation of a resource’s profitability (von Frisch 1967; Seeley et al. 1991; Seeley 1995; Seeley et al. 2000) and GLY can disrupt the underlying functions of gustation, associative learning, and memory, we predicted that sublethal GLY exposure would reduce the incidence of each of these behaviors. Next, we conducted a follow-up experiment with a subset of the same experimental bees to examine the effect of sublethal GLY exposure on raw and relative brain supply (Lim et al. 2016) of certain key biogenic amines involved in foraging, recruitment, and the neurological signaling associated with these functions, including octopamine, tyramine, and dopamine (reviewed in Bicker et al. 1999; Scheiner et al. 2002; Farooqi 2012; and Blenau and Baumann 2016), as well as their biosynthetic precursor, the amino acid tyrosine (Karlson and Herrlich 1965; Roeder 2005; Matsuyama et al. 2015).

Material and Methods

Study Organism

We studied three queenright honey bee colonies of mixed European race, mainly *Apis mellifera ligustica*, each containing brood and approximately 5,000 workers. Each experimental colony was housed indoors in observation hives consisting of three American Standard Deep frames and transparent Plexiglas walls to enable direct observation of activity on the comb. Colonies were connected to the outdoors via a plastic tube measuring approximately 5 cm × 30 cm. Our experimental trials worked with one hive at a time.

We managed colonies throughout the project to avoid overcrowding and maintain availability of empty cells for incoming nectar storage. This aspect of management is important in feeder training experiments where experimental bee cohorts are recruited by nestmates because insufficient availability of empty cells may result in suppression of recruitment related to nectar unloading delays (Seeley 1989; Seeley and Tovey 1994; Seeley 1995).

We collected data from July 14, 2021, to August 1, 2021, on days with favorable foraging conditions. We chose to work in high summer because we have found it easier to train bees to feeders when there is a relative dearth of natural forage in the surrounding landscape (Couvillon et al. 2014; Ohlinger et al. 2022a). It was sometimes necessary to provide colonies with supplemental sugar solution, which we removed at least 24 hours before the beginning of data collection.

Training Forager Bees to Feeders and Glyphosate Treatment

We trained an initial cohort of worker honey bees from our study colonies to visit artificial feeder stations containing 2 M sucrose solution scented with lavender (10 μL/L) using our step-wise training method (Ohlinger et al. 2022). Briefly, on the day prior to the experiment (Day -1), we loaded a Petri dish with a few drops of scented solution, placed that Petri dish inside a small box, placed the box on a platform mounted on a tripod (approx. 1 meter in height), then used the box to cover the exit tube such that exiting worker bees would be able to explore the box and encounter the sugar solution. Importantly, this method prevented bees from the nearby apiary from discovering the

Petri dish. As soon as there were at least three bees drinking the solution from the Petri dish, we removed the box and gently transferred the dish to the tripod platform just outside the tube entrance. Stepwise training began as soon as any bee made a new visit to the feeder (i.e., landed on the Petri dish and began to collect the sucrose solution).

Two researchers participated in Day -1 training, one marking visiting foragers with numbered plastic discs (BetterBee, Greenwich, NY, USA), with the other confirming the membership of marked foragers to the experimental colony. To exclude bees from the nearby apiary, we removed any marked bee that returned to the feeder but whom we could not confirm inside the observation hive. As soon as 3-5 marked bees from the experimental colony had made at least three visits to the Petri dish, we swapped out the dish for the full feeder setup, which consisted of a 4-oz. Mason jar on a plastic base with collection wells that contained the same scented training solution.

In this stepwise training phase, we progressively moved the feeder across an open field in sequential steps (10-12 steps, with 10-15 meters between steps) until reaching the penultimate location five meters in front of and centered between what would ultimately be the final two experimental feeder locations, referred to as “left” and “right” from the bee’s-eye-view. At each step along the way, new, unmarked bees arriving at the feeder were marked, confirmed, or removed as before. We allowed marked bees to visit at least three times before making the next step to ensure they had learned its current location. We remained at this penultimate location until 5-15 confirmed foragers had made at least three visits each, typically by mid- to late afternoon on Day -1. The accumulation of multiple visits at the final Day -1 feeder location provided a highly rewarding experience for these foragers, thus increasing the likelihood that they would return to the feeder the next day (Al Toufailia 2013).

The next morning (Day 0), we positioned two feeders containing the Day -1 solution side-by-side on a tripod at the end location from Day -1. We allowed trained foragers from Day -1 to self-assemble across the two feeders. A forager was considered ‘committed’ once she had visited one of the two feeders at least five times. When multiple (approximately 3-5) committed foragers were observed simultaneously collecting sucrose solution, we delicately relocated the feeders, along with the drinking bees, to the designated experimental feeder locations five meters apart and equidistant from the colony: we placed one feeder on the “left” tripod and the other on the “right” tripod, each with a randomly-assigned yellow or blue background color cue, which we alternated between right and left in each trial). The bees trained on Day -1 were then free to forage and to recruit to the feeders. These Day -1 training bees were not ultimately destined to be part of our experiment but rather served to recruit bees that would.

As newly recruited (Day 0) foragers arrived at either feeder, we marked them and confirmed their colony membership as before. The color of the plastic discs assigned to each Day 0 forager corresponded to the feeder they visited first, while also distinguishing them from the Day -1 training foragers. We recorded each visit made by the Day 0 foragers as a training visit. Once a consistent pattern of feeder visits and recruitment emerged among the Day 0 foragers, we steadily removed the Day -1 foragers from the experiment to prevent overcrowding at the feeder. As soon as 5-15 Day 0 foragers had made at least three training visits to only one feeder (either “left” or “right”), we began the experimental phase.

At the start of the experimental phase, we simultaneously removed both training phase feeders and replaced them with experimental feeders, each containing unscented 1 M sucrose solution, either unaltered (control) or with 5mg/L GLY (treatment) ([*N*-(phosphonomethyl) glycine], Product # 89432-100MG, TraceCERT®, Sigma-Aldrich Production GmbH, Switzerland). We selected this concentration of GLY because it is consistent with label application rates and those found in natural environments and semi-field experiments, which range between 1.4 to 7.6 mg/L (Goldsborough and Brown 1988; Feng et al. 1990; Giesy et al. 2000; Thompson et al. 2014). We assigned treatment and control solutions to the "left" and "right" feeders randomly with a coin toss, and thereafter alternated between trials. Researchers collecting feeder visitation data were blinded to treatment assignment between feeders.

For the next three-hour experimental phase, we allowed the marked bees to forage freely at their designated feeder. We recorded the bee identity and time of every feeder visit ("foraging frequency", see below). Any unmarked bee arriving at a feeder during the experimental phase was captured away. During these three hours, we also filmed the observation hive (see below). At the end of the experimental phase, both feeders were simultaneously and promptly removed.

To study the effect of GLY exposure on bees' persistency to a previously rewarding, now-unrewarding food source, on the following two days (Day 1 and Day 2), we re-erected feeder stations identical to those for the Day 0 experimental phase, but this time empty and unrewarding. We then recorded all visits in which a marked bee made physical contact with any part of the feeder itself ("persistency", see below). We did not consider it a visit if the bee contacted the platform but not the feeder. We monitored the feeders for the 8-hour period between 8:00 and 16:00 on Day 1 and Day 2.

In order to confirm that our experimental bees experienced the dosage of glyphosate as sublethal, we performed mortality censuses on the mornings of Day 1 and Day 2 to determine which marked bees were still alive in the colony. Any bee that could not be visually confirmed during a 1-hour search or that did not make a persistency feeder visit was presumed dead. Any bee presumed dead via mortality census data was excluded from persistency analysis to avoid zero-inflation in our data.

Video Recording and Monitoring of Observation Hives

We filmed each observation hive continuously during the experimental phase to record Day 0 bees' recruitment behaviors ("dance propensity," "dance frequency," and "waggle run repetitions" – see below). To do this, we set up two Canon Vixia HF R82 camcorders mounted on tripods and equipped with SanDisk Extreme SD cards recording at 30 frames per second, one on either side of the observation hive, with the lens of each positioned 1 m from the Plexiglas surface of the observation hive. We focused the camera on the "dance floor," or the portion of the bottommost frame of comb where most waggle dance activity occurs (approx. 25 x 20 cm). After the completion of each experimental phase, we backed up all SD cards to a Google Team Drive (GTD) for analysis.

Behavioral Response Variables

Honey bees are exquisitely sensitive to the profitability of a resource, which depends on the net benefit of its sucrose reward versus the energetic cost required to reach it on the wing, and they will tune their foraging and waggle dance behavior accordingly (von Frisch 1967; Seeley 1995; Seeley, et al. 2000; Couvillon 2012; Couvillon et al. 2015). In this experiment, both feeders presented equal profitability in that they offered identical sucrose reward (1 M) and identical energetic cost (equal flight distance from the colony). Therefore, any difference in measured behaviors between treatment and control would indicate an effect of GLY.

Foraging frequency, or the number of visits to a food source per unit time, is one such behavioral metric that correlates positively with a resource's profitability (von Frisch 1967; Seeley 1995; Seeley et al. 2000). Once treatment (with GLY) and control (without GLY) feeders were in place, we measured foraging frequency as the number of foraging trips that each experimental bee made to her 1 M feeder for the 3-hour duration of the experimental phase on Day 0. We counted visits for analysis according to a specific and consistent set of criteria. First, the marked bee must alight on the experimental feeder, extend her proboscis, and collect the sucrose solution. Second, we only counted a visit for each bee if at least three minutes had elapsed following that bee's previous visit. We implemented this condition because a foraging bee might occasionally visit the feeder a second time after a short period of flight but before returning to the colony, and previous work has shown that even highly motivated foragers take at least three minutes to make the return trip to the colony, unload their collected sucrose solution, and then return to the feeder (Couvillon et al. 2015) at a foraging distance similar to ours (145 m).

