EFFECTS OF SOCIAL CUES ON MONARCH BUTTERFLY ORIENTATION AND OVIPOSITION BEHAVIOR

by

CAROLINE G. AIKINS

(Under the Direction of Sonia Altizer and Takao Sasaki)

ABSTRACT

Decisions made during oviposition and migration have critical fitness implications for monarch butterflies (Danaus plexippus). The presence of conspecifics or cues left behind can indicate desirable resources and provide energetically inexpensive information. We examined female monarch response to conspecific eggs across swamp milkweed (Asclepias incarnata) stalks of differing physical quality. Additionally, we asked whether monarchs reared under different conditions would orient more accurately as conspecific group size increased. Females did not change their oviposition behavior in response to social cues, neither avoiding nor preferring stalks containing conspecific eggs regardless of host-plant quality. During flight trials however, group size had a significant, positive effect on orientation, suggesting that conspecifics improve monarch navigation. These results provide novel evidence of social information use by monarchs, and indicate that social cue use may be situational, or dependent on larval rearing environment. Future work could further examine the link between rearing environment, genetics, and social behavior.

INDEX WORDS: *Danaus plexippus*, social information, orientation, migration, conspecific, oviposition, host-plant, ovipositor, *Asclepias incarnata*

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May 2022

DEDICATION

I dedicate this work to my parents. Because of you, I was never afraid to fail.

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I would like to thank Sonia Altizer and Takao Sasaki, as well as committee members Richard Hall and Andy Davis for their critical feedback on project design, execution, and data analysis. Andy Davis and Sonia Altizer allowed me to conduct research on their property in Athens, GA and provided valuable training in data collection and management practices. I also thank Kenneth Donaldson, Isabella Ragonese, Christopher Brandon, Amitesh Anerao, Valentina Suárez and Alexa Robles for their assistance in data collection and preparing monarchs for experiments. Finally, I thank my family for their continual support in all of my pursuits, and for their constant encouragement every step of the way.

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CHAPTER 1

INTRODUCTION

Social behavior has been observed in a variety of taxa (Allen, 2019), and its overwhelming prevalence suggests critical fitness benefits to individuals utilizing social information. Known as the costly information hypothesis (Boyd & Richerson, 1988), time spent personally assessing a resource or local environment, while resulting in reliable information, may be evolutionarily unfavorable compared to energetically inexpensive social information. Social information use by invertebrates is a growing field of research, and has been documented within both colonial and non-colonial species (Chittka & Leadbeater, 2005; Colasurdo & Despland, 2005; Langridge et al., 2004; Provecho & Josens, 2009; von Frisch, 2013). In fact, the physical presence of conspecific individuals or the chemical, visual, and olfactory cues they produce can influence choices made during mating (Cory & Schneider, 2018; Valone & Templeton, 2002) and oviposition site selection (Duménil et al., 2016; Otake & Dobata, 2018; Raitanen et al., 2013; Sarin & Dukas, 2009). Conspecific presence can also help inform navigation decisions made by migratory species over the course of long-distance travel (Berdahl et al., 2018; Dalziel et al., 2016; Guttal & Couzin, 2010; Németh & Moore, 2007, 2014).

With their vibrant orange wings, monarch butterflies *(Danaus plexippus L.)* are a unique example of an iconic and charismatic invertebrate species, attracting a high degree of public interest and conservation concern (Preston et al., 2021). Two of the most universally fascinating components of monarch biology are their obligate relationship with milkweeds *(Asclepias* spp.) as larval host plants, and their annual, long-distance fall migration (Gustafsson et al., 2015).

Inspired by shared fascination, a number of community science programs like Journey North and the Monarch Larva Monitoring Project ask volunteers to record observations of monarch eggs, larvae, and adults. Thanks to these programs, we know that monarchs are often observed in large numbers while nectaring in patches of dense resources, visiting plants during oviposition, and during peak windows of their fall migration. Despite these opportunities for conspecific interaction, monarch social behavior remains understudied.

All stages of monarch development take place on *Asclepias* species, with larvae often remaining on their natal stalks. Because of this, oviposition site selection by females has critical fitness implications- impacting offspring survival, growth, and sequestration of toxic cardenolides for defense (Jones & Agrawal, 2019; Ladner & Altizer, 2005). Female monarchs are known to preferentially oviposit on plants within an ideal range of cardenolide concentrations (Haribal & Renwick, 1998; Ladner & Altizer, 2005; Pocius et al., 2018), and prefer plants with certain attractive physical characteristics, like flowering and height (Cohen & Brower, 1982; Zalucki et al., 1990). Despite their inclination to selectively lay on certain plants, it is unknown whether female monarchs use conspecific cues to help inform their host-plant selection.

Eggs laid by late-summer generation adults are exposed to a shortening photoperiod, cooler temperatures, and the declination of the autumnal sun, which serve as cues to emerge in reproductive diapause ready to migrate (Goehring & Oberhauser, 2002; Reppert & de Roode, 2018; Zhu et al., 2009). During their annual fall migration, monarchs display a striking example of collective behavior. Each night, migrating monarchs stop to rest and refuel, clustering in roosts numbering anywhere from 12 to upwards of 1,000 individuals (Brower et al., 2006; Davis et al., 2012; Urquhart & Urquhart, 1979). What remains unknown to science is whether monarchs utilize social information to help inform their flight bearings once they break roost in the morning. Roosting behavior is unique to migratory individuals, which are also physiologically different from sedentary spring and summer generations of monarchs (Davis, 2009; Flockhart et al., 2017). Captive rearing has known impacts on physical characteristics (Davis et al., 2020) as well as migratory orientation abilities (Tenger-Trolander et al., 2019; Tenger-Trolander & Kronforst, 2020), but if there is a connection between rearing condition and social behavior in monarchs, it remains unexplored.

The overarching goal of my thesis work was to examine the role of conspecific individuals and cues during monarch oviposition and migratory orientation. Specifically, my first goal was to observe how females responded to the presence of conspecific eggs across three *A. incarnata* stalk types of varying quality, and to examine any change in oviposition strategy corresponding to host plant condition. My second goal was to investigate the impact of conspecific group size and rearing conditions on monarch migratory orientation in outdoor flight cages.

Collectively, this thesis work expands the knowledge of social behavior and information use in monarchs, a solitary insect for which relatively little behavioral research has been conducted. Work that quantifies the effect of conspecific attraction on monarch oviposition site selection and navigation is crucial to better understand the implications of recent monarch population decline (Brower et al., 2012; Thogmartin et al., 2017). If monarch reproduction and migration is improved upon repeated interaction with others and the cues they leave behind, there may be a threshold population density required to maintain these benefits, which could help inform future management and conservation decisions.

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CHAPTER 2

NEITHER COPY NOR AVOID: NO EVIDENCE FOR SOCIAL CUE USE BY MONARCH BUTTERFLIES DURING OVIPOSITION SITE SELECTION[1]

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[1] Aikins, C.G., T. Sasaki, and S. Altizer. To be submitted to *PLOS One.*

Abstract

Time spent assessing host-plant quality during oviposition for phytophagous insects represents an energy-fitness tradeoff that can affect future reproduction and offspring survival. In situations where resources are scarce or undesirable, organisms can utilize social information, including cues left behind by conspecific individuals, to inform decisions. In invertebrates, eggs deposited by previous females could signal a desirable resource for oviposition, or alternatively females might avoid ovipositing close to conspecific eggs to reduce competition for her offspring. Here, we show that monarch butterflies (*Danaus plexippus L.*) display the same pattern of oviposition across milkweed host plant (*Asclepias incarnata L.*) stalks of differing quality, ignoring potential social cues and competition pressure imposed by conspecific eggs. Female monarchs preferentially laid eggs on healthy milkweed stalks relative to water-stressed or herbivory-damaged plants and exhibited differential post-alightment behavior (including foreleg drumming) on the three stalk types. Despite this apparent assessment of host-plants for oviposition suitability, females failed to respond to social information in the form of conspecific eggs. Contrary to our expectations that females would avoid healthy stalks with high egg numbers and would prefer less desirable stalks with moderate egg numbers, the number of eggs laid by focal females did not depend on the number of prior conspecific eggs per stalk, regardless of stalk type. Our results suggest that monarchs neither avoid nor prefer stalks with conspecific eggs- perhaps due to low egg densities and larval survival in the wild, making discrimination based on conspecific eggs impractical when milkweed is abundant. Alternatively, egg clustering by females may be an adaptive oviposition strategy when host plant options are few, regardless of quality.

Index Words: *Danaus plexippus*, social information, oviposition, host-plant, ovipositor, *Asclepias incarnata*

Introduction

Organisms must constantly make choices that either improve or reduce their fitness in unpredictable or changing environments. Environmental information used to inform decision-making can be gathered by individuals using visual, olfactory, or other cues. Some animals also rely on the continual collection of social information in the form of cues from conspecific or even heterospecific individuals (Seppänen et al., 2007). These cues can inform predator avoidance, foraging, mate choice, nest site selection, and more. Social information use and learning by invertebrates is extensive, and its impact on behavior has been observed across a multitude of species, including social insects like ants and bees, and gregarious species such as tent caterpillars (Colasurdo & Despland, 2005; Provecho & Josens, 2009; von Frisch, 2013). Utilization of these social cues is often an adaptive behavior, as it allows for informed decision-making with less individual investment in the collection and processing of information. However, relying solely on socially-acquired information can lead to incorrect decisions and can be costly in other ways (Dall et al., 2005; Rieucau & Giraldeau, 2011). There is growing evidence that organisms are selective about their use of social information over individual information, and the type of information they prefer can depend on environmental context, resource quality, or other factors (Grüter & Leadbeater, 2014; Otake & Dobata, 2018; Papaj & Messing, 1996; Sorci, 2009). For example, guppies have been observed to use personally-acquired foraging information to select feeding sites, but chose to copy conspecifics when the use of personal information was perceived as costly (Kendal et al., 2004).

Some of the most important fitness decisions organisms make surround reproduction, and can benefit from social information. This includes assessing the quality of potential mates (Valone & Templeton, 2002), the number of mates chosen (Cory & Schneider, 2018), and selecting nesting sites and egg-laying substrates (Duménil et al., 2016; Otake & Dobata, 2018; Raitanen et al., 2013; Sarin & Dukas, 2009). In Lepidoptera, since larvae typically remain on their natal host plant from hatching until eclosion, the site that an organism chooses for oviposition can impact survival, competition, growth rate, and defenses of offspring from predation and parasitism (Jones & Agrawal, 2019; Ladner & Altizer, 2005).

Monarch butterflies (*Danaus plexippus L.*) are milkweed specialists, utilizing only plants from the family *Asclepiadaceae* as larval host plants. Females typically lay eggs singly on the underside of leaves, with each female laying around 300-400 eggs in the wild over a period of 2-4 weeks (Oberhauser & Solensky, 2004). All larval development and pupation takes place on the milkweed host, and nutrient consumption during this time affects adult size, longevity and reproduction. The protein required for egg development in females is derived from larval reserves or is transferred from males during mating (Boggs & Gilbert, 1979; Oberhauser, 1997). Since larval host-plants have such important fitness implications, it was thought that females oviposit in line with the preference-performance hypothesis (PPH) (Jaenike, 1978), wherein options would be assessed carefully using all available information to maximize offspring success. However, observed oviposition behavior in monarchs often varies from the PPH (Jones & Agrawal, 2019; Thompson & Pellmyr, 1991).

Monarchs are characterized as solitary insects during the breeding season in North America: sightings of multiple caterpillars on the same plant, or multiple adults together are relatively rare (Kasten et al., 2016; Pleasants et al., 2017). In some circumstances, monarchs can be observed collectively under patchy resources, meaning there is opportunity for conspecific interaction, particularly in urban and suburban areas, and at locations where monarchs breed year-round (Lindsey et al., 2009; Majewska, Satterfield, et al., 2019; Satterfield et al., 2015). A large body of research suggests that female monarchs use host plant chemistry, particularly milkweed cardenolides (plant secondary metabolites that deter herbivory) in host plant choice (Cohen & Brower, 1982; Zalucki et al., 1990). Monarch caterpillars can sequester cardenolides and use them to deter predators, but high cardenolide concentrations are toxic to caterpillars (Malcolm, 1994). Other work shows that females preferentially oviposit on smooth-leaved low-latex species such as *Asclepias incarnata L.* over *Asclepias* species with high latex and trichomes (Haribal & Renwick, 1998; Ladner & Altizer, 2005; Pocius et al., 2018). Other physical characteristics like plant height, size, and flowering also affect host plant choice by female monarchs (Cohen & Brower, 1982; Zalucki et al., 1990). Using chemo-receptor sensilla on the antennae and forelegs, females have been observed to exhibit distinct postalightment behavior on plants of differing characteristics and quality (Haribal & Renwick, 1998; Zalucki et al., 1990), suggesting that female monarchs spend time assessing host-plants for suitability. However, it is not understood whether monarchs utilize social information, in the form of cues from other females, to help gauge the quality of host-plant options.