A bee may not advertise all food sources, however: there is a range of middling profitability in which a bee will continue to forage upon a resource but will not dance to recruit nestmates to it. The higher the profitability, the higher the likelihood a bee will dance at all for that resource, or the higher her **dance propensity** (von Frisch 1967; Seeley 1995; Couvillon et al. 2015). We measured each bee's dance propensity as a binomial outcome reflecting whether she performed at least one waggle dance ("1", dancers) or foraged but did not dance ("0", non-dancers) during the experimental phase.

When a bee does dance to advertise a resource, she tunes her dance behavior according to her perception of its profitability in several measurable ways. For instance, bees will dance more frequently when foraging upon a highly profitable resource, and those dances tend to contain greater numbers of waggle runs, the repeating subunit of the waggle dance behavior that encodes the vector information (von Frisch 1967; Seeley et al. 2000). We therefore monitored video data to quantify each dancer's **dance frequency**¸ defined here as the number of waggle dances performed during the 3-hour experimental phase, as well as **waggle run repetitions**, defined as the mean number of waggle runs per dance for each recruiter bee.

Finally, another behavior that a forager bee tunes according to her perception of a resource's profitability is the frequency with which she will reinvestigate a formerly rewarding food source after it has become unrewarding, called **persistency**. Persistency is adaptive in natural settings because floral resources that have gone dry one afternoon may yet be re-rewarding within hours to days, as plants replenish their floral nectaries or open fresh blooms. The more profitable the resource, the more persistently a forager bee will reinvestigate it (Seeley et al. 1991; Seeley 1995; Al Toufailia 2013). Furthermore, it is known that the presence of pharmacologically active substances in nectar can modulate persistency (Couvillon et al. 2015**,** caffeine, persistency increased; Ohlinger et al. 2022, a neonicotinoid, persistency decreased). We therefore measured bees' persistency to the experimental feeders for two days (Day 1 and 2) following the experimental phase.

Brain Dissection and Quantification of Analytes via Ultra High Performance Liquid Chromatography with Tandem Mass Spectrometry

To investigate the effect of GLY on the brain supply and balance of tyrosine, tyramine, dopamine, and octopamine, we recaptured all available experimental bees on Day 3 after the behavioral portion of the experiment was complete. We did this by temporarily capping the colony entrance for approximately 10 minutes between 14:00 – 17:00, then capturing all marked bees that accumulated into individual sterile Falcon tubes and immediately stored them over dry ice. Then we flash-froze the bees in liquid nitrogen and stored them at -80 °C.

Brain dissection and analyte quantification occurred April-May 2023. We dissected each frozen brain, one at a time, using sterile dissection tools under a dissecting microscope and on chilled dissecting plates set on a bed of dry ice to prevent brains from thawing during dissection, which degrades analytes. Additionally, we rotated our use of dissecting tools, with one set chilling over dry ice while the other was in use, and swapping back in the re-chilled tools every 15-30 seconds. We dissected entire brains, including optic and antennal lobes, but excluding the suboesophageal ganglia. After dissection, we immediately transferred each brain to a sterile 150 μL microcentrifuge tube and returned it to storage at -80 °C.

We performed individual chemical extractions in batches of six. For each brain, we added 100 μL of extraction solution containing all four isotopically labeled internal standards in methanol and 1% formic acid (v/v), and immediately sonicated the sample at 35,000 Hz for two 10-second bursts with a QSonica Q55A-110 Sonicator using a 2mm probe (Genesee Scientific Corporation, USA). After sonication, we re-sealed each microcentrifuge tube and stored it over dry ice until the batch of six was complete. We then centrifuged that batch of sonicates in a refrigerated centrifuge at 10,000 g for 20 minutes. If any particulates were still visible in suspension via visual inspection, we repeated the centrifugation step for those tubes, balancing the centrifuge with blanks. We transferred each supernatant into a brown glass microvial, which we stored at -80 °C until all extractions were complete.

We analyzed the samples at the Virginia-Maryland College of Veterinary Medicine Analytical Chemistry Research Laboratory. Analyte concentrations in bee brain samples were determined by UPLC-MS/MS.

We obtained reference standards of dopamine hydrochloric (DA), racemic p-octopamine hydrochloride (OA), tyramine (TA), L-tyrosine sodium salt hydrate (TS), isotopically labeled dopamine-d4 hydrochloride (Dd4), and L-tyrosine-13C915N (TSIS) from Cayman Chemical. We obtained isotopically labeled octopamine-13C215N acetic acid (OIS) and tyramine-d4 hydrochloride (TAIS) from Toronto Research Chemicals (Ontario, Canada). Stock solutions of the dopamines, octopamines, and tyramines were made up in methanol (MeOH) with 1% v/v formic acid (FA) to acidify and solubilize, and 0.1% w/v ascorbic acid (AA) as an antioxidant to stabilize the analytes in solution. The tyrosines, with the most acidic pKa, required 1% v/v hydrochloric acid (HCl) + 0.1% AA in MeOH for dissolution. We made up all stock solutions individually at a concentration of 1 mg/mL and then were diluted in 50/50/0.5% MeOH/H2O/FA to their final standard concentrations.

Sample extracts were subjected to chromatographic separation performed on a Waters H-Class UPLC system with an HSS T3 reverse phase column (Waters Acquity UPLC HSS T3, 100 mm length x 2.1 mm ID x 1.8 μm) and matching guard column (Waters Acquity UPLC HSS T3 VanGuard Pre-Column, 5 mm length x 2.1 mm ID x 1.8 μm) maintained at 40°C. We injected five microliters of sample onto the column using a refrigerated autosampler maintained at 5 °C. Mobile phase A consisted of 0.02% heptafluorobutyric acid (HFBA) + 0.02% trifluoroacetic acid (TFA) in water (H2O), mobile phase B consisted of 0.025% HFBA + 0.05% TFA in MeOH. The mobile phase was delivered to the UPLC column at a flow rate of 0.4 mL per min. The gradient elution program is shown in Table A1.

To reduce MS contamination, we used the divert valve to transfer the column effluent to the MS from 1.0 to 2.00 minutes. From 0 to 0.99 and 2.01 to 4.25 minutes, all the column effluent was transferred to waste. Simultaneous and efficient separation of all analytes of interest was achieved using this ion pairing methodology with octopamine, dopamine, tyrosine, and tyramine eluting at approximately 1.13, 1.32, 1.50, and 1.54 minutes, respectively. The UPLC column effluent was pumped directly without any split into a triple-quadrupole mass spectrometer (Waters Xevo TQD) equipped with a Zspray ionization source which was operated in positive-ion electrospray mode (ESI+) using multiple reaction monitoring (MRM). The parent and product ion transitions for the compounds of interest are shown in Table A2.

We used commercial software (MassLynx) to analyze the data. Tuning was performed on each analyte by direct infusion of standard solution (5 ng/μL) at a rate of 20 μL per min. Mass spectrometer parameters used for the detection of the analytes of interest are shown in Table A3.

We then made up a nine-point calibration curve in solution containing the same isotopically labeled internal standard as the extracts with a range of approximately 1 to 1,000 ng analyte / mL solution for all analytes. Using these standards, calibration curves were constructed for each of the individual analytes using the MassLynx software to determine analyte concentration in samples based on the sample / IS ratio.

Amino Acid and Biogenic Amine Response Variables

We quantified brain supply of the biogenic amines octopamine (OA), dopamine (DA), tyramine (TA), and their amino acid precursor tyrosine (Tyr) in terms of brain supply (ng analyte / brain homogenate) on the individual brains for the experimental bees that could be recaptured on Day 3 and which were also eligible for behavioral analysis (i.e., only visited either the treatment or control feeder during the experimental phase, see below, n = 25).

Statistical Analysis

Foraging and Waggle Dance Response Analysis

We performed all statistical analyses in R 4.2.3 (R Core Team 2023). We present in graphical form both the original data as well as data summarized by treatment and trial phase (experimental phase and persistency phase). In these summaries, we present both means (and 95%-CI) and medians (and upper and lower quartiles). We used generalized linear mixed effect models to infer any differences between treatment groups, and report point estimates for any treatment differences, including two-sided 95% confidence intervals (95%-CI) and associated *P*-values. We selected for analysis only those bees that foraged exclusively at one or the other feeder (i.e., collected only treatment or control sucrose solution) for the entire duration of the three-hour experimental phase (N = 86). In other words, we excluded all bees who visited both feeders at any point during the experimental phase from this analysis (N = 18), as those bees experienced both treatment and control conditions.

Unfortunately, when we began our video analysis of recruitment behavior, we found that the videos for hives 2 and 3 were of poor quality, and this precluded us from reading bee tag numbers. We therefore could only analyze dance propensity, dance frequency, and mean waggle run repetitions for the bees belonging to the first colony (trial 1). Since tag color was still readily identifiable, and tag color indicated for each colony the identity of the feeder to which a bee was initially trained and admitted to the experiment, we initially considered analyzing dance data for these latter two hives according to the intention-to-treat (ITT) principle (Gupta 2011). ITT analysis accepts noncompliance and protocol deviations as often occur in field trials, and accordingly avoids overoptimistic estimates of any effect of treatment. In our case, doing so would admit greater signal noise into the waggle dance analysis from feeder-swapping behavior, which we had observed during the trials. This was because some bees trained to one feeder and assigned the corresponding tag color ultimately swapped to the other feeder before the beginning of the experimental phase, and would therefore be assigned to the incorrect treatment group based on tag color in video data. While the ITT principle accepts this kind of signal noise, we opted to carry out an exploratory analysis to determine whether a comparable number of bees would be incorrectly assigned to their respective treatment group for both treatment and control – that is, whether the introduction of this signal noise would be equal in both directions. We found that levels of feeder-swapping were not even in each direction for either of the latter two colonies. Furthermore, the inability to discern bees' individual identities would have prevented us from excluding those bees that visited both feeders during the experimental phase as we did in the foraging frequency and persistency analysis. We therefore ultimately decided to analyze only the video data from the first experimental colony, and present dance data as secondary outcomes due to the lower statistical power available for the analysis of these waggle dance response variables.

We analyzed count data for foraging frequency during the experimental phase and for persistency visits during the persistency phase using Poisson generalized linear mixed-effect models. We modeled these data using the glmer() function from the lme4 package (Bates et al. 2015). For dance frequency, we used instead the Quasi-Poisson model family as we detected overdispersion in those data. We modeled dance propensity as a binomial response variable, with a binomial generalized linear mixed-effect model (function glmer(), package lme4) (Bates et al. 2015). We used the emmeans package to calculate treatment group contrasts and extract 95%-CI (Lenth et al. 2024). For each, we modeled the response variable with respect to categorical treatment group, feeder color cue (blue or yellow), and feeder position (left or right, from the outbound forager bee's view) as fixed effects, and included hive as a random effect to control for possible inter-colony differences (Harris and Woodring 1992), and generated a set of models for each outcome variable using backwards elimination of nonsignificant terms. We then selected the final model for each outcome according to the second-order Akaike Information Criterion (Portet 2020), the use of which is indicated when the number of modeled terms (k) exceeds the number of observations divided by 40 (n/40), as is the case with our dataset. To do this, we first used the AICc() function in the MuMIn package (Bartoń 2023) to compare identical models with and without hive as a random effect, selecting the model with the lowest AICc value as the most parsimonious model, and proceeding from there with backwards elimination of nonsignificant terms.

Amino Acid and Biogenic Amine Analysis

We analyzed the brain supply of each of our four analytes of interest as continuous numeric data using Gaussian generalized linear mixed-effects models (function glmer(), package lme4), specifying the following as fixed effects: treatment group, feeder color cue (blue or yellow), feeder position (left or right from the outbound forager bee's view), total number of feeder visits made during the experimental phase, and finally an interaction term combining treatment group with number of feeder visits. We included this interaction term to account for the incremental accrual of exposure to glyphosate on a per-feeder-visit basis; that is, bees in the GLY treatment group accrued an additional unit of exposure each time they collected sucrose solution containing GLY. We therefore included this interaction term to account for this variability in exposure arising from unequal numbers of feeder visits between bees within each treatment group. We obtained regions of significance for interactions by supplying the model object to the sig_regions() function from the package reghelper (Hughes and Beiner 2023).

We also conducted exploratory analysis of the brain analyte data using the pairs() and pairs.panels() functions in ggplot2, which allowed us to visualize and identify correlative relationships between analyte levels between treatment and control bees. Finally, when these exploratory analyses indicated a significant correlation between analytes, we then performed post hoc analysis of correlations between analyte levels by treatment group using the corr.test() function in R to quantify Pearson correlations, 95% confidence intervals, and p values. In a scenario where there may be large variability in baseline values of analytes or where sample size is limited, examining relationships between levels of precursors and products rather than raw amounts can be more informative of biosynthetic pathway activation (Lim et al. 2016) and physiological outcomes (Latshaw et al. 2023).

Results

GLY Decreased Foraging Frequency

The number of foraging visits estimated by our mixed model approach significantly differed between control bees (N = 40) and treatment bees (N = 46) (mean (95%CI)):

control: 31.76 (26.43 to 38.16), treatment: 27.52 (23.06 to 32.84); ratio: 1.15 (1.02 - 1.30); p = 0.022. In other words, bees collecting sucrose solution containing GLY foraged 13.34% less compared to control bees (Fig. 4.1). This was our primary outcome.

Feeder color also had a significant effect on foraging in our mixed model: bees foraging at a blue feeder visited 26.91 (22.61 to 32.03) times during the experimental phase, while bees foraging at a yellow color cued feeder visited 32.47 (26.99 to 39.06) times; ratio: 0.82 (0.74 to 0.93); p = 0.002. In other words, bees collecting from a feeder with a yellow color cue foraged 20.64% more than bees collecting from a feeder with a blue color cue. The model selected as most parsimonious, with the lowest relative adjusted Akaike's Information Criterion (AICc) via methods described above, also retained hive as a random effect, as well as feeder position as a nonsignificant fixed effect.

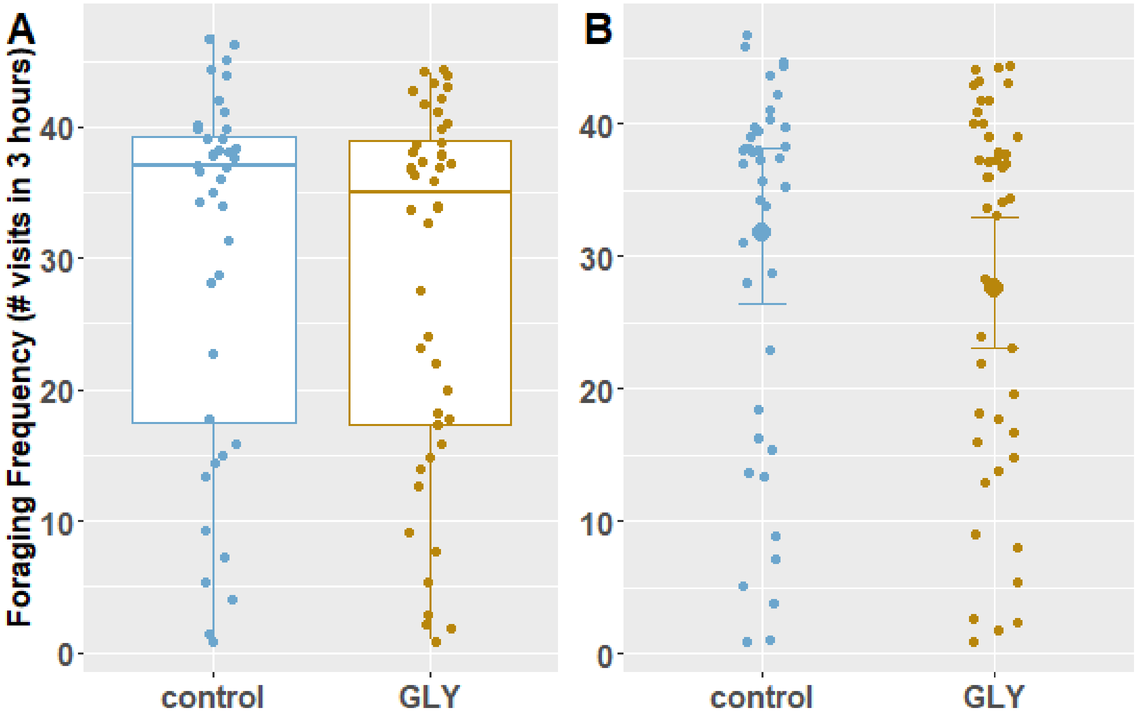

Figure 4.1. Foraging frequency was reduced in honey bees collecting 2 M sucrose solution containing GLY at 5 mg / L (n = 46) compared to bees collecting unaltered equimolar solution (n = 40). (A) Foraging frequency, medians and interquartile ranges over raw data points; (B) Foraging frequency, estimated marginal means and 95% confidence intervals over raw data points. Bees foraging on GLY foraged 13.34% less than control bees (p = 0.022).

GLY Did Not Affect Recruitment Behaviors or Persistency

Dance Propensity

Of the 26 bees belonging to the first experimental replicate that was suitable for video analysis, 16 performed at least one waggle dance during the experimental phase; control: 6 dancers, 6 non-dancers; treatment: 10 dancers, 4 non-dancers.

The predicted probability of a bee performing a waggle dance, or dance propensity, did not differ between control and treatment bees (estimated marginal probability (95% CI));

control: 0.50 (0.19 to 0.80); treatment: 0.65 (0.30 to 0.88); odds ratio: 0.54 (0.08 to 3.54); p = 0.519 (data not shown).

Dance Frequency

For the bees that did perform at least one dance, we first investigated their dance frequency qualitatively (geometric mean (95% CI)): control bees danced 5.32 (1.75 to 16.20) times during the experimental phase, and treatment bees danced 8.66 (5.37 to 14.00) times during the same period. The median number of dances was 4 for control bees, and 7.5 for treatment bees.

However, when compared via our mixed modeling approach, the number of dances performed did not differ between control and treatment bees (mean (95% CI)); control: 8.50 (3.99 to 18.11); treatment: 10.80 (6.42 to 18.16); ratio: 0.79 (0.31 to 1.98); p = 0.617 (Fig. 4.2A).

Waggle Run Repetitions

We then examined the mean number of waggle run repetitions performed by each dancer (geometric mean (95% CI)) by treatment group. Control bees' dances contained 14.90 (7.49 to 29.70) waggle runs, and treatment bees' dances contained 16.20 (10.90 to 24.20) waggle runs. The median number of was 14.4 for control bees, and 15.9 for treatment bees.

Among bees who danced during the experimental phase, the predicted mean number of waggle run repetitions per dance did not differ between control and treatment bees in our mixed modeling approach (mean (95% CI)); control: 17.62 (10.85 to 28.59); treatment: 18.49 (12.71 to 26.91); ratio: 0.95 (0.52 to 1.76); p = 0.919 (Fig. 4.2B).