The goal of our study was to assess whether or not female monarchs utilize social information in the form of conspecific eggs to inform host-plant selection, and to examine if monarchs preferentially lay eggs on milkweed stalks with conspecific cues as host-plant quality decreases. In this study, stalks of *Asclepias incarnata L.* (swamp milkweed) were physically manipulated to simulate herbivory damage or water stress, as two states that might be less preferred by ovipositing females. In the first experiment, stressed and damaged stalks were presented to novel mated females alongside healthy (undamaged, unstressed) stalks to gauge female oviposition preference, based on behavior and number of eggs laid per stalk. In a second experiment, three stalks of the same treatment, one of which contained social information in the form of conspecific eggs (focal stalk), were presented to mated females, and we again recorded the number of eggs laid per stalk. We hypothesized that among the three stalk treatments, female monarchs would preferentially oviposit on healthy stalks over damaged and water-stressed stalks, to increase the survival and development of future offspring. When presented with three healthy stalks (one containing eggs), we predicted that females would avoid stalks with higher numbers of conspecific eggs, to reduce resource-based competition. Based on findings from other insect oviposition choice studies, we expected this to change as milkweed quality decreased, with females utilizing social information and exhibiting conspecific attraction in situations where their host-plant options are undesirable. The presence of conspecific individuals or cues left behind by those individuals (eggs in this case) can be an indicator of desirable resources (Otake & Dobata, 2018; Papaj & Messing, 1996; Raitanen et al., 2013)

Methods

Monarch and milkweed sources

To obtain adult butterflies for the experiments, monarch caterpillars were reared indoors at 26-28°C under ambient light supplemented by overhead fluorescent lighting set to a 16-hour day. Caterpillars hatched from eggs laid by non-inbred F1 descendants of wild-caught spring migrants collected from Athens, GA in Apr-May 2021. We obtained 8-10 outcrossed genetic lineages of monarchs per experiment. Prior to mating and oviposition, adults were examined for the protozoan parasite Ophryocystis elektroscirrha, and any infected adults were excluded from further study (Altizer et al., 2000).

After eggs hatched, caterpillars remained on natal stalks until late second instar, and were then transferred to 1.4 L plastic containers with mesh screen lids, 6 caterpillars/container, and fed fresh cuttings of greenhouse raised Asclepias incarnata daily. Following emergence, uninfected adults were kept individually in glassine envelopes at 12°C for 5-14 days. To obtain mated females for the oviposition experiments described below, adult males and females were placed into $0.6m^3$ mesh screen cages for 5-7 days, until each female had mated (10 M and 10 F per cage, from outcrossed parentage). Mated females were removed from cages and held at 26°C in glassine envelopes prior to oviposition choice studies and fed 20% honey water to satiation every second day.

The milkweed used in these experiments was cultivated from seed (Prairie Moon Nurseries) in a greenhouse set to 31°C day and 26°C night. Greenhouse lights were set to a 14-hr day length. Milkweed (A. incarnata) was sown in a bulk pine bark soil mix, in 6” diameter pots, and plants were 6 months of age when trials began in late June. One tablespoon per pot of time-release fertilizer 15-9-12) was applied every 3 months. Seedlings were also given Jack’s Professional Peat Lite 20-10-20 liquid feed at a rate of 200 ppm after sprouting, which was repeated monthly.

Plant treatments

Oviposition trials took place in $0.6m^3$ mesh cages and were conducted indoors in a room exposed to ambient light, with four artificial fluorescent tube lights (set to 16-hr day) at 26-28°C. Cuttings of *A. incarnata* were immediately placed into 0.6L plastic bottles and provided as an egg-laying substrate in each cage. Stalks selected for the experimental trials were standardized in height (range 18-22 inches from the bottom of each bottle to the top of the stalk) and leaf number (16-24 leaves per stalk). Prior to trials, the milkweed stalks were manipulated to create one of three treatments: (i) healthy stalks (fresh and with no signs of herbivory or water stress), (ii) herbivory damage, or (iii) water stress (Figure A1). To simulate herbivory damage, we punched round holes in milkweed stalks (3mm diameter) after cuttings were placed in plastic bottles, with 4-6 holes per leaf, depending on leaf size. Water stress was simulated by removing cut stalks from water approximately 5 hours before presenting to females, to allow wilting; water removal was continued throughout the duration of oviposition (24 hours).

Experiment 1: Plant treatment preference

To establish oviposition preference among the three *A. incarnata* stalk treatments, three stalks in total, one from each treatment (healthy milkweed, water-stressed, and herbivory damaged), were placed in each laying cage (0.6m^3 mesh screen). The position of each stalk type within each cage was randomized. We placed a single female monarch into each cage at 9 AM, and allowed females to oviposit for 24 hours, after which the number of eggs laid by each female on each milkweed stalk was recorded. We set up 10 cages per day, over 6 days, to test a total of 60 females. If fewer than 10 eggs were laid across all three stalks (combined), data from that female was excluded from further analysis (N = 11). Females were randomly chosen from one of 10 genetic lineages (full-sib family lines), with 5-7 females per lineage.

Oviposition behavior of each female was observed for one hour, following a min 1-hr acclimation period. During the hour of observation, we recorded each time a female alighted on a stalk, drummed forelegs or antennae on a leaf (drumming), or curled the abdomen to reach the underside of leaves (from here on referred to as dabbing; Figure A2; (Zalucki et al., 1990). If a female did not land, drum, or dab on any plants during the observational period, that female was excluded from behavioral analyses (N = 18).

Experiment 2: Social cue use during oviposition site selection

Here, we sought to establish whether or not female monarchs utilize social cues in the form of conspecific egg presence on milkweed stalks during oviposition, and if females display a differential use of social cues corresponding to environmental condition. We provided novel females in this experiment with three *A. incarnata* stalks of the same treatment type. One of the 3 stalks contained eggs laid by another female the day before. Since we found no significant difference between the control and herbivory stalk treatments during experiment 1, we conducted experiment 2 using only control and water-stressed stalks. To create stalks with conspecific eggs (focal stalks) prior to the start of this experiment, females from the previous preference trials were presented with healthy milkweed stalks, and allowed to lay for 24 hours, until 10-100 eggs were present on each focal stalk. These focal stalks were then assigned to their treatment (healthy or water-stressed), and water was removed from wilted stressed treatment stalks 5 hrs prior to trials. The position of the focal stalk in each cage was randomized, and we recorded the number of eggs on the focal stalk prior to adding a single test female to each cage. As before, after 24 hours, the number of eggs laid by each test female on each milkweed stalk was recorded. We set up 10 cages per day, over 6 days, for a total of 55 females. If fewer than 10 eggs were laid across all three stalks (combined), data from that female was excluded (N = 10). Females were randomly chosen from one of 10 genetic lineages (full-sib family lines), with 2-12 females per lineage.

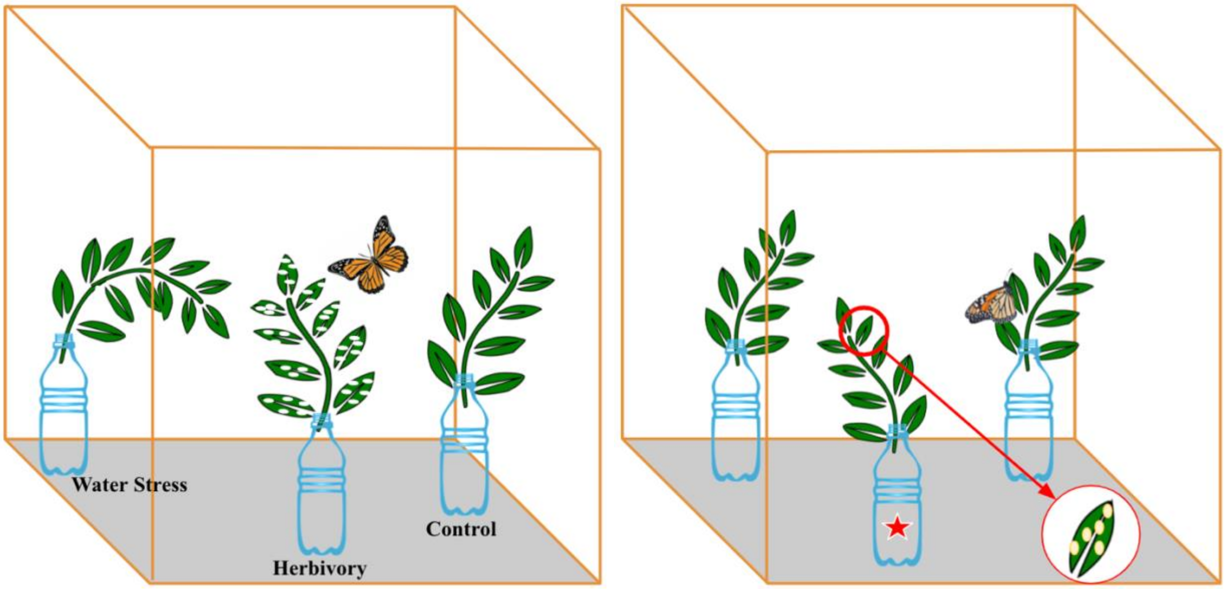

Figure 2.1. Illustration of cage set up for experiments 1 (plant treatment preference, left) and 2 (social cue use, right). Each cage in experiment 1 contained a water-stressed stalk (left), herbivory-damaged stalk (middle), and healthy stalk (right). In experiment 2, three stalks of the same treatment type (either herbivory or healthy) were placed in each cage. The focal stalk (with eggs from a different female) is indicated with a star.

Data Analyses

In experiment 1, to test whether females discriminate based on host-plant quality when laying eggs on milkweed, we examined the proportions of eggs laid on each of the three stalk treatment types. Proportions were square-root transformed prior to analysis to normalize error variance. We used a linear mixed model (LMER) with transformed proportions of eggs on each stalk type as our response variable, stalk treatment type as a fixed effect, and female ID as a random effect. To examine the behavior of females across milkweed treatments, we conducted three linear mixed models with the proportions of landings, drumming, or dabbing events per stalk as the response variable, stalk treatment type as a fixed effect, and female ID as a random effect. Proportions were again square-root transformed prior to analysis.

In the second experiment, the effect of monarch egg cues (number of initial starting eggs on each focal stalk) was tested by examining the proportion of eggs each female laid on the focal stalk, relative to the total number of eggs laid across all stalks combined. Proportions were square-root transformed prior to analysis. With transformed proportions as the response variable, we included plant treatment type (fixed effect), the starting number of eggs on the focal stalk (continuous covariate), and the interaction between the two as predictor variables.

Results

Experiment 1: Treatment preference trials

In the treatment preference experiment, stalk treatment type was a significant predictor of the proportions of eggs laid by females (F=4.50, df=2, p=0.0127). A post-hoc test showed that monarchs laid significantly more eggs on the healthy milkweed stalks over the water-stressed stalks (Figure 2; p=0.0129). More eggs were laid on the control stalks than herbivory-damaged stalks, but the difference between these two treatments was not significant (p=0.0773), and there was not a significant difference between numbers of eggs laid on the two damaged stalk types (p=0.7697). The laying propensity of each female (the total number of eggs laid across all stalks) did not have a significant effect on treatment preference (Figure A3; F=0.347, df=2, p=0.7076).

Stalk treatment type was not a significant predictor of the proportion of landings per stalk (Figure 2.3; F=1.443, df=2, p=0.2401), meaning females visited all stalk types relatively equally. The frequency of drumming antennae or forelegs differed significantly among stalk types (F=5.650, df=2, p=0.0045). Females were observed drumming significantly more often on herbivory-damaged stalks than control stalks (p=0.0040). Water stressed plants were also drummed upon more often than control stalks (p=0.0460), but we found no significant difference in drumming behavior between the two damaged stalk treatments (p=0.680). Treatment type was also a significant predictor of the number of abdomen dabs by females (Figure 3; F=5.973, df=2, p=0.0033). Monarchs dabbed (oviposition attempt) significantly more on control plants than on water-stressed plants (p=0.0029) and herbivory-damaged plants (p=0.0480). There was no significant difference between female dabbing rates on water-stressed vs. herbivory stalks (p=0.5970).

Experiment 2: Focal stalk trials

Females did not show evidence for avoidance (fewer eggs on egg-laden focal stalks) or attraction (more eggs on egg-laden focal stalks) in either the healthy or water stressed stalk treatments (Figure 4; F=0.0001, df=1, p=0.9907). The number of initial conspecific eggs per focal stalk was not a significant predictor of the proportions of eggs laid by females on the focal stalk (Figure 5; F=0.0520, df=1, p=0.8208), regardless of treatment (healthy stalks: R2=0.009; Beta=-0.0009; F=0.19; p=0.6710) (water stressed stalks: R2=0.000; Beta=0.0002; F=0.01;

p=0.9441).We also found no effect of an interaction between starting focal egg number and stalk treatment (F=0.1216, df=1, p=0.7291) on the proportion of eggs laid on the focal stalk.