Persistency

We performed an initial qualitative examination of persistency visits using arithmetic means and 95% CIs for persistency data, since some experimental bees were confirmed to be alive in the observation colony but made no persistency visits, and geometric means are inappropriate for data including values of zero.

Day 1: 72 of our 86 analysis-eligible Day 0 bees were still alive in the colony, and 66 of these (92%) made at least one persistency visit. Control bees made 4.26 (3.03 to 5.50) persistency visits, and treatment bees made 5.55 (4.39 to 6.71) persistency visits. The median number of Day 1 persistency visits was 3.5 for control bees, and 5 for treatment bees. The mean number of Day 1 persistency feeder visits predicted by our mixed model approach was not significantly different between our control and treatment bees (mean (95% CI)); control: 4.79 (3.95 to 5.82); treatment: 5.46 (4.77 to 6.27); ratio: 0.88 (0.68 to 1.12); p = .30 (Fig. 4.2C).

Day 2: 66 of our 86 analysis-eligible Day 0 bees were still alive in the colony on the morning of Day 2, and 31 of these (47%) made at least one persistency visit. Control bees made 0.85 (0.39 to 1.31) persistency visits, and treatment bees made 1.09 (0.54 to 1.65) persistency visits. The median number of Day 2 persistency visits was 0 for both treatment and control bees.

For Day 2 persistency, treatment was the least significant predictor in the full model, and we therefore removed it from the model first according to our backwards-elimination method. However, the most parsimonious model indicated a significant effect of the Day 0 feeder color cue on Day 2 persistency.

The mean number of persistency feeder visits predicted by our mixed model approach differed significantly between bees who had foraged at a feeder with a yellow color cue versus a blue color cue (mean (95% CI)); blue: 0.77 (0.55 to 1.06); yellow: 1.47 (1.02 to 2.13); ratio: 0.52 (0.32 to 0.85); p = 0.009 (data not shown). In other words, bees that on Day 0 had foraged at a feeder with a yellow color cue rather than blue during the experimental phase on Day 0 made 92.40% more persistency visits on Day 2 than bees foraging at a feeder with a blue color cue.

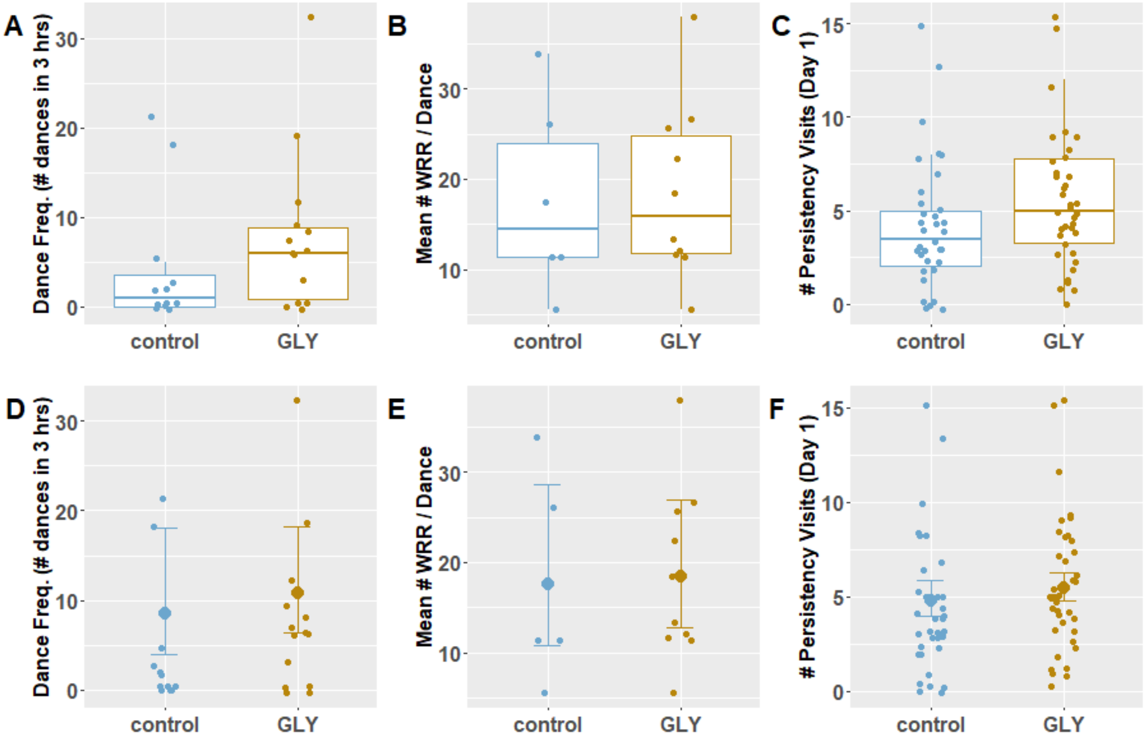

Figure 4.2. GLY exposure did not affect dance frequency, waggle run repetitions (WRR), or persistency (all NS). (A-C): Boxplots showing (A) dance frequency, (B) mean number of waggle run repetitions per dance, and (C) number of persistency visits,

showing medians and interquartile ranges over raw data points. (D-F): Estimated marginal means and 95% CI's over raw data points for (D) dance frequency, (E) mean waggle run repetitions per dance, medians and interquartile ranges; and (F) number of persistency visits.

Brain Supply of Tyramine was Modulated by Interaction of GLY Exposure and Foraging Frequency, but Tyrosine, Octopamine, and Dopamine Were Unaffected

In total, we quantified brain supply of tyrosine, tyramine, octopamine, and dopamine in the brains of 25 recaptured experimental bees with 12 bee brains for control, and 13 for treatment. We present descriptive, untransformed results (medians and min-max range (ng analyte / brain homogenate)), estimated marginal means, and p-value outputs of mixed effect models for each analyte in Table 1.

Our mixed model approach indicated a significant effect of the interaction between the number of feeder visits and treatment group (GLY versus control) on brain supply of tyramine ($p = 0.004$; Fig. 4.3), with the significance of this effect occurring between 25.13 and 45.42 feeder visits (given by the sig_regions() function in the reghelper R package, as described above). The visualization of the data indicates that it is likely a crossover interaction. In these circumstances, because the two-way interaction is driving the significance of the effect, interpreting the fixed effects (GLY treatment or foraging frequency) alone is not appropriate (Schielzeth 2010).

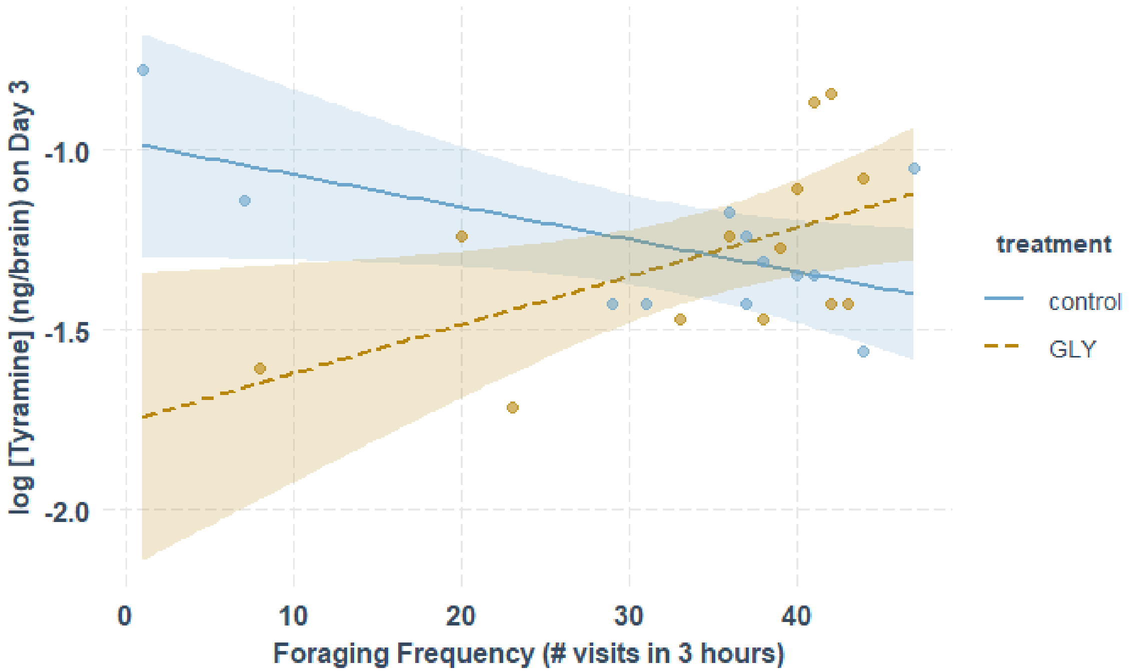

Figure 4.3. There is a significant interaction between treatment (GLY or control) and foraging frequency on the brain supply of TA, with the region of significance ranging from 25.13 - 45.42 foraging visits (foraging frequency; p = 0.004). Tyramine levels were measured for bees that were recaptured and flash frozen approximately 72 h after the experimental phase concluded (afternoon of Day 3).

Octopamine significantly correlates with Tyrosine and Tyramine in GLY bees

We found a significant correlation between log-transformed brain supply of octopamine (OA) and its two precursors, tyrosine (Tyr) and tyramine (TA), in the bees exposed to GLY (**Tyr**: Pearson’s product-moment correlation, for GLY bees: T = 2.77, p = 0.018 versus control bees: T = 0.18, p = 0.864; **TA:** Pearson's product-moment correlation, for GLY bees: T = 3.08, p = 0.011 versus control bees: T = -0.68, p = 0.512; Figure 4.4).

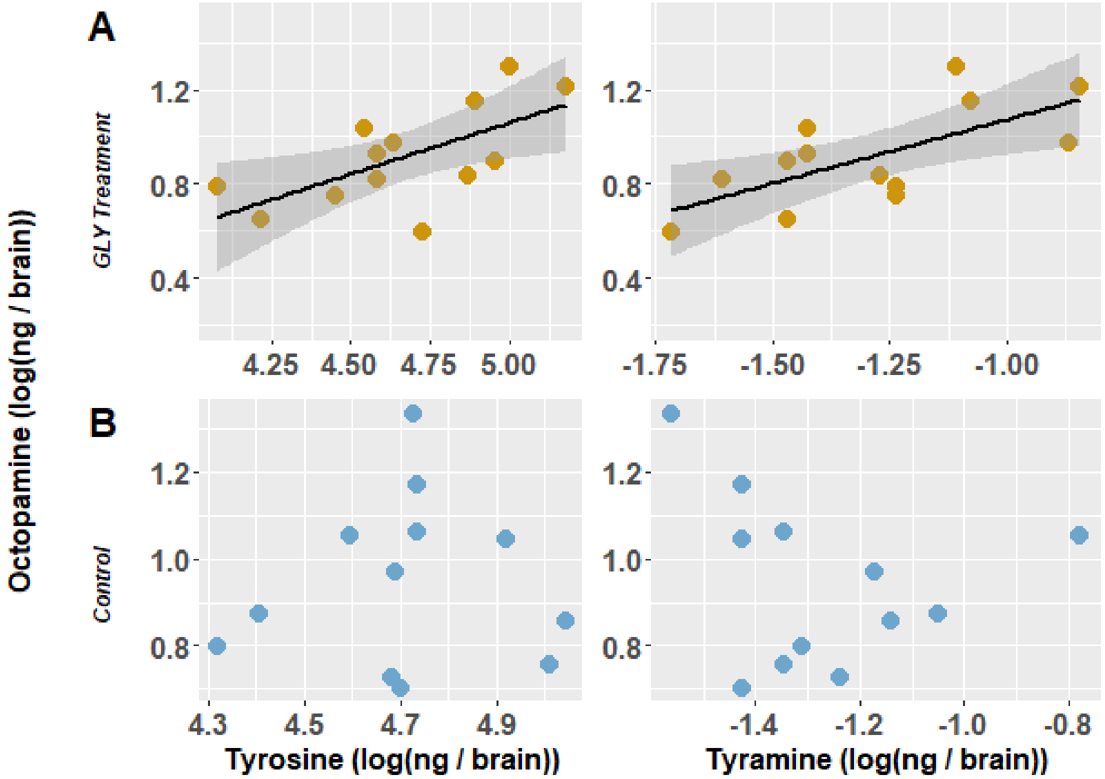

Figure 4.4. The brain supply of octopamine significantly correlated with its precursors tyrosine (left, p = 0.018) and with tyramine (right, p = 0.011) in (A) treatment but not (B) control bees. Each point represents the brain of a bee that experienced the three-hour experimental phase (Day 0) and was collected at the end of the persistency phase (Day 3).

GLY Exposure Was Confirmed as Sublethal

Across the three trials, a total of 86 bees foraged at an experimental feeder and remained site-specific to that feeder for the duration of the three-hour experimental phase and were therefore eligible for analysis (control: 40 bees; treatment: 46 bees).

The probability of survival to the morning of Day 2 predicted by our mixed model approach did not differ significantly between control bees and treatment bees (mean (95%)): control: 0.84 (0.65 to 0.94), treatment: 0.73 (0.53 to 0.86); odds ratio: 1.94 (0.63 to 5.98); p = 0.251. In other words, our GLY exposure was sublethal, not lethal, as we expected and in accordance with previous studies (Herbert et al. 2014; Thompson et al. 2014; Balbuena et al. 2015)

Discussion

Here we report that honey bee foragers experiencing a field-realistic, sublethal exposure to glyphosate (GLY) foraged significantly less (-13.34%) compared to control bees (Figure 1). GLY did not affect recruitment (dance propensity, dance frequency, and waggle run repetitions) in our trial 1 bees, nor were bees' persistency altered by the treatment (Figure 2). However, our data also indicate that exposure to GLY can affect the neurochemical state of the forager bee brain. We found that the brain supply of tyramine was modulated by an interaction between GLY treatment and foraging frequency, such that tyramine was higher in bees that foraged on GLY sucrose solution at least 25 times during the experimental phase (Figure 3). Lastly, we found that octopamine levels correlate with the levels of its precursors tyrosine and tyramine (Figure 4), but only in the bees exposed to GLY. Overall, these data indicate that the most widely used herbicide worldwide generates subtle but significant effects on the behavior and physiology of exposed foraging honey bees.

Bees foraging on sucrose solution containing glyphosate at 5 mg/L decreased their foraging by 13.34% compared with control bees. Interestingly, this result is in contrast with previous work that showed no effect on foraging frequency under GLY exposure conditions (Herbert et al. 2014). However, the foraging experiment in that study used a lower GLY concentration than we did (2.5 mg GLY–/ L), with double the molarity of sucrose solution (2 M). Sucrose solution at 2 M offers a highly rewarding foraging experience, which is why we used this concentration for training bees when we wanted to maximize their commitment to that particular feeder. However, such a highly concentrated sucrose reward may mask any effect of treatment if the effect is not sufficient to overcome such a strong reward, which is why we swapped to 1 M during the experimental phase. Additionally, the study by Herbert et al. (2014) examined a bee's foraging cycle time for three foraging trips before and after the addition of GLY, rather than studying separate side-by-side treatment groups, as we did. Since foraging and recruitment scale positively with a bee's experience at a rewarding food source (Seeley 1995, Couvillon et al. 2015), any effect of treatment may have additionally been impacted by the order effect (with all GLY visits happening after all control visits, concurrent with the foragers having more experience at the feeder).

In our study, GLY did not affect recruitment (waggle dance propensity, waggle dance frequency, waggle run frequency) or persistency. This was surprising because the foraging and recruitment behaviors in these studies typically scale positively together for a given food source, according to the bee's perception of its profitability. In other words, the higher her assessment of a resource's profitability, (1) the more frequently she will visit it to collect food, (2) the more likely she is to dance to advertise it to nestmates, (3) the more frequently she will perform independent bouts of dances, (4) the greater the number of waggle runs she will perform per dance (von Frisch 1967; Seeley 1995; Couvillon et al. 2015), and finally, (5) the more persistently she will revisit that food source after it has become unrewarding (Al Toufailia et al. 2013). In our study, however, GLY elicited a reduction only in foraging, while the other behaviors remained unaffected.

This apparent uncoupling of behaviors that are expected to increase or decrease in tandem may be due to the effect of treatment being subtle enough that our statistical power was insufficient to capture any difference. In the case of our analyses of waggle dance behaviors, this may well be the case, since only a subset of dance data was suitable for analysis because of insufficient video quality. It is also worth noting that any effects on persistency might be more difficult to detect compared to effects on foraging frequency, since we would expect the range distribution of persistency visit counts to be smaller. A forager bee working a highly profitable resource may visit as frequently as she can, limited only by her physiological constraints, the distance of the resource, and any nectar unloading delays. So long as her previous trip was sufficiently rewarding, she has incentive to return. In contrast, a forager bee making a visit to a previously-rewarding but now unrewarding food source would be ill-served by continuing to visit at such a high frequency, as the likelihood of resource replenishment would not outweigh the cost of such frequent repeated visitation. In other words, the maximum visit frequency that is adaptive is higher for rewarding food sources, and lower for spent food sources that may or may not become rewarding again. Thus, the range distribution of persistency visits would be expected to be smaller than that of rewarded visits, and any change in persistency may therefore be more difficult to detect. Nevertheless, our results are consistent with a scenario in which a honey bee foraging workforce collecting GLY-laced nectar in the field might reduce its foraging frequency while maintaining recruitment. Such a scenario could reduce the total nectar inflow while still maintaining a steady inflow of GLY residue to the colony, which could ultimately produce deleterious health outcomes at the colony level.

We found that bees exposed to sublethal GLY experienced alterations in both brain supply and relative levels of tyrosine, tyramine, and octopamine. First, we found that tyramine levels in bee brains were related to an interaction effect between treatment group (GLY or control) and foraging frequency, and that the region of significance for this effect was from 25.13 - 45.42 feeder visits. (Since feeder visits cannot be fractional, we therefore interpret the biologically relevant region of significance of this effect as 26 – 45 visits, or all the integer values contained in that range.) In other words, once treatment bees accrued at least 26 increments of exposure to GLY during the experimental phase, their brain supply of tyramine increased more per additional feeder visit than did that of control bees.