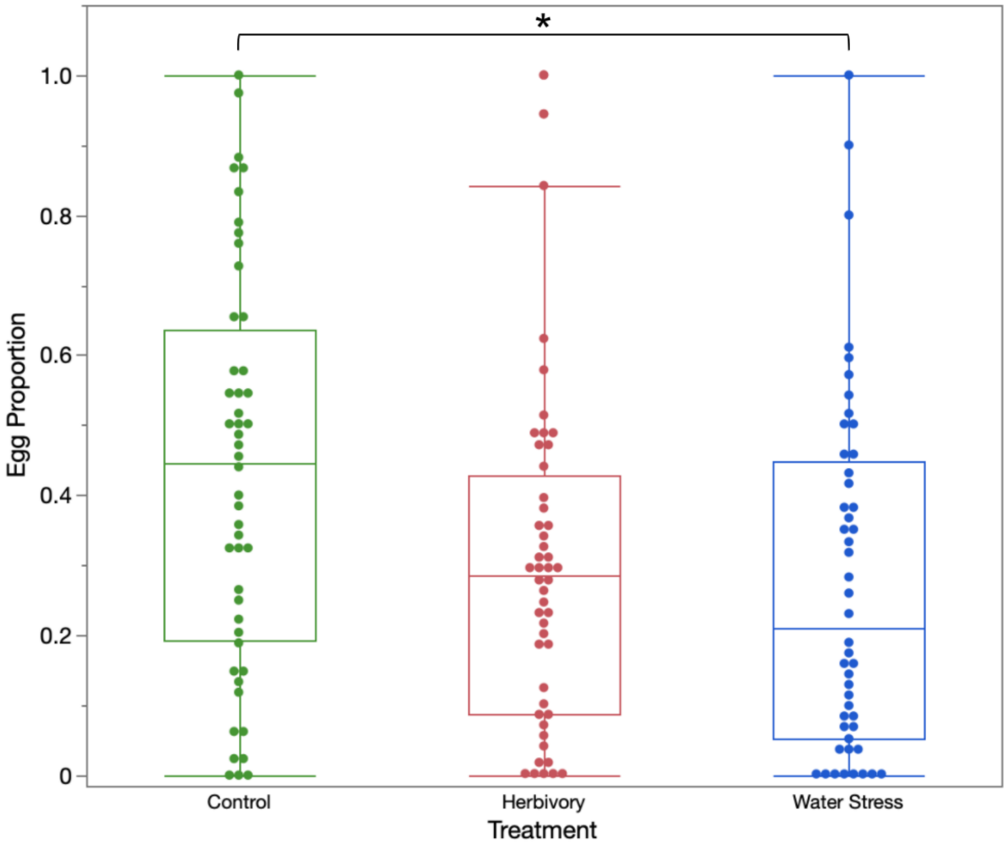

Figure 2.2. Proportions of eggs laid by each female across each milkweed treatment type in Experiment 1. Control stalks had significantly more eggs than water stressed stalks, but no other pairwise differences between treatments were significant.

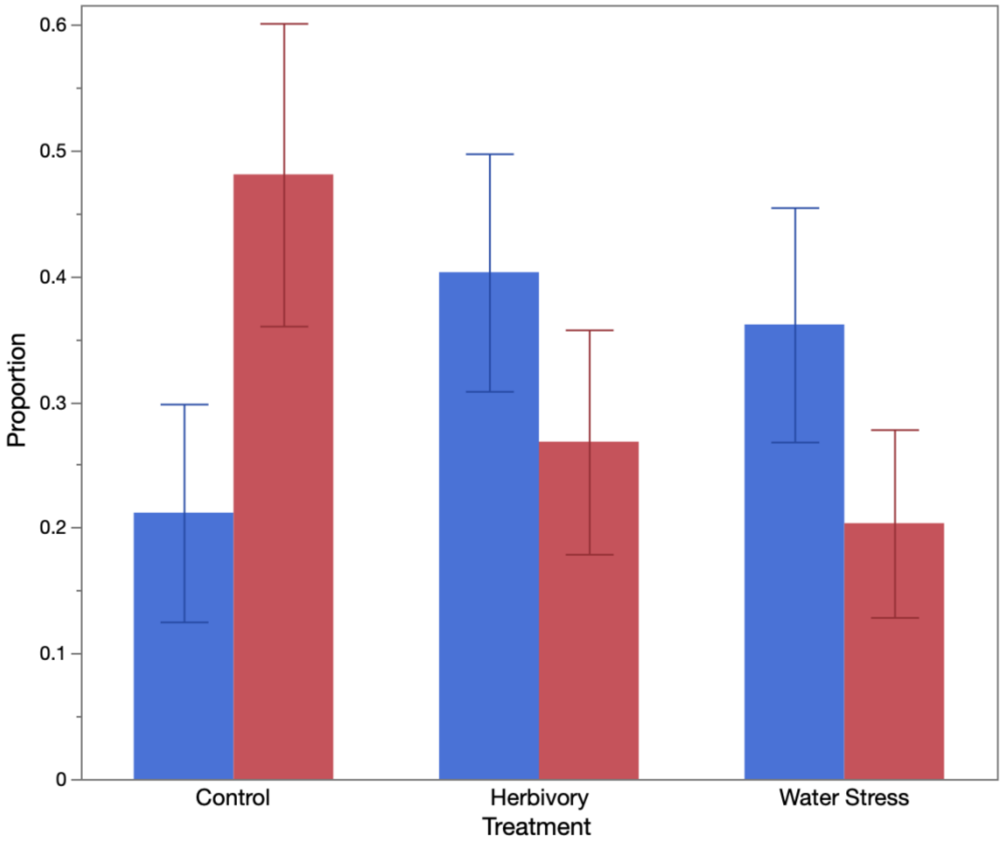

Figure 2.3. Mean proportions of observed drumming (blue) and dabbing (red) behavior by females after landing across each milkweed treatment type in Experiment 1. Control stalks were drummed on with antenna and forelegs significantly less than the other stalk treatments and were dabbed on significantly more often.

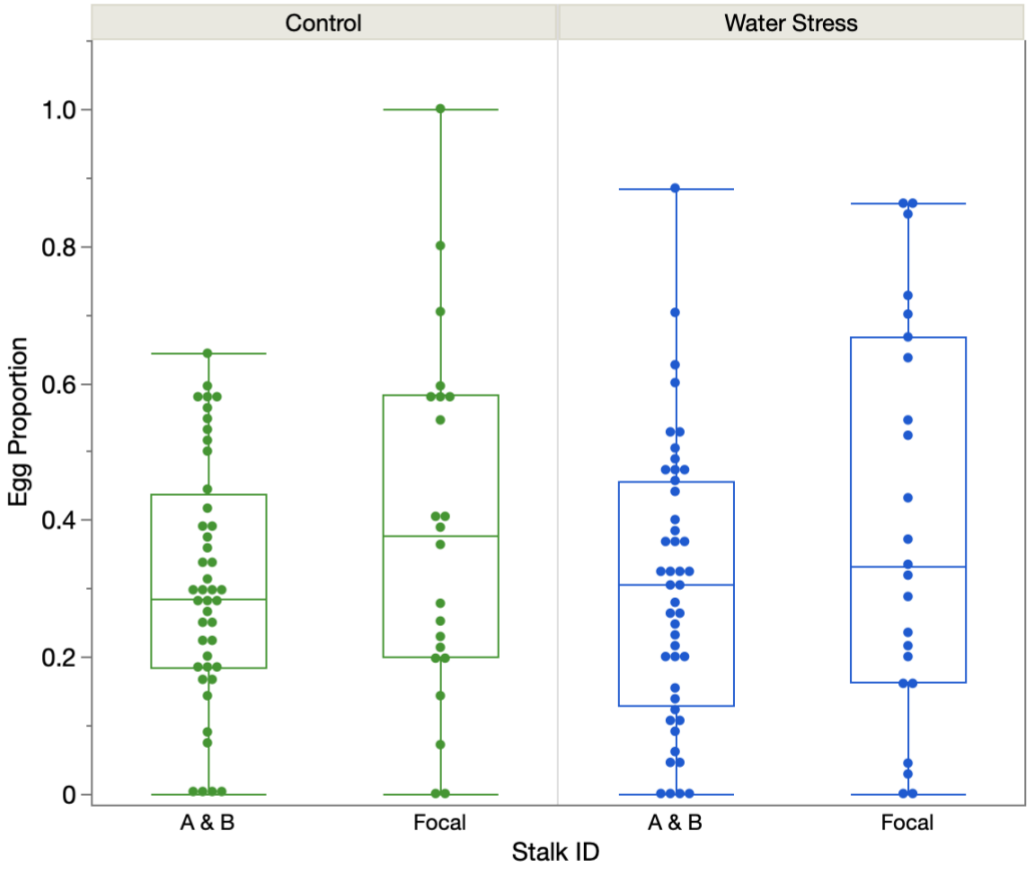

Figure 2.4. Proportions of eggs laid by each female across each of the three milkweed stalks in Experiment 2. Females did not display avoidance of or attraction to egg-laden focal stalks in either stalk treatment type. There was no significant difference in the proportions of eggs laid by females on focal stalks versus the two plain stalks (A and B). Each point represents the proportion of total eggs per stalk type by one female.

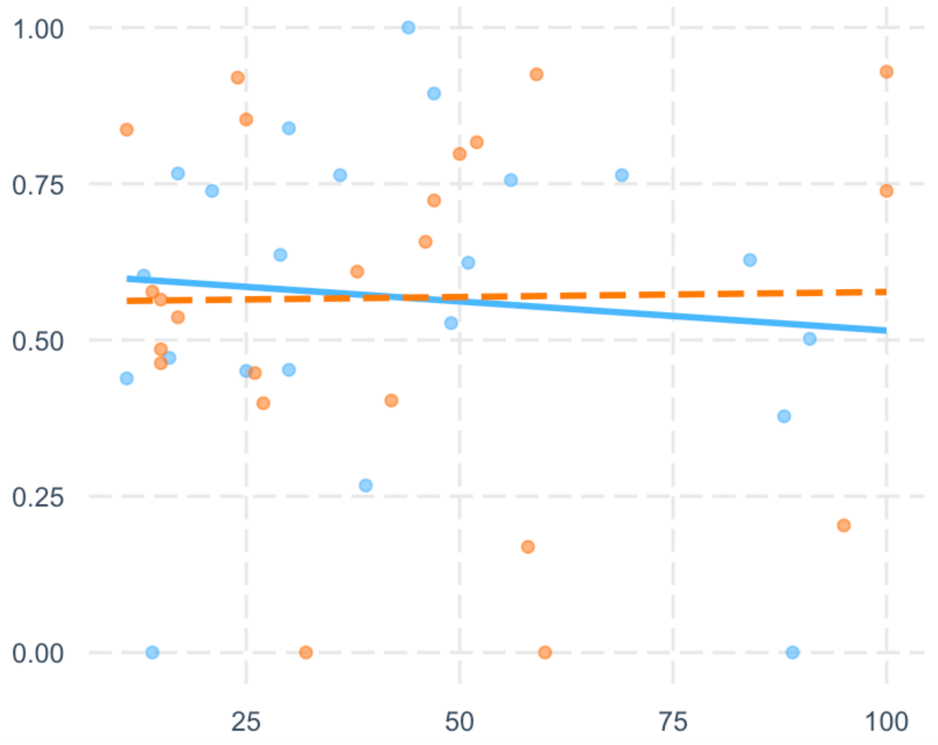

Figure 2.5. Proportions of eggs laid on focal stalks (y-axis) did not depend on the starting number of eggs on focal stalks (x-axis) in either the control (blue) or water stress (orange) treatments in Experiment 2. Each point represents the proportion of total eggs per focal stalk by one female.

Discussion

Our study showed that female monarchs preferentially laid eggs on healthy-looking milkweed stalks over those that were water stressed or with simulated herbivory. Females did not change their oviposition behavior in response to social cues, and neither avoided nor preferred stalks containing the eggs of conspecifics. The lack of social cue response was similar for both preferred (healthy looking) and nonpreferred (water stressed) stalk types. This suggests that while monarchs likely use the visual appearance of stalks and other plant traits in selecting oviposition sites, they do not appear to copy the host plant selection behavior of other females when presented with poor resources, nor do they avoid plants selected by previous females when presented with high quality host plants.

Females were observed visiting all stalk types relatively equally in Experiment 1 but exhibited differential post-alightment behavior between preferred and nonpreferred stalk types. On healthy-looking stalks, females were often observed dabbing to lay an egg almost immediately after landing, with infrequent or brief drumming of antennae or forelegs on the leaf surface. On the lower quality water-stressed and herbivory-damaged stalks, however, females spent more time assessing the suitability of the plants by drumming chemoreceptor sensilla on antennae and forelegs against the leaves. Despite being landed on an equal amount, stalks of lower quality received fewer eggs, particularly water-stressed stalks. This suggests that these stalks were rejected (female leaves the plant without laying) more frequently than healthy stalks. Past research also found that females used antennae, midlegs, and forelegs most extensively on the host-plant options that ultimately received the fewest eggs (Haribal & Renwick, 1998), which aligns with our observations. This suggests that visual cues may not be the primary tool used by females when selecting a host plant at a close range, and that host-plant suitability is investigated after landing. This further differentiation between host plant options involves female examination of leaf nutrient availability, latex levels, cardenolide concentrations, and water content (Zalucki et al., 1990). Discrimination based on the water content of leaves is likely why the wilted, water-stressed stalks consistently contained the fewest eggs after 24 hours.

Interestingly, herbivory-damaged stalks were drummed on most frequently and contained nearly as many eggs as healthy control stalks after 24 hours. The high levels of drumming by females suggests a lengthy period of plant assessment, but ultimately most females were not dissuaded from depositing eggs. In past research, female monarchs have been observed to exhibit a strong avoidance of plants containing conspecific larvae, but it was unclear whether this was in response to visual or chemical cues produced by the larvae itself, or to plant volatiles released through physical herbivory damage (Jones & Agrawal, 2019; Wason et al., 2013). When milkweed leaves are damaged, the plant exudes latex, which is known to developmentally hinder early-instar larvae and increase mortality (Ladner & Altizer, 2005; Van Zandt & Agrawal, 2004; Zalucki et al., 1990; Zalucki & Malcolm, 1999). It is possible that female monarchs exhibit competition avoidance only when caterpillars are physically present on host-plants, and don't respond to plant volatiles left behind by larvae.

Although the presence of high latex levels is generally thought to deter monarch oviposition, latex production of *A. incarnata* (0.20–50 µl) is lower than other milkweed species (Zalucki et al., 1990). Physical damage to milkweed plants is also known to induce a spike in cardenolide concentrations up to 24 hours after the damage occurs (Malcolm & Zalucki, 1996). Since monarchs are known to preferentially oviposit on milkweed plants within a cardenolide concentration ranging from 200-500 g/0.1g dry weight, and *A.incarnata* typically ranges from 42-199 g/0.1g (Zalucki et al., 1990), increased cardenolide concentrations on herbivory damaged stalks could have outweighed the deterrent properties of exposed latex, especially since stalks were damaged the night before trials took place. There was likely necrosis beginning in the plant tissue surrounding the damage, and latex had time to begin drying. The use of cut stalks as opposed to intact plants likely limited latex flow and cardenolide response, but exposed latex was still present on leaves after the mechanical damage was completed.

Despite evidence that females assessed host plants prior to oviposition, we found no indication that females either avoided or were attracted to stalks with conspecific eggs. Assuming that females could, in fact, detect the conspecific eggs on focal stalks, results observed here suggest that oviposition strategies of monarchs do not utilize social information. There is growing documentation of vision and learning in Lepidoptera, particularly as it pertains to mate recognition, nectar foraging, and host-plant detection (Arikawa, 2003; Kinoshita et al., 1999; Stavenga & Arikawa, 2006). In fact, monarchs have demonstrated the cognitive ability to learn and retain color and olfactory information pertaining to foraging (Blackiston et al., 2011; Gegear, 2021; Rodrigues, 2016; Rodrigues et al., 2010). Monarchs are thought to use visual cues hierarchically, most often relying on high-contrast color information, with object shape secondary in informing decisions (Cepero et al., 2015; Mackay & Jones, 1989; Papaj, 1986). It is possible that female monarchs did not recognize conspecific eggs present on the underside of leaves. Many lepidopteran species have excellent distance-based vision and estimated perceptual ranges varying between 8m to 50-100m (Haddad, 1999; Harrison, 1989; MacDonald et al., 2019; Merckx & Van Dyck, 2007; Schtickzelle et al., 2007; Schultz & Crone, 2001), but may not be able to perceive certain visual cues at a close range. The small size of our experimental cages ($0.6m^3$) may have limited the monarchs' ability to visually detect physical differences in the host-plants or the presence of conspecific eggs. This could also explain why monarchs landed on all treatment types equally, despite the distinct physical variation between healthy stalks and those that were heavily wilted or damaged by herbivory. Their discrimination between treatments occurred largely post-alightment, after drumming of antennae and forelegs on the tops of leaves.

It is equally likely that monarchs were able to detect the conspecific eggs but did not utilize egg-based social information in their oviposition process. Female monarchs typically deposit eggs singly, and milkweed plants in core regions of the migratory breeding range are highly abundant, such that numbers of eggs per milkweed rarely exceed 1 egg per stem, and egg densities are often much lower (Stenoien & Nail, 2015). In the field, females have been shown to travel an average distance of 23m between landing on milkweed plants, passing many viable options in the process (Zalucki et al., 1990). In areas with abundant resources, monarchs may choose to disperse eggs widely, since the probability that any given host-plant will receive more than 2 eggs is very low, and therefore conspecific eggs will have a minimal impact on overall fitness.

In areas with limited or highly patchy milkweed resources, monarchs might show a different oviposition strategy. An observational study in southwestern Ontario, Canada saw females deposit more eggs per plant in areas with a low density of milkweed plants (Pitman et al., 2018). Another study conducted in Queensland, Australia reported a decrease in eggs per plant as the number of plants per patch increased, with isolated plants receiving the most eggs (Zalucki & Kitching, 1982). This suggests that females may be more likely to ‘egg dump’ when their host-plant options are few. Such conditions could occur in urban settings and in regions like the southeastern United States where milkweed occurs at much lower densities. When host-plant options are limited, females might need to lay eggs on milkweed stalks already containing eggs from a previous female, a practice observed in *Pieris rapae L. (Ives, 1978)*. Because females in our experiments were presented with only three milkweed stalks, this could explain why some females chose to lay additional eggs on stalks already containing high densities. For example, one female deposited an additional 91 eggs on a stalk that contained 51 conspecific eggs at the beginning of the trial. Our focal stalks contained a range of 10-100 eggs prior to trials, a number similar to that used by Zalucki and Kitching (1982), who also found no evidence for female monarch avoidance of milkweed plants containing eggs. It therefore seems likely that the high fecundity of monarchs, combined with low egg and early-instar survival rates, make egg clustering by multiple females an appropriate alternative to searching for unexploited resources when milkweed abundance is low.

It is important to note that the transmission of a debilitating protozoan parasite (*Ophryocystis elektroscirrha*) in monarchs occurs during oviposition, and infection risk to offspring increases when females lay eggs on plants previously visited by infected monarchs. This parasite forms spores on the outside of the body of adults, and infection occurs when caterpillars ingest parasite spores (McLaughlin & Myers, 1970). Parasite spores can be transmitted from females to their offspring during oviposition (Altizer & Oberhauser, 2004). In addition, adults can scatter spores onto milkweed leaves which are later consumed by unrelated caterpillars (de Roode et al., 2009; Majewska, Sims, et al., 2019). Analysis of volunteer- and field-based monitoring data show that parasite transmission increases with greater egg, larval, and adult densities in milkweed patches (Bartel et al., 2011; Majewska et al., 2022). Thus, females could benefit from avoiding milkweed already visited by other females when monarch densities and parasite infection risk is high. Further work could examine whether healthy females might respond differently to the presence of conspecific eggs laid by infected females, which would carry the benefit of disease avoidance.

In sum, our study showed that monarchs prefer to oviposit on healthy milkweed stalks and exhibit behaviors post-alightment assessment behaviors. However, monarchs did not use social information in the form of conspecific eggs to help inform oviposition and showed no preference or avoidance of stalks with eggs laid by prior females. That conspecific eggs did not deter monarch oviposition is surprising, given the strong competition pressure that would likely occur between caterpillars feeding on the same host plant. Further work is needed to examine the mechanisms by which females detect the presence of conspecific eggs and larvae (see Jones & Agrawal, 2019; Wason et al., 2013), and to explore whether other aspects of monarch reproduction, such as mate choice, might respond to social information.

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CHAPTER 3

REARING CONDITIONS ALTER THE USE OF SOCIAL CUES TO INFORM DIRECTIONAL ORIENTATION IN MONARCH BUTTERFLIES[2]

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[2] Aikins, C.G., S. Altizer, K. Donaldson, A.K. Davis, and T. Sasaki. To be submitted to *Insects*.

Abstract

Long-distance migration is often energetically costly and risky, and survival during migration requires successful foraging and navigation. To help inform migratory decisions, inexpensive, socially-acquired information could be used together with individually-acquired sensory information. In particular, the orientation and navigation decisions of conspecifics can be indicative of a desirable heading. Here, we show that rearing condition and the number of conspecifics present influence monarch butterfly *(Danaus plexippus L.)* directional orientation in outdoor cages during fall migration. We followed traditional studies of migratory birds and insects by placing animals in outdoor cages to record compass orientation based on visual observation. Our results showed that wild-caught adult migrants oriented towards the correct S/SW heading (towards wintering sites in Mexico) with significantly more accuracy than cohorts reared indoors (from lineages bred in captivity) and outdoors (from both wild and captive bred sources). In both the wild migrant and outdoor-reared cohorts, monarchs in groups of 4 and 8 showed greater accuracy in compass angle than single monarchs, suggesting use of social information in the form of conspecific cues by individuals to inform directional orientation. These results confirm previous findings that indoor-reared and captive-bred individuals exhibit poor migratory performance, and present novel evidence of conspecific cue use in monarchs. Different use of social cues among the four rearing conditions tested here implies that collective decision-making is phenotypically plastic in monarchs, and could depend on environmental cues experienced during immature stages, together with adult experiences and underlying genetic background.

Index Words: *Danaus plexippus,* orientation, migration, conspecific, social information

Introduction

Migratory animals face a unique set of challenges during their annual journeys, which can take individuals thousands of kilometers across entire continents and often over inhospitable terrain. Traversing long distances means high energetic demands and continual assessment of unfamiliar stopover and foraging sites (Dingle & Drake, 2007; Wikelski et al., 2003). Many migratory species rely on multiple sensory-based compasses, including those that use celestial and geomagnetic cues, to navigate along their migratory route (Alerstam & Bäckman, 2018; Gould, 1998; Hobson, 2013). Importantly, animals can also utilize social cues to help inform movement decisions. In migrating caribou, conspecific interaction has been shown to alter migratory movement (Dalziel et al., 2016), with individuals displaying conspecific attraction and copying behavior, following nearby individuals (Torney et al., 2018). Given the seasonal nature of most migrations and the use of migratory pathways, large numbers of individuals often move in a similar direction at the same time and place each year. While this clustering of migrating animals alone is not evidence of social information use, it does provide opportunity for conspecific interaction and information exchange- a relatively understudied aspect of animal migration (Couzin, 2018). Broadening the scope of research beyond individual migratory mechanisms has revealed that many different taxa utilize some form of social information during migration (Berdahl et al., 2018; Guttal & Couzin, 2010; Németh & Moore, 2007, 2014) and that the presence of conspecifics can improve migratory performance (Mueller et al., 2013) .

Monarch butterflies (*Danaus plexippus L.*) undertake a massive, multi-generational migration each fall to escape freezing winter temperatures and track favorable environmental conditions. Traveling from as far north as southern Canada, eastern North American monarchs reach overwintering sites in the oyamel fir forests of central Mexico after a roughly 6-week journey. While smaller populations west of the Rocky Mountains travel shorter distances to overwinter at sites on the California coast (Nagano et al., 1993), eastern migratory monarchs travel up to 4,000 km on their journey to Mexico, with 500 million or more monarchs embarking on the fall journey (Flockhart et al., 2017). This great migration is guided primarily by a time-compensated sun compass and secondarily by an inclination-based magnetic compass (Mouritsen & Frost, 2002; Reppert & de Roode, 2018), which informs flight during overcast conditions. Sun-compass orientation in monarchs has been extensively researched- utilizing tethered flight simulators (Froy et al., 2003; Guerra & Reppert, 2013; Merlin et al., 2009; Mouritsen & Frost, 2002), vanishing-bearings during releases (Guerra & Reppert, 2013; Kanz, 1977; Perez et al., 1997), and outdoor flight cages (Kanz, 1977). Antennal circadian clocks allow monarchs to compensate for the ever-changing azimuthal position of the sun throughout the day and maintain a southerly bearing (Merlin et al., 2009; Reppert & de Roode, 2018).