The tyramine receptor AmTYR1 is expressed in multiple regions of the honey bee brain (Mustard et al. 2005; Sinakevitch et al. 2017; Thamm et al. 2017), and both AmTYR1 and the cellular sources of tyramine are localized most particularly in the olfactory learning and memory neuropils regions (Sinakevitch et al. 2017). Accordingly, the gene that encodes this receptor exerts a pleiotropic suite of effects on behaviors and physiologies related to foraging and recruitment, including effects on sucrose sensitivity (Pankiw et al. 2001; Thamm et al. 2017; Scheiner et al. 2017), proclivity to forage nectar or pollen (Page et al. 2000; Arenas et al. 2021), division of labor as it relates to foraging (Scheiner et al. 2014; Cook et al. 2019), and latent inhibition (Cook et al. 2019; Latshaw et al. 2023). Importantly, activation of the AmTYR1 receptor by tyramine results in a reduction in levels of 3’-5’-cyclic adenosine monophosphate (cAMP) in a dose-dependent manner (Blenau et al. 2000). We might therefore expect that activation of AmTYR1 would reduce the excitability of those axon terminals throughout the bee brain, which would be consistent with a suppression of the activation of the behavioral syndrome associated with increasing foraging and recruitment alongside increasing perceived profitability. Treatment with tyramine can also produce behavior changes that align with a reduction in foraging: worker bees injected with tyramine spend less time flying (Fussnecker et al. 2006) and are less apt to initiate precocious foraging (Schulz and Robinson 2001). Altogether, our observed increase in tyramine in treatment bees at higher increments of exposure therefore seems consistent with our observed reduction in foraging frequency.

It is important to note that this interaction between treatment group and foraging frequency could be interpreted in the other direction: a positive relationship between brain supply of tyramine and higher foraging frequency values could have also emerged in a scenario where individual bees that naturally have higher tyramine levels (e.g., scout bees have higher tyramine than recruit bees; see Cook et al. 2019) might tend to forage more frequently. However, this interpretation is inconsistent with the documented functions and effects of tyramine in bees as an overall suppressor of foraging behaviors (Scheiner et al. 2002; Pankiw et al. 2003; Schutzler et al. 2019; Latshaw et al. 2023). It is also unlikely that greater foraging frequency alone contributed to higher tyramine levels, since tyramine has been shown to be unrelated to cumulative foraging experience (Peng et al. 2021; Latshaw et al. 2023).

Our experimental bees that were exposed to sublethal GLY also experienced alterations in the relative brain supply of octopamine with both tyramine and tyrosine. Specifically, we found that GLY exposure can induce a positive correlative relationship between the brain supply of octopamine and TA, as well as between octopamine and tyrosine three days following the three-hour sublethal exposure. We found no such correlations between any of our studied analytes in control bees (Figure 3). The emergence of significant correlations between levels of octopamine with tyramine and tyrosine in treatment bees should be interpreted in light of the fact that these three molecules are biosynthetically linked; indeed, the amino acid tyrosine ultimately serves as the backbone molecule for the biosynthesis of all three catecholamines studied. Briefly, tyrosine is converted to tyramine via decarboxylation by tyrosine decarboxylase (Karlson and Herrlich 1965); tyramine is then acted upon by tyramine-beta-hydroxylase and converted to octopamine (Roeder 2005). Relative brain supply between constituents of this biosynthetic pathway can therefore be indicative of pathway activation, and may even be more informative than total brain supply when there exists wide variability in precursor concentrations between individuals (Lim et al. 2016), as was the case for tyrosine brain supply in our bees (Table 1). The emergence of correlative relationships between octopamine and both its immediate precursor tyramine and its ultimate precursor tyrosine in bees exposed to GLY therefore indicates an effect of treatment on the neurochemical state of the bee brain as it pertains to tyramine and octopamine synthesis. Although our analysis did not reveal significant differences in the amount of tyrosine in the bee brains (Table 1), the emergence of correlations between levels of co-metabolites tyramine and octopamine in treatment bees (Figure 4) would be consistent with a scenario where their precursor tyrosine becomes more limiting with GLY exposure. The idea that available tyrosine may limit tyramine and octopamine is also supported by evidence that dietary supplementation with tyrosine can increase brain levels of tyramine and octopamine (Matsuyama et al.

2015). In our study, it is possible that total bioavailable tyrosine might have been limited, even if such limitation was not detectable in brain tissue (Table 1).

How might GLY affect a reduction in available tyrosine in the honey bee, particularly outside of the brain? Although honey bees themselves do not endogenously possess the molecular target of GLY, namely EPSPS within the shikimate pathway, some members of the gut microbiota do. Honey bees possess a relatively simple and conserved microbiome (Martinson et al. 2011; Moran et al. 2012) organized into five core lineages clustering within the genera *Bifidobacterium*, *Bombilactobacillus* (previously *Lactobacillus* Firm-4 (Zheng et al. 2020)), *Lactobacillus* (previously Lactobacillus Firm-5 (J. Zheng et al. 2020)), *Snodgrassella*, and *Gilliamella*, with additional non-core bacteria of the genera *Bartonella*, *Commensalibacter* and *Frischella* commonly present (Kwong et al. 2017). Previous work has demonstrated that the bee gut microbial community is susceptible to perturbations by GLY. For example, bees fed GLY at concentrations ranging from 0.01 – 0.1 mM show not only a reduced total abundance of beneficial bacteria (Motta et al. 2018; Motta and Moran 2020), but they also demonstrate impacts to microbial biodiversity via changes in the relative abundance of some bacterial groups (Motta et al. 2018; Motta and Moran 2020; Blot et al. 2019; Castelli et al. 2021). Notably, most *Snodgrassella alvi* strains express a susceptible class I EPSPS (Motta et al. 2018), and usually drop in relative abundance in the presence of GLY while other groups increase, though there is some between-strain variation (Motta et al. 2018).

Under normal conditions, *S. alvi* forms a biofilm on the chitin-layered wall of the bee ileum and rectum (Callegari et al. 2021; Motta and Moran 2023) and enriches the lumen with amino acids, notably tyrosine and other products of the shikimate pathway (Zheng et al. 2017). While it is not confirmed whether microbe-produced tyrosine specifically is bioavailable to the bee host, microbial provisioning of an insect host with tyrosine is documented in other insect taxa (Anbutsu et al. 2017, with beetles), and the gut microbiome is known to donate other amino acids to bee nutrition and physiology (including tryptophan, another product of the shikimate pathway) (Motta and Moran 2023). In a GLY exposure scenario in which the shikimate pathway is inhibited in the gut microbiome, microbial production of these amino acids and any contribution thereof to the host physiology would likewise be expected to decrease. Importantly, tyrosine is the biosynthetic precursor of multiple, important insect neurotransmitters, including octopamine (Livingstone and Tempel 1983; Monastirioti et al. 1996; Lehman et al. 2000), tyramine (octopamines precursor and a neurotransmitter in its own right) (Kononenko et al. 2009; Lange 2009; Blenau and Baumann 2016), and dopamine (Murdock et al. 1973; Owen and Bouquillon 1992). Octopamine in particular is intricately involved in a diverse suite of insect physiological processes (reviewed in Farooqui 2012). Of particular interest is its involvement in foraging and recruitment behaviors: honey bees with higher levels of octopamine show increased propensity for initiation of flight behavior (Fussnecker et al. 2006) and precocious foraging (Barron et al. 2002), increased sucrose sensitivity (Scheiner et al. 2002; Pankiw and Page 2003), decreased thresholds of olfactory response (Mercer and Menzel 1982; Barron et al. 2002), improved olfactory memory formation and retrieval (Menzel et al. 1999), and higher propensity to reinvestigate previously rewarding food sources (or persistency, as we call it here) (Linn et al. 2020).

Possible evidence for a downstream limitation of tyrosine in bee physiology resulting from GLY exposure also arises from studies of GLY's impact on melanization in insects. Tyrosine is also a biosynthetic precursor of melanin in the melanization cascade (Rzepka et al. 2016), which serves a key role in immune function via nodulation and encapsulation of invading pathogens and parasites (Nappi and Vass 1993; Zhao et al. 1995; Carton and Nappi, 1997; Nappi and Ottaviani 2000; Dubovskiy et al. 2016). GLY exposure inhibits melanization and increases susceptibility to infection in two non-Hymenopteran taxa (Smith et al. 2021). While there is evidence that this inhibition occurs via oxidative effects on melanogenesis (Smith et al. 2021), such inhibition might plausibly also be achieved by a reduction in tyrosine availability as a required precursor. Further evidence comes from beetles, in which tyrosine provisioned by the endosymbiont *Nardonella* is necessary for proper cuticle melanization and sclerotization, and endosymbiont-suppressed individuals show a lighter-colored phenotype with a soft cuticle (Anbutsu et al. 2017). It would be informative to investigate the effect of GLY on melanization in honey bees as well to further explore whether inhibition of the shikimate pathway in the bee gut microbiome could reduce tyrosine availability in such a way that it becomes limiting in its various functions within the bee host physiology.

Intriguingly, we now see an emerging picture of overlap between the lists of behaviors and physiologies that (1) underpin foraging, (2) are disrupted by GLY exposure, and (3) are modulated by neurotransmitters biosynthesized from Tyr, an amino acid produced in the gut microbiome and plausibly donated to the bee host by the very pathway that GLY inhibits. There is even evidence that the bee gut's colonization status can directly affect levels of certain neurotransmitters, including dopamine, in the bee brain (Zhang et al.

2022). However, as far as we are aware, the effects of glyphosate exposure on levels and ratios of tyrosine and its product neurotransmitters octopamine, tyramine, and dopamine in the bee brain have never been studied. Recent work supports the existence of the gut microbiota - brain axis in insects (Cryan and Dinan 2012; Liberti and Engel 2020; Liberti et al. 2022; Motta and Moran 2023). The honey bee gut microbiome contributes to growth and development (Zheng et al. 2017), immunity (Emery et al. 2017; Kwong et al. 2017), endocrine function (Kešnerová et al. 2017; Zheng et al. 2017; Kwong et al. 2017), nutrition (Zheng et al. 2019), and even the colony's social network (Liberti et al. 2022). The microbiome also contributes to important physiological and behavioral underpinnings of foraging and recruitment behavior: microbiota-free or -depleted bees demonstrate not only reduced sucrose sensitivity compared to normally-colonized bees (Zheng et al. 2017), but also diminished olfactory learning (Zhang et al. 2022a; Cabirol et al. 2023) and navigation capabilities (Zheng et al. 2017). Lack of a normal gut microbiome can also directly alter levels of neurotransmitters GABA, 5-HT, and dopamine in the brain (Zhang et al. 2022). Therefore, it will be valuable to investigate more thoroughly how GLY impacts the gut microbiome as a possible route of pesticide exposure in insect pollinators.