Since monarch migration depends on the sun's azimuth, and temperature thresholds of 13-16° C are required for monarch flight (Masters et al., 1988), migratory flight typically occurs between the hours of 1000 and 1400 h on days ranging from overcast to sunny (Kanz, 1977). During the night, monarchs cluster in trees, forming roosts that allow individuals to rest and refuel (Brower et al., 2006; Davis, Nibbelink, et al., 2012; Urquhart & Urquhart, 1979), with monarchs taking off once the temperature warms the following morning. Social cues facilitate the formation of these collective roosts, and larger roosts at overwintering sites have been shown to afford protection against predation to roost members (Calvert et al., 1979). Large-scale community science programs like Journey North (http://www.learner.org/jnorth/), which asks volunteers to record monarch sightings throughout the year, have informed scientific understanding of monarch fall migratory flyways (Howard & Davis, 2009) as well as migratory roosting and nectaring behavior. Monarchs are often observed in high densities during windows of peak migration, but it remains unknown whether monarchs use social cues to help inform migratory navigation.

Monarchs show evidence for a trans-generational physiological shift at the end of the breeding season that is thought to be important for the initiation and success of migration. In eastern North America, monarchs emerge at the end of the summer and early fall in a hormonally-mediated state of reproductive diapause (Herman, 1981). This delayed reproduction in monarchs is thought to facilitate the energy storage and greater longevity needed to complete this successful two-way migration (Herman & Tatar, 2001). Previous work on reproductive diapause in monarchs shows that the induction of diapause depends on multiple environmental cues experienced during pre-adult development, including decreasing daylength, cooler temperatures, and decreasing milkweed quality (Batalden et al., 2014; Goehring & Oberhauser, 2002; Zhu et al., 2009). Migratory generation individuals showcase a darker red wing hue as adults (Davis, 2009) and larger, more elongated forewings (Flockhart et al., 2017), both of which are associated with individual quality and migratory success (Davis, Chi, et al., 2012; Freedman & Dingle, 2018; Hanley et al., 2013). Physiological superiority of migrant monarchs is especially clear when compared to captive-reared monarchs (Davis et al., 2020), which also display poor migratory orientation abilities (Tenger-Trolander et al., 2019; Tenger-Trolander & Kronforst, 2020). Importantly, trans-generational, or seasonal differences in migratory physiology could covary with propensity for social cue use in directional orientation.

In this experiment, we observed monarch migratory orientation behavior in outdoor flight cages. We first compared the compass directional orientation of individual monarchs from four different cohorts, representing different genetic origins and rearing conditions: (i) wild adults captured outdoors during autumn; (ii) wild-collected caterpillars reared outdoors during autumn; (iii) third generation lab-reared monarchs raised outdoors during autumn; and (iv) third-generation lab-reared monarchs raised indoors under summer-like conditions. Furthermore, we compared the orientation performance of different group sizes (1, 2, 4, and 8 individuals housed together in outdoor cages) within each cohort. We predicted that wild adults and both cohorts of outdoor-reared monarchs would show evidence for compass orientation at approximately 180°S-225° SSW (towards central Mexico), and that indoor-reared monarchs would orient more towards the direction of the sun, or in no consistent direction. We further predicted that monarchs would show less variation, and more accurate compass orientation, when held in larger groups (4 and 8 individuals per cage) than when held singly or in pairs. Finally, we predicted that wild-caught adults, which had already been migrating towards their wintering sites in Mexico prior to capture, would show the strongest evidence for social cue use in their directional orientation.

Figure 3.1. Individual monarchs may vary in the migratory flight path they take from Athens, Georgia to the oyamel fir forests in central Mexico (indicated by the lower red point near several of the best studied overwintering sites). In these experiments, an accurate heading was classified as one between 180°S and 225°SW.

Methods

Monarch sources

Monarchs used in this experiment were obtained from four different groups, corresponding to different origins and rearing conditions, as described below.

Lineage Indoor (LI)

Third-generation lab-reared caterpillars were hatched from eggs laid by non-inbred F3 descendants of wild-caught spring migrants from Athens, GA (wild monarchs collected in Apr-May 2021). Eggs from 5 distinct genetic lineages were laid on stalks of swamp milkweed (*Asclepias incarnata)* that were cultivated in greenhouse conditions. Larvae were reared indoors at 26-28°C under ambient light supplemented by overhead fluorescent lighting set to a 16-hour day, simulating summer conditions. Caterpillars remained on natal stalks until late second instar and were then transferred to 1.4 L plastic containers with mesh screen lids, 6 caterpillars/container, and fed fresh cuttings of *A. incarnata* daily. Following eclosion, adults were held in the summer condition incubator for 4-8 days and fed 20% honey-water every 3-4 days until placed outdoors in field cages.

Lineage Outdoor (LO)

Third-generation lab-reared caterpillars (eggs obtained as described above) were moved to potted plants in late September 2021 and held outdoors until the adult stage. During the early second instar (roughly 6 days after eggs were laid), larvae were transferred from the laboratory onto potted *A. incarnata* plants, 2-3 larvae/plant. Plants were covered with fine mesh netting and sealed at the bottom using rubber bands (Figure B2). Plants were in a small, open field bordered by trees with full sun and were replaced as needed (when larvae consumed nearly all leaves). Upon pupation, leaves containing chrysalises were taped into 1.4 L plastic containers with mesh screen lids, 6 chrysalises/container. All containers remained outdoors until adults eclosed (in early October). Adults were held in glassine envelopes in the fall condition incubator for 6-10 days and fed 20% honey-water every 3-4 days until experiments began.

Wild Reared (WR)

Monarchs of this cohort were collected from the field as late-instar (4th and 5th) larvae during late September 2021. Caterpillars were obtained from several dozen swamp milkweed (*A. incarnata*) plants at the Georgia State Botanical Gardens (SBG) in Athens, GA, USA. Larvae were transferred to 1.4 L plastic containers with mesh screen lids, 6 caterpillars/container, and fed fresh cuttings of *A. incarnata* daily. Larvae remained in outdoor tubs (exposed to ambient photoperiod and temperature) until eclosion. Adults were held in the fall condition incubator for 7-13 days and fed 20% honey-water every 3-4 days until experiments began.

Wild Migrants (WM)

Wild fall migrants were caught in nets as adults while nectaring at the University of Georgia Trial Gardens (TG) and the SBG between Oct 11-25, 2022. Adults were held in glassine envelopes in the fall condition incubator for 5-10 days, and fed 20% honey-water every 3-4 days until experiments began.

Monarch treatments

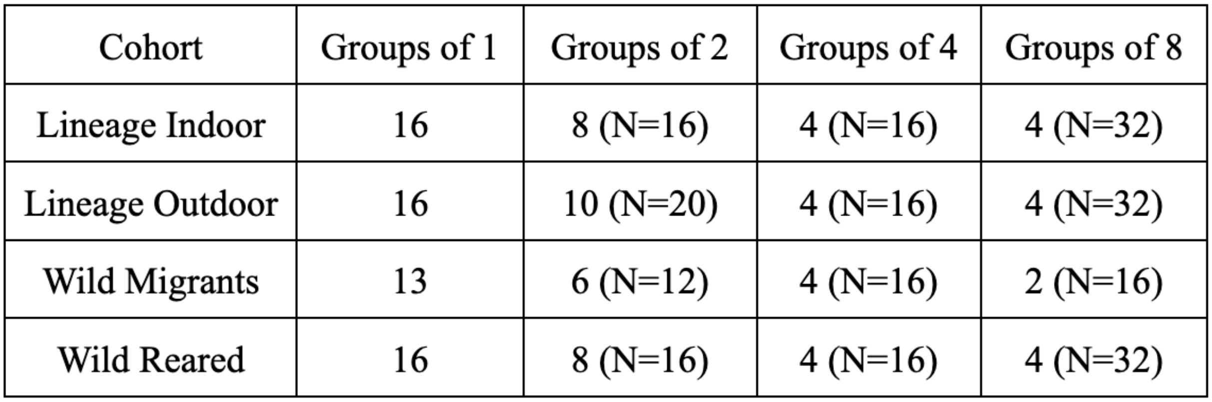
Each adult monarch used in this experiment was examined for the protozoan parasite *Ophryocystis elektroscirrha* upon eclosion or capture (using protocols outlined in (Altizer et al.,

2000) and any infected adults were excluded from further study. Each adult monarch was scanned with wings spread on the dorsal side with a standard computer scanner to capture morphological characteristics (using protocols and software outlined in Davis et al. 2020). Monarchs were labeled with a numerical ID in the discal cell on the ventral side of both hindwings. A small, colored sticker (2 mm diameter) was glued to the ventral side of both hindwings behind the discal cell to better identify individuals within outdoor cages (Figure B1). Adults were kept individually in glassine envelopes in incubators set to either autumn-like or summer-like conditions until experiments began. The autumn condition incubator was set to 21°C day and 16°C night, with a 12.4 hr day (sunrise was set to 0715 h, and sunset at 1940 h, to mimic outdoor conditions during early October in Athens, GA, USA). The summer condition incubator was set to 21°C day and 16°C night, and 15.5 hr day (sunrise was set to 0600 h, and sunset at 2135 h, to mimic conditions in Athens, GA during early August).

Experimental methods

All experiments took place between October 11-27, 2022, during the window of peak monarch migration through Northeast Georgia (estimated to be between October 1-31 based on Journey North observations). Experiments were conducted at the University of Georgia's Horseshoe Bend (HSB) ecological research field site (33°57' N, 83°23' W), in a clearing roughly an acre in size (4,000 m2). Experiments took place in 4 (1.83 m3) BioQuip mesh cages, which were placed roughly in the center of the field and evenly spaced (Figure B3). Monarchs of the same cohort were assigned to experimental group sizes of 1, 2, 4, or 8 individuals per cage (Table 1). Prior to the start of trials, monarchs were placed outdoors in (1m2) pop-up mesh cages to acclimate for 1 hour with the other individuals of their assigned group. After the hour of acclimation (at roughly 1100 h, or when the ambient temperature reached at least 17°C), pop-up cages were placed into the center of the BioQuip cages and unzipped, allowing monarchs to fly into the larger experimental cage without additional handling.

Approximately 10 minutes following release into field cages, an observer entered the BioQuip tent, and sat motionless on the ground at the center point of the tent. The compass orientation of each monarch was interpreted as the migratory heading an individual would have taken if free flying. Each monarch's heading was recorded with a calibrated iPhone compass every 1-2 minutes until 5 headings were captured per individual. From Athens, Georgia, USA the optimal migratory bearing is south/southwest, ranging from 180°-225° (Figure 3.1). Each monarch was only used once (for one observation interval). Weather conditions (temperature, humidity, cloud cover, wind speed and direction, and sun angle) were recorded at the start of each trial, and trials were conducted on clear to partly cloudy days (less than 75% cloud cover) above 17°C, to limit the impacts of cloud cover on sun-compass orientation.



Cohort	Groups of 1	Groups of 2	Groups of 4	Groups of 8
Lineage Indoor	16	8 (N=16)	4 (N=16)	4 (N=32)
Lineage Outdoor	16	10 (N=20)	4 (N=16)	4 (N=32)
Wild Migrants	13	6 (N=12)	4 (N=16)	2 (N=16)
Wild Reared	16	8 (N=16)	4 (N=16)	4 (N=32)

Table 3.1. Number of replicates for each experimental group size across all four cohorts, including sample size.

Data Analyses

To investigate the effect of local weather conditions, rearing condition (cohort), and group size on monarch migration, we examined each monarch's deviation from the ideal flight bearings. We first took the circular mean of the 5 values per individual to obtain an average direction per monarch. The average heading of each individual was used to determine that individual's deviation from the ideal flight bearings which, in all models, represent 180°S-225°SW. Monarchs within the range of 180°S-225°SW were given a deviation score of 0, and any monarch outside the range had a deviation score corresponding to their distance from the closest end of the range (either 180°S or 225°SW). Degree deviation was square-root transformed prior to analyses to normalize error variance. We used a linear model with transformed degree deviation from the ideal flight bearings as our response variable, sex and cohort as fixed effects, and wind speed, sun direction, and group size as continuous covariates. We also included an interaction term between group size and cohort. Next, we ran four one-way ANOVA models to examine the effect of group size on the transformed degree of deviation from the ideal flight bearings, testing each cohort separately. Finally, we conducted an additional one-way ANOVA to examine the impact of rearing condition (cohort) on monarch wing hue, as a confirmation of differences in migratory state among the cohorts.

Results

For the fitted regression model, cohort (F=14.145, df=3, p<0.0001) and group size (F=6.263, df=3, p=0.0004), and the interaction between the two (F=4.514, df=3, p=0.0041) significantly predicted deviation from the ideal flight bearings. Sun direction (F=0.560, df=1, p=0.4547), sex (F=0.415, df=1, p=0.5199), and wind speed (F=3.1969, df=1, p=0.0748) were not significant predictors of deviation from the ideal flight bearings. Directional trends resulting from cohorts and group sizes are described in detail below.

Differences among cohorts

Wild Migrants (WM) oriented in a compass direction that more closely matched the ideal predicted heading (S-SW; Figure 3.2) than monarchs from the other 3 cohorts (n= 57; x̄=166.88; 95% CI [156.11, 177.66]). The orientation of the WM cohort was significantly different than all other cohorts (Wild Reared: p=0.0146, Lineage Outdoor: p<0.0001, Lineage Indoor: p<0.0001). The difference in compass orientation between the Wild Reared (WR) (n= 80; x̄=148.15°; 95% CI [132.23°, 164.08°]) and Lineage Outdoor (LO) (n= 84; x̄=142.31°; 95% CI [114.04°, 170.57°]) cohorts was not significant (p=0.256), and both of these groups oriented in a SE direction on average. The Lineage Indoor (LI) cohort displayed an easterly orientation (n= 80; x̄=109.04°; 95% CI [91.69°, 126.38°]), and was significantly different in compass orientation than the WR (p=<0.0001), LO (p=0.0203), and WM (see above) cohorts.

Group size effects

One-way ANOVAs examining the effect of group size on compass orientation within each cohort showed that group size significantly predicted orientation in the Wild Migrant (F=11.380, df=3, p<.0001) and Lineage Outdoor cohorts (F=2.970, df=3, p=0.0367) (Figure 3.2). Group size was not a significant predictor of orientation in the Wild Reared (F=0.756, df=3, p=0.522) and Lineage Indoor (F=1.186, df=3, p=0.321) cohorts. Within the Wild Migrant cohort, a post-hoc Tukey's HSD test revealed that single monarchs oriented significantly more poorly (in a direction farther from the optimal SW compass angle) than groups of 4 (p<.0001) and groups of 8 (p<.0001). Groups of 2 were also significantly less accurate than groups of 8 monarchs (p=0.022). We found a non-significant improvement trend between groups of 2 to 4 (p=0.055), and no difference between single monarchs to groups of 2 (p=0.212). In the Lineage Outdoor cohort, groups of 4 showed significantly improved orientation relative to single monarchs (p=0.0241), but no other pairwise differences between groups were significant.

Wing Hue

One-way ANOVA showed a statistically significant difference in orange wing hue among cohorts (Figure 3.3; F=22.781, df=3, p=<.0001). A Tukey's HSD test for multiple comparisons found that the mean wing hue of the Wild Migrant cohort was significantly redder than every other cohort (Wild Reared p=<.0001, Lineage Outdoor p=0.0001, Lineage Indoor p=<.0001). The Wild Reared and Lineage Outdoor cohorts had a similar mean wing hue (p=0.9954), and both of these outdoor-reared cohorts were significantly redder than the Lineage Indoor cohort (p=0.0002 and p=<.0001, respectively). Finally, we observed a very weak positive correlation between wing hue and degree deviation (Figure 3.4; R2= 0.025; Beta = 0.3641; F = 7.625; p = 0.0061). Monarchs with redder wings showed compass orientation closer to the optimal angle, relative to monarchs with yellower wing hue.

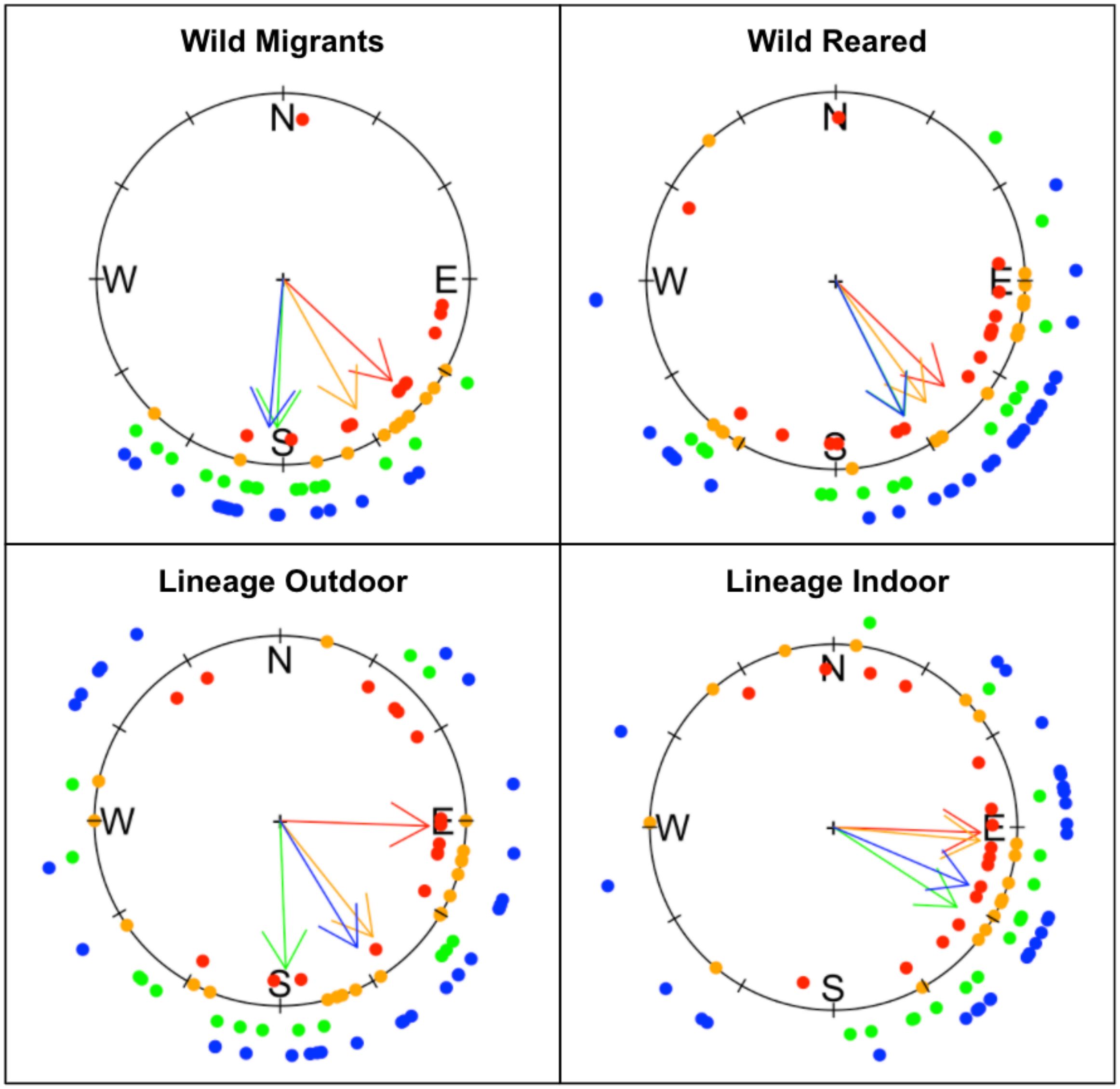


Figure 3.2. Orientation of monarchs across all four cohorts and all four group sizes within each cohort. Each data point represents a single monarch's average heading. Data points are color coded by group size- single monarchs (red), groups of two (orange), groups of 4 (green), and groups of eight (blue). Corresponding colored arrows display the average heading of all monarchs from a given group size.

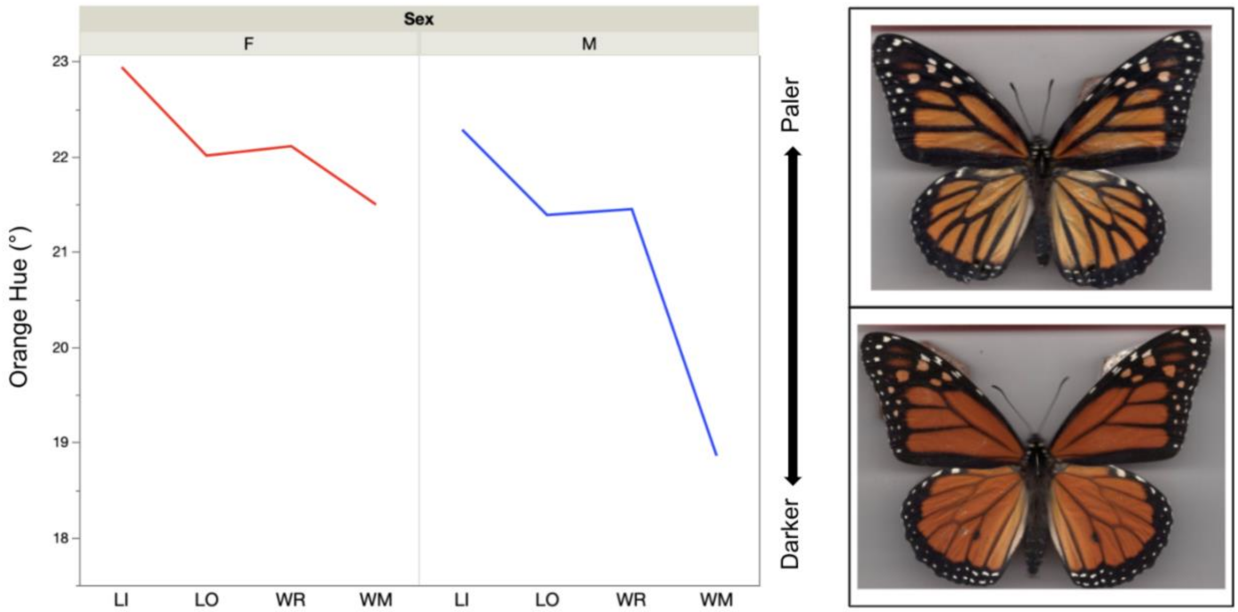


Figure 3.3. Left- mean orange hue on monarch forewings (vertical axis) was significantly different across individuals between each cohort (horizontal axis), corresponding to orientation flight performance. Here, lower values indicate redder wing coloration, and larger values indicate yellower wing hue. Wild Migrant wings (bottom right) were significantly redder than every other cohort, particularly Lineage Indoor monarchs (top right).

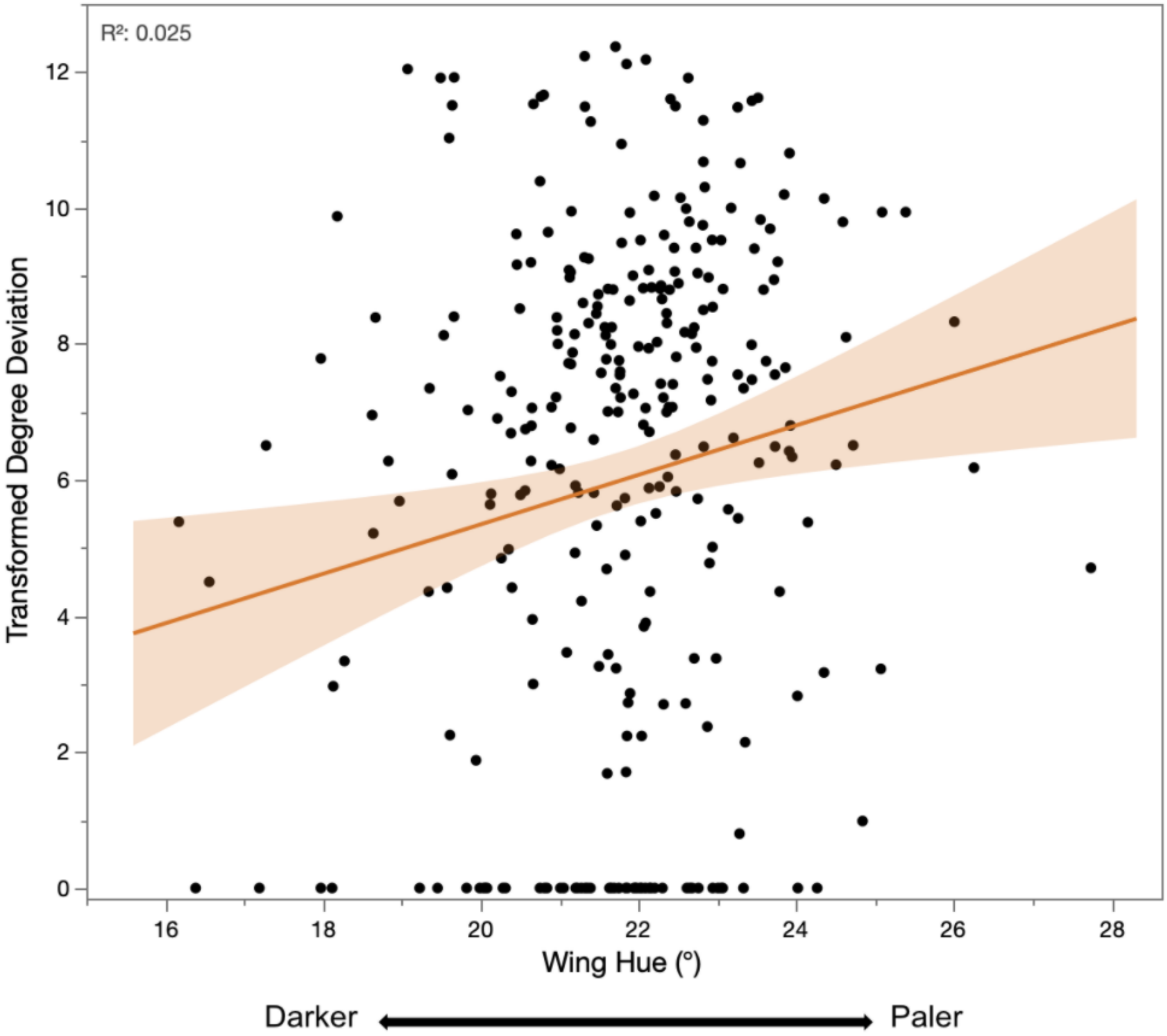


Figure 3.4. Degree of orange hue in monarch wings (x axis) is positively correlated with degree deviation from the ideal flight bearings (y axis). As monarch wings grow paler in hue, orientation tends to become less accurate, although the relationship is weak (R2= 0.025).