Taken together, our results demonstrate that sublethal GLY exposure impacts honey bee foraging behavior concurrent with the exposure, and that it affects the neurochemical balance of octopamine, tyramine, and tyrosine in the bee brain on the scale of days post-exposure. This finding highlights the importance of characterizing the effects of GLY on the bee brain, particularly along the axes of dosage, and timing of/since exposure. Further work is needed to clarify the extent and character of possible causal linkages between perturbations of the gut microbiome associated with GLY exposure (Motta et al. 2020; Motta and Moran 2020, 2023), and downstream impacts on bee physiology and behavior. Furthermore, it is possible and even likely that downstream impacts of disruption to the gut microbiome could work additively or synergistically alongside other mechanisms of action. Therefore, there is a critical need to elucidate the whole-body impacts of GLY in insect systems, which will then better equip us to make informed pesticide regulatory decisions that protect non-target beneficial insects like pollinators.

Tables

Analyte	Group	Median (Range)	EMMEAN (95% CI)	Predictor, P-value
Tyrosine	Treatment (GLY)	103.00 (91.30 - 132.00)	4.67 (4.52 - 4.83)	Treatment, 0.694
	Control	114.00 (98.60 - 129.00)	4.71 (4.55 - 4.88)	
Tyramine	Treatment (GLY)	0.28 (0.24 - 0.33)	NA	Treatment*Foraging Frequency, **0.004****
	Control	0.27 (0.24 to 0.33)	NA	
Octopamine	Treatment (GLY)	2.45 (2.22 - 2.89)	2.50 (2.23 - 2.81)	Treatment, 0.635
	Control	2.52 (2.28 - 2.96)	2.57 (2.28 - 2.91)	
Dopamine	Treatment (GLY)	2.43 (2.10 - 2.81)	2.54 (2.16 - 2.98)	Treatment, 0.758
	Control	2.55 (2.13 - 2.70)	2.42 (1.94 - 3.03)	

Table 4.1. There was no effect of GLY exposure on brain supply for tyrosine, octopamine, or dopamine (ng / brain homogenate), but brain supply of tyramine was modulated by the interaction between treatment group and foraging frequency. Estimated marginal means (EMMEANS) are excluded for TA, since they may be misleading in the presence of a significant interaction.

Symbols and Abbreviations (Listed Alphabetically)

AA: Ascorbic acid
DA: Dopamine
Dd4: Dopamine-d4 hydrochloride
FA: Formic acid
GABA: Gamma-aminobutyric acid
GLY: Glyphosate
HFBA: Heptafluorobutyric acid
HSS: High strength silica
MRM: Multi-reaction monitoring
MS: Mass Spectrometry
MS/MS: Tandem Mass Spectrometry
OA: Octopamine
OIS: Octopamine-13C215N acetic acid
TA: Tyramine
TAIS: Tyramine-d4 hydrochloride
TFA: Trifluoroacetic acid
TS: L-tyrosine sodium salt hydrate
Tyr: Tyrosine
UPLC: Ultra High-Performance Liquid Chromatography
5-HT: 5-hydroxytryptamine (serotonin)

Appendix A

Time (mins)	**%A (0.02%HFBA + 0.02%TFA in H_2O)**	**%B (0.025%HFBA + 0.05%TFA in MeOH)**
0.00	80	20
1.00	0	100
2.50	0	100
2.51	80	20
4.25	80	20

Table A1. UPLC gradient method used for the ion-pairing analysis.

Analyte	**Parent Ion (amu)**	**Product Ion (amu)**	**Cone Energy (V)**	**Collision Energy (eV)**	**Quant/Qual Transition**
Dopamine	137.0 $[M+H]^+$	91.0	40	18	Quantifier
	137.0 $[M+H]^+$	118.9	40	12	Qualifier
Dopamine-d4 (IS)	141.1 $[M+H]^+$	94.8	46	18	Quantifier
Octopamine	136.0 $[M+H]^+$	91.0	32	16	Quantifier
	136.0 $[M+H]^+$	118.9	32	12	Qualifier
Octopamine-$^{13}C_2$,^{15}N (IS)	139.1 $[M+H]^+$	92.0	30		Quantifier
Tyramine	121.0 $[M+H]^+$	77	40	20	Quantifier
	121.0 $[M+H]^+$	103	40	15	Qualifier
Tyramine-d4 (IS)	125.1 $[M+H]^+$	106.3	40	16	Quantifier
Tyrosine	182.1 $[M+H]^+$	136.0	22	16	Quantifier
	182.1 $[M+H]^+$	165.1	22	10	Qualifier
Tyrosine-$^{13}C_9$,^{15}N (IS)	192.1 $[M+H]^+$	145.1	24	12	Quantifier

Table A2. MRM transitions and specific mass spectrometry tuning parameters for the quantification of the analytes of interest.

Parameter	Value
Capillary (kV)	0.50
Cone (V)	40
RF (V)	2.50
Extractor (V)	3.00
Source Temperature (°C)	150
Desolvation Temperature (°C)	600
Cone Gas Flow (L/Hr)	10
Desolvation Gas Flow (L/Hr)	750

Table A3. Mass spectrometer tuning parameters for the detection of the analytes of interest.

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Competing Interests

The authors declare no competing or financial interests.

Author Contributions

L.C.M. and M.J.C. conceived and designed the feeder training experiment, and conducted it with assistance from R.S. and B.D.O. L.E.J. assisted with feeder data entry and collected dance data from video recordings. L.C.M. analyzed the data with assistance from R.S.

L.C.M. conceived the molecular portion of the experiment, and A.D.G. advised on its design and provided invaluable laboratory space and assistance. L.C.M. performed brain dissections and sonication. M.C.T. conducted the UPLC and wrote the associated portion of the materials and methods. L.C.M. and M.J.C. wrote the manuscript, and all co-authors contributed valuable discussions and comments throughout.

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Data Availability

All data and analysis code are available at XXX (a permanent DOI will be provided after final acceptance of paper).

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CHAPTER 5

OVERALL DISCUSSION AND FUTURE DIRECTIONS

In the course of my graduate school experience, I was frequently asked what my dissertation was about in a nutshell. As someone who leans verbose but is at least self-aware about it, I often began “How much explanation are you after? My dissertation won’t really be a cohesive thing -- it’ll be more like three raccoons stacked on top of each other in a trench coat, pretending to be one thing.” I have found the effort to neatly unite the components of my Ph.D. research – (1) my studies of individual waggle dance calibrations and their impact on communication, (2) my description and examination of waggle dance recruitment networks, and (3) my testing of the effect of an herbicide toxicant on foraging, recruitment, and the balance of biogenic amines in the bee brain – into a tidy bundle for this overall discussion to be about as straightforward as stacking raccoons. The work presented in this dissertation pushes the boundaries of our knowledge outward in a few directions at once, like little trash bandits chasing different shiny things. At the same time, the multi-directionality of the work has enriched my intellectual growth and analytical capabilities as a scientist, not to mention preventing me from burning out on any one topic. I thrive on variety. The exercise in mental and analytical flexibility brought on by such wide-ranging work has been deeply rewarding.

In Chapter 2, we showed that the range of individual calibrations among forager bees (Schürch et al. 2016) does affect the likelihood that a given dancer-follower pair will communicate successfully, and that this likelihood depends not on either member, but on the relationship between the two communication partners' calibrations. We now know that a follower bee is more likely to locate successfully a dance-advertised food source in the landscape if she expects it to be farther away than it is. This contribution in turn then opens us up to new questions and directions (Figure 5.1): what underpins the variation in calibration in a colony, and is it adaptive or a result of physiological constraint? With a sufficient sample size, would we discern clustering of calibration values along the lines of patriline, of which there are many and in varying relative abundances in a honey bee colony (Withrow and Tarpy 2018)? While recent years have seen scientific forays into the existence and role of conserved inter-individual differences, or individuality, in animal societies, the whole field is still in its infancy, and this is even more true for the eusocial insects. To my mind, the exploration of the proximate and ultimate mechanisms of individuality in eusocial insect societies can only add fascination and nuance to our understanding of social living.

In Chapter 3, we first combined an adapted a deterministic compartmental model (DCM) of the "systemic-infected" (SI) type (Anderson and May 1991) with network simulation to estimate a biologically-informed empirical upper bound for network density. While other researchers had previously applied SI DCMs to study and to simulate upper-bounds for trophallaxis-mediated infection networks in honey bees (Gernat et al. 2018), this dissertation is the first effort to adapt such a model to study information flow in the waggle dance. We found that using our biologically-informed complete graph (BICG) as the upper bound for network density rather than the complete graph yielded biologically-informed network density values that, while still sparse, were larger than what was calculated using classical density by three orders of magnitude. This result strongly emphasizes the importance of implementing network analysis methods with a critical lens toward the biology of the organism in question. Had we simply calculated network density without considering that a complete graph in the classical sense is a biological impossibility in the context of honey bee foraging, we would be reporting density that understated the networks' connectivity by a factor of 1000. I do not suggest the abandonment of the complete graph as an upper bound for network density across the board, since classical density calculated thus enables cross-species density comparisons. However, I do propose that future studies of animal social networks will glean more meaningful insights into the biology of the species in question when descriptive statistics are also calculated using biologically-informed reference points.