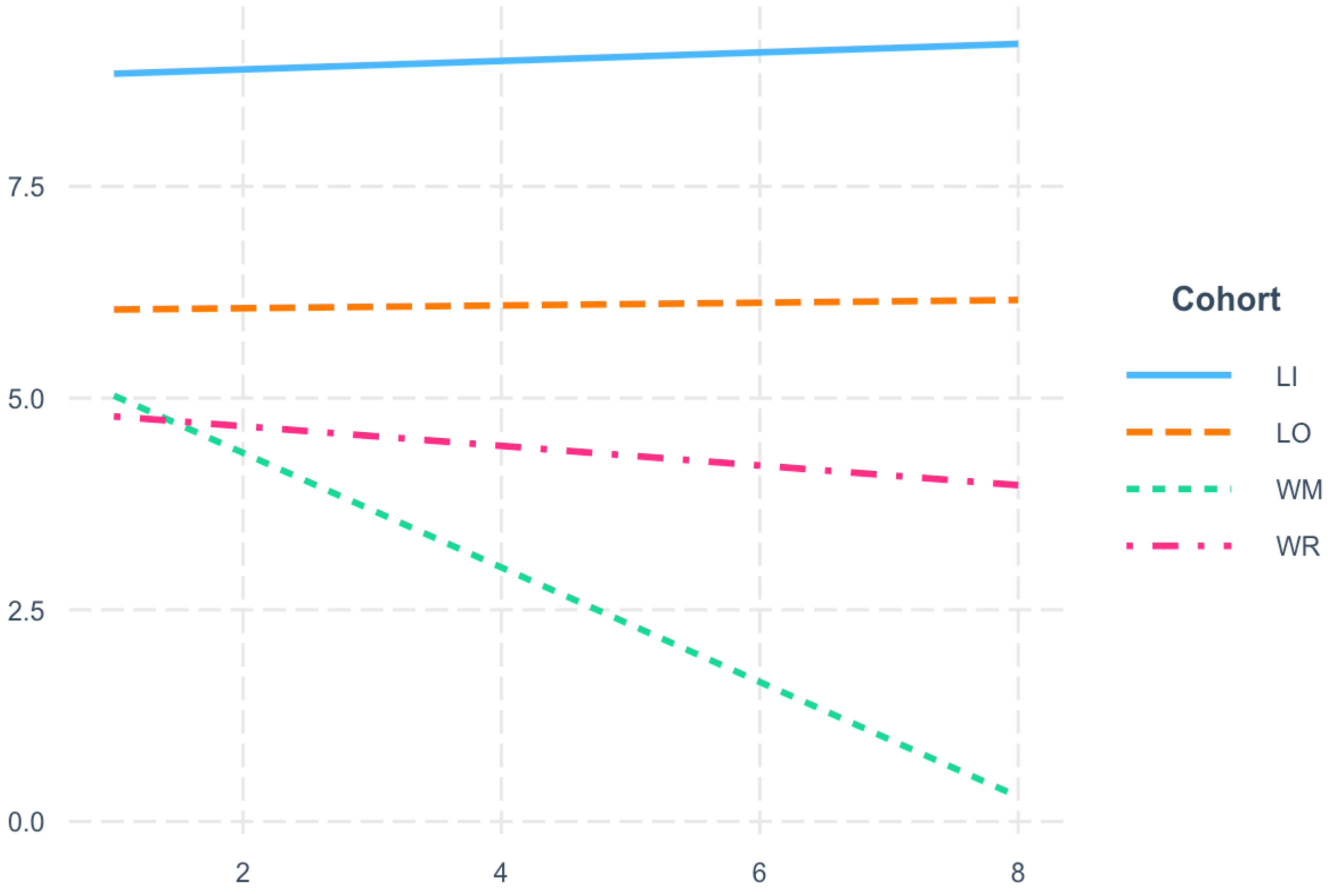


Figure 3.5. Transformed degree deviation from the ideal flight bearings (y-axis) was significantly predicted by an interaction between group size (x-axis) and rearing condition (cohort) during experimental flight trials. Wild Migrants (WM) experienced the strongest, positive effects of increasing group size.

Discussion

In these experiments, we compared the orientation and social information use of Wild Migrant monarch butterflies to those from three other rearing conditions to examine the effects of rearing environment and social cues on migratory accuracy. We found that rearing condition had the largest impact on navigational accuracy, with Wild Migrants significantly outperforming monarchs from every other rearing condition during orientation. Wild Migrants were also significantly darker in mean wing hue than every other cohort (Figure 3.3), a known predictor of fitness and flight performance (Davis, Chi, et al., 2012). However, across all four cohorts, individual monarchs oriented more poorly on average than groups of 4 and 8 (Table B1, Figure 3.2). Our results show the largest benefit as group size increases from 1 to 4 or 1 to 8, but increasing from a group of 4 to 8 had very little additional impact- suggesting a maximum benefit at a density of around 4 monarchs/1.83 m3, above which social cues provide no further improvement. The navigational benefit experienced by monarchs in groups could be an example of public information use, a form of indirect social information wherein individuals observe the performance or decisions of conspecifics, and utilize it to help inform their own behavior (Chittka & Leadbeater, 2005; Valone, 2007; Valone & Templeton, 2002). Our results reflect patterns observed in migrating salmon, where increasing conspecific density resulted in faster and more accurate navigation through dam fishways (Okasaki et al., 2020). However, the positive effect of group size on orientation accuracy was only significant in the Wild Migrant and Lineage Outdoor cohorts.

Of the four cohorts, the Lineage Indoor individuals had the palest mean wing hue, weakest ability to orient towards the optimal range of 180°S-225°SW and did not significantly benefit from the presence of conspecifics. Instead, the LI cohort displayed a sun-orienting response towards the southeasterly azimuth during trials. This lab-laid and -raised cohort's poor orientation performance was expected, as even genetically wild larvae are known to orient poorly when reared inside under autumnal conditions (Tenger-Trolander et al., 2019; Tenger-Trolander & Kronforst, 2020). Past research has also shown that summer condition monarchs do not display accurate migratory orientation abilities (Kanz, 1977; Zhu et al., 2009). By rearing our LI cohort indoors and under summer conditions, we were aiming to create a baseline 'non-orienting' group, which was reflected in our results. The pale wing hue of LI individuals reflects previous research that found paler wings among both captive-reared (Davis et al., 2020) and summer breeding generation monarchs (Davis, 2009) when compared to migratory individuals.

Interestingly, there was not a significant difference in orientation accuracy or mean wing hue between our Lineage Outdoor and Wild Reared cohorts. While both of these groups were significantly darker in hue and more navigationally accurate than the LI cohort, neither displayed orientation capabilities or dark wing hue on par with Wild Migrants. The LO cohort was laid by lab-lineage females, and their poor orientation performance confirms work by Tenger-Trolander et al. (2019) and Tenger-Trolander and Kronforst (2020), who found that monarchs from commercial sources failed to orient even after outdoor rearing. These LO individuals were the offspring of non-inbred F3 descendants of wild-caught spring migrants, suggesting that habituation to an artificial environment and negative genetic effects of captive rearing on orientation can occur in as few as four generations. Outdoor rearing and exposure to the declination of the autumnal sun, cooling temperatures, and shortening photoperiod, while beneficial, was not enough to completely offset these effects, despite supposition that some combination of these factors is responsible for the induction of migratory status in larvae (Goehring & Oberhauser, 2002; Reppert & de Roode, 2018; Taylor et al., 2019).

More surprising was the poor orientation performance of the wild-laid, outdoor reared larvae in the Wild Reared cohort. Adults in this cohort were laid by late-summer generation wild females on wild *A. incarnata*, and while they were moved into tubs as late instar larvae, they remained outdoors until eclosion. We had hypothesized that the orientation performance of this group would be nearly identical to that of the Wild Migrant cohort, since there would be no genetic effects of lab-lineages, and the larvae were exposed to all celestial and weather cues thought to induce migratory status (Goehring & Oberhauser, 2002; Reppert & de Roode, 2018; Taylor et al., 2019). Their failure to accurately orient could be explained by the physiological effects of human handling and/or the absence of natural host-plant cues during late instars, as this cohort was physically placed into tubs and briefly handled daily during feeding. Short bouts of human handling are known to cause a heart rate spike in monarch larvae and pupae (Davis, 2020). Little is known about monarch habituation to continual stressors, but previous work suggests a diminishing in their cardiac response (Davis et al., 2018). More research is needed to examine the potential impacts of human handling and prolonged stress on orientation ability and general ‘wildness’.

It seems that the LO and WR cohorts are each halfway to migratory status- but as a result of different variables. The Lineage Outdoor cohort was exposed to all the environmental cues that should have induced migratory status (dark wing hue and accurate orientation), but may have been limited by genetic adaptation to a captive environment. The Wild Reared cohort was genetically unmanipulated, and were reared outside, which should have produced a monarch of migratory status, but they were still second-rate when compared to Wild Migrants. Again, this could have been the effect of handling or being reared in non-natural outdoor plastic tubs. As a result, individuals from both the WR and LO cohorts were darker hued and more accurate during flight than LI individuals (who were genetically and environmentally limited), but were paler and less accurate than Wild Migrant individuals. Together, the WR and LO cohorts are further evidence of the cryptic combination of genetics and environmental cues required to produce a true, navigationally proficient monarch of migratory status.

When monarchs reared in a controlled setting (either indoor or outdoor) are released, they are instantaneously thrust into an unfamiliar and variable environment. Their survival depends on physiological and genetic fitness coupled with their ability to perform foraging, anti-predation, and navigation behaviors, particularly during intensely selective events like migration. There are known differences in physiology and migratory ability between captive reared and wild monarchs (Davis et al., 2020; Tenger-Trolander et al., 2019; Tenger-Trolander & Kronforst, 2020), but the effects of rearing environment on social behavior are understudied. In addition to the Lineage Indoor cohort (lab genetics), our study included genetically wild outdoor-reared individuals (WR), neither of which exhibited significant use of social information.

A similar non-response across genetically different cohorts suggests that social behavior may be phenotypically plastic in monarchs, largely influenced by larval or adult experiences. Fisheries research has found stark differences in migratory behavior and social behavior between hatchery-bred and wild individuals, suggesting that early social environment (Chapman et al., 2008) and relevant life experiences (Brown & Laland, 2001; Suboski & Templeton, 1989) are critical for developing and learning adaptive social behaviors. Here, the Wild Migrant (WM) monarchs had already successfully completed a portion of their migration, gaining critical navigation experience before their use in experiments. These migrant individuals were under intense selection pressure, while all the other cohorts were reared (at least partially) under artificial conditions with relaxed selection, maintained by experimenters. It is possible that social information use in monarchs has a genetic basis that is selected for in a wild environment, and the maintained survival of unfit individuals ‘diluted’ the strength of the relationship in our other cohorts.

Despite their lab-lineage genetics and only partial migratory status, the Lineage Outdoor cohort displayed use of conspecific cues during orientation, which strongly suggests the importance of outdoor rearing for producing ‘social’ monarchs. The Lineage Outdoor cohort was laid in a controlled, lab setting and moved onto outdoor plants at second instar with 1-2 other larvae. Predation was prevented with plant covers, but otherwise larvae faced natural selection pressures until after pupation. The Wild Reared cohort was opposite the LO cohort- the genetically-wild larvae had a natural early-social environment, until their collection at fourth instar when they were moved to experimentally controlled tubs before pupation. Since only the Lineage Outdoor cohort displayed a significant relationship between group size and orientation accuracy, it could be that a ‘wild’ larval environment during fifth instar and pupation represents an important window of monarch social development.