In Chapter 3, we also explored burstiness, or temporal heterogeneity in the performance of waggle dance bouts. We found strong evidence of clustering, which we interpret as evidence for the existence of two burstiness behavioral types: non-bursty bees that perform dances at regular intervals, and bursty bees that perform many dance bouts in quick succession, followed by periods of longer gaps between dances. It may be that our bimodal burstiness distribution plays an adaptive role in the regulation of collective foraging, which would make most sense under a "repeat-exposures" strategy of information transmission (Karsai et al. 2011; Min et al. 2013; Rocha and Blondel 2013; Evans et al. 2020)). In such a strategy, multiple exposures to the same information can push an individual above an information acceptance threshold. It seems plausible that the repeat-exposures learning strategy might be at play in a colony, since the waggle dance is, in its most basic form, a repeating informational subunit. A dance follower who follows a dance in earnest is repeatedly exposed as she follows multiple waggle runs (Judd 1994, **mean 8 runs**; Grüter et al. 2008, **17**; Wray et al. 2012, **15.5**; Menzel et al. 2011, **20-23**) by one nestmate advertising the same location before she rapidly peels off from the dancer, having accepted the received information, and scurries out of the colony (Seeley 1995). One level up, however, an unoccupied bee on the dance floor may encounter repeated exposures to multiple separate waggle dances that may advertise different foraging locations (Seeley 1995). We know that bees are equally likely to follow a waggle dance, and equally likely to search for the resource it advertises regardless of the relative duration of the waggle run, or distance indicated (Hasenjager et al. 2022). Nor do bees ignore dances for "implausible" locations (Wray et al. 2008). What, then, flips the switch between half-hearted, languid dance-following and the enthusiastic, energetic dance-following that precedes a hive exit?

I speculate that unoccupied followers on the dance floor (particularly those that are not in possession of private information that they will act upon after dance-following (Biesmeijer and Seeley 2005; Grüter et al. 2008; Grüter and Ratnieks 2011) may respond to a nested system of repeat exposures leading to the overcoming of information acceptance thresholds, where repeated exposures to waggle dances occurring within perceptive range on the dance floor eventually produce a change in volitional state towards the earnest acquisition of waggle run information. Then, once this has occurred, the repeated exposures to location information via following multiple waggle runs ultimately supersedes that bee's information acceptance threshold and results in her exiting the hive in short order to search for the resource. In other words, repeat exposures to waggle dances on the dance floor can overcome a threshold for accepting the information that changing activities to foraging is indicated, and a concomitant change in volitional state toward earnest dance-following; then, repeated exposure to waggle runs advertising a particular location overcomes an acceptance threshold for where to direct those foraging efforts. It also may be possible that some attribute(s) of the signal itself may modulate either of these information acceptance thresholds, if they exist. For instance, noisier waggle runs with higher angular scatter, or directional imprecision (Al Toufailia et al. 2013; Klein et al. 2018), might take more repeat exposures to be accepted. Perhaps more energetic, vigorous dances (Seeley 1995) with shorter return phases might reduce information acceptance thresholds. Future work should examine the role of the bursty dancers as they sporadically and temporarily heighten the "volume" of the waggle dance signaling on the dance floor. A coincidence of bursts of waggle dancing with new waves of recruits to an advertised food source would support the model of repeat exposures in recruitment.

Finally, in Chapter 4, we learned that sublethal GLY exposure produces a small but significant reduction in foraging. Intrigued by this result and puzzled by the lack of any described mechanism of action in insect physiology, I developed the idea that maybe tyrosine levels and its downstream molecular products tyramine and octopamine, both important insect neurotransmitters, might be limited. While we did not find any effect on absolute levels, we did learn that GLY can induce significant correlations in the levels of octopamine with both tyramine and tyrosine in the bee brain three days following a three-hour exposure period, where no correlations existed in control bees. My idea to test tyrosine, tyramine, and octopamine was admittedly based on a series of unconfirmed hypotheses that I myself did not have capacity to test in my program: first, that the bee gut microbiota, which did have the shikimate pathway, were producing tyrosine that was bioavailable to the host; second, that foraging on GLY-laced sucrose solution could inhibit the shikimate pathway in those microbes to produce a meaningful reduction in tyrosine, which would then make tyrosine more limiting to the bee. Our data don't seem to support this model of indirect impact. However, since we did not test tyrosine levels in the gut lumen, hemolymph, or any other part of the body besides the brain, I remain unconvinced that tyrosine limitation via gut microbiome perturbation is not underlying the changes we found in the balance of precursor with product(s).

Our results here have contributed an important piece to the model of impact for this herbicidal chemistry on the insect physiology. The revelation that GLY exposure can not only produce changes in biogenic amine balance in the bee brain, but that this effect is observed on the scale of three days post-exposure emphasizes the critical need to elucidate its mechanism of action in insects. My hunch is that indirect effects of gut microbiome perturbation via limitation of tyrosine has a part to play, but future work should also focus on whether there may be one or more endogenous targets in insects as well, whether genomic, transcriptomic, or proteomic (Fig. 5.1). Only armed with specific knowledge about its real-world impacts will we be better-equipped to make informed and strategic pollinator protection decisions.

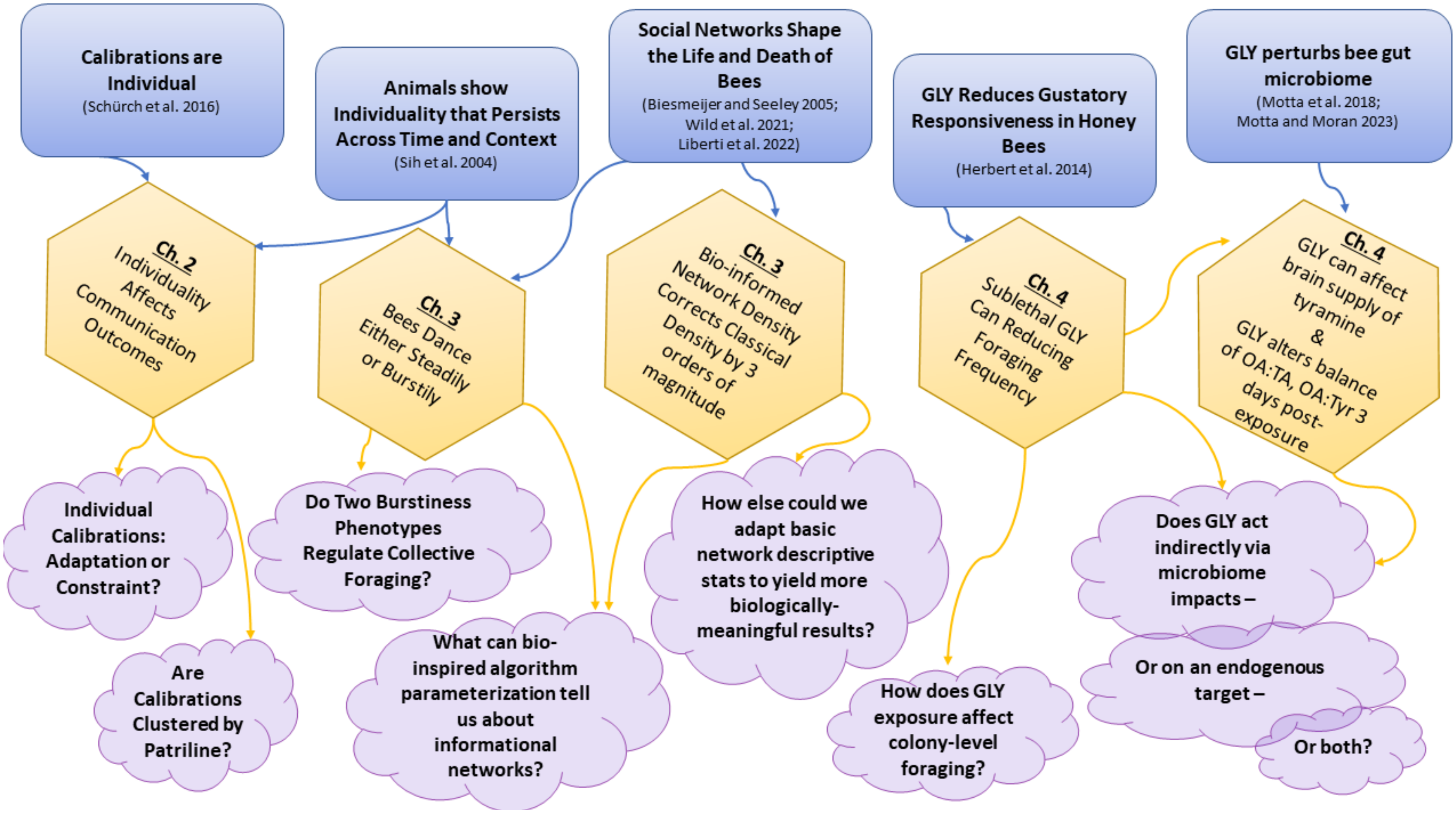

Figure 5.1. Overview of this dissertation (hexagons) and how it fits into the larger field of honey bee foraging and recruitment, including both the foundational research that inspired and informed it (blue rectangles) and future research questions arising from my contributions (purple clouds).

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Individually-marked bees on the comb in the observation hive. Credit: L. McHenry.

Laura applies a BetterBee tag to a cold-anesthetized worker. Credit: R. Schürch.

Bee-Marking Party. Facing camera: L. McHenry. Back row, from left to right: L. Johnson, H. Stilwell, B. Ohlinger, M. Couvillon, and R. Schürch's plaid shoulder.
Credit: L. McHenry.