In summary, larval rearing environment has severe implications for monarchs- impacting their wing hue, migratory orientation, and adaptive social behaviors. Here, we found novel evidence of social information use by monarchs during navigation and saw that the ‘ingredients’ required to produce a dark-winged, accurate navigator are not necessarily the same as those required to produce a social monarch. These results suggest that the ability to utilize social information during orientation depends mostly upon environmental or social cues during larval stages, while wing color and navigational accuracy has an additional genetic basis. However, more research could help dissect whether social behaviors in monarchs are a result of natural, outdoor rearing, or are genetically predisposed in all monarchs and were revealed at a significant level in this experiment as a by-product of natural selection pressures faced by Wild Migrant adults and Lineage Outdoor larvae. Given the critical fitness implications of foraging, navigational, or stopover decisions during migration, social information use represents an adaptive strategy, even in an otherwise solitary species. Since there are navigational benefits of conspecifics during migration, these results also suggest a possible threshold population density required to maintain successful migration, which could further inform critical conservation decisions.

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CHAPTER 4

GENERAL CONCLUSIONS

Relatively energetically inexpensive, socially acquired information can result in rapid, more accurate decision making. Social information use is relatively understudied in solitary insects, including monarchs, representing a critical gap in our understanding of these species. Conspecific cues can be indicative of desirable resources or environments, especially in uncertain or unfamiliar situations. Here, we saw evidence for social information use during monarch migratory orientation, but not during oviposition. Given the different fitness implications of these two biological processes, social information use by monarchs may represent an energy-fitness tradeoff that is not always adaptive.

Despite previous research that suggested monarchs differentiate based on physical plant characteristics, female monarchs in these experiments did not change their oviposition behavior in response to social cues, and neither avoided nor preferred stalks containing the eggs of conspecifics, regardless of host-plant quality. Monarchs are known to egg cluster when milkweed resources are few, and with three stalks per experimental cage, we may have created an environment where ‘egg dumping’ is ideal for females. However, it is likely that the high fecundity of monarchs combined with low egg and early-instar survival rates explains female non-response to social cues. No one host-plant typically receives more than 3-4 of any one female’s 200+ eggs, so carefully assessing each host stalk would represent an overwhelming energetic cost, with a relatively insignificant fitness benefit.

During migratory orientation, we found that rearing condition had severe implications for both accuracy in flight bearings and use of conspecific cues. Artificial rearing environments, even when outdoors, were unable to produce adults orienting with the navigational accuracy observed in Wild Migrants. Wild Migrants significantly out-performed individuals from every other rearing condition during orientation. Despite all other cohorts not orienting as accurately, there was a trend of improvement in orientation as group size increased across all four cohorts, particularly as groups increased from 1 to 4 individuals. However, the relationship between experimental group size and accuracy was only significant in the Wild Migrants and the outdoor-reared Lineage Outdoor cohort. This provides novel evidence of social information use in monarchs, and strongly suggests a link between larval ontogeny and the development of social behavior.

Based on these experiments, we can conclude that monarchs do use social information, but that the type of information they rely on (individual or social) may be situational. It is also possible that monarch use of social cues is not situational, but is developed during larval ontogeny or adult experiences. In our experiment on monarch oviposition, we utilized only lab-lineage F2 descendants of wild-caught monarchs, which were reared in an artificial environment. Since we saw an effect of rearing environment on social information use during orientation, we recommend replications of our oviposition experiment using wild females to examine the effects of rearing condition and genetic adaptation to a captive setting on social behavior during host-plant selection. Research into the visually perceptive range of monarchs and/or the mechanisms by which they detect conspecific larvae, eggs or adults would also lend further insight into these results. Overall, future research is needed to examine if social cues are used situationally and to determine the combination of genetics, environmental cues, and larval or adult experiences required to ‘turn on’ social behavior in monarchs.

APPENDIX A

CHAPTER 2 SUPPLEMENTAL MATERIALS

Figure A1. Cage setup during experiment 1, where all three physical manipulations of A. incarnata stalks- water-stressed (left), herbivory-damaged (center) and healthy (right)- were presented to female monarchs.

Figure A2. Female monarch "dabbing", or curling the abdomen to push the ovipositor against the underside of a leaf and lay an egg.

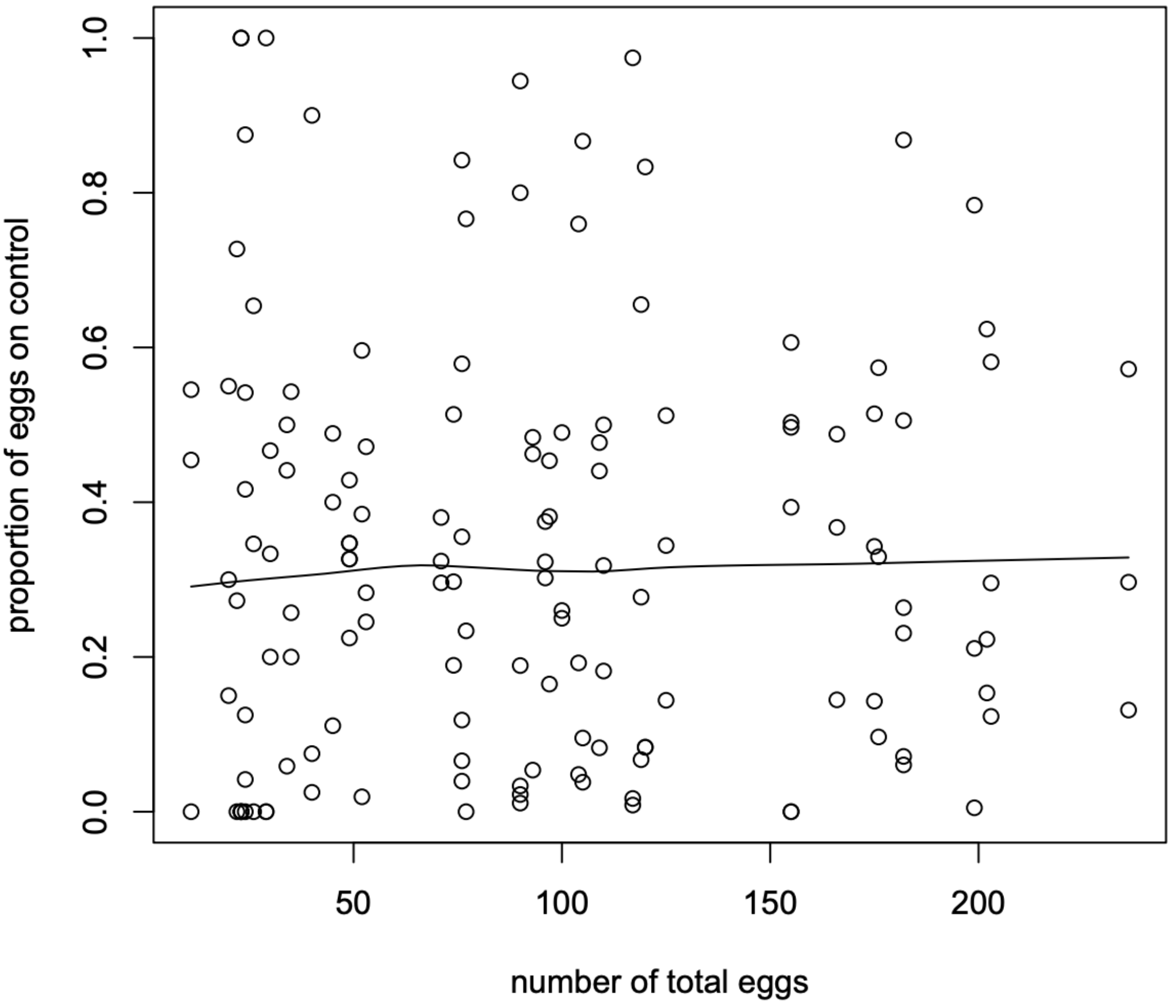


Figure A3. Eggs laid on control stalks as a proportion of the total number of eggs laid across all three stalks by each female. Female laying propensity had no significant impact on treatment preference, as the proportion of eggs laid on control stalks was similar between females with a low number of total eggs (less than 50) and a high number of total eggs (150 or more).

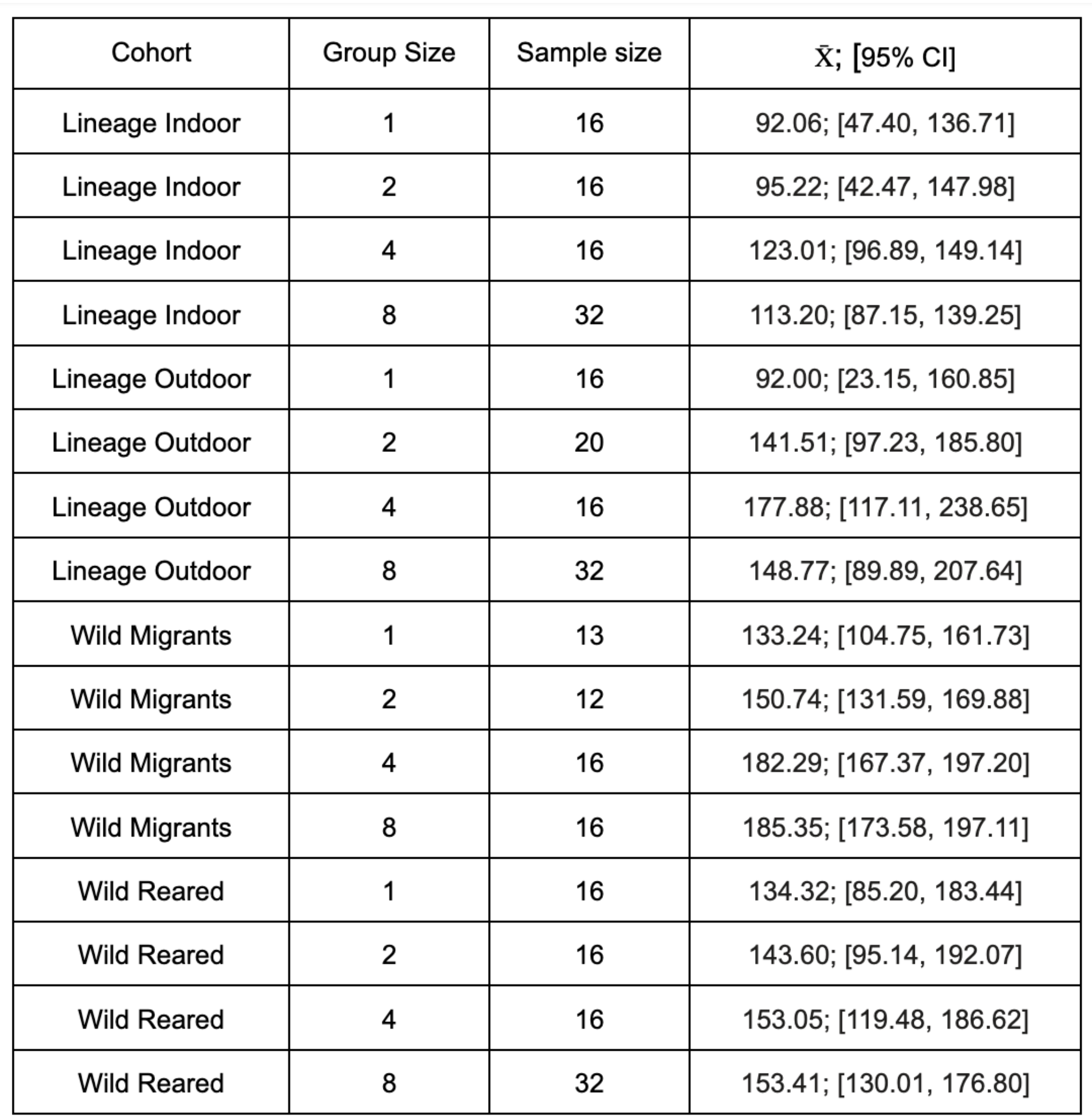
APPENDIX B

CHAPTER 3 SUPPLEMENTAL MATERIALS

Figure B1. Placement of unique ID numbers in the discal cell and randomly assigned colored stickers on the ventral side of the hindwing behind the discal cell. Pictured here on two deceased monarchs after outdoor flight trials.

Figure B2. Outdoor rearing set-up for the Lineage Outdoor (LO) cohort larvae. A. incarnata plants with 2-3 larvae/plant were covered with mesh plant protectors and sealed at the bottom using rubber bands to prevent larvae from leaving their host plant.

Figure B3. Experimental setup of outdoor orientation cages at HSB ecological field site. Four (1.83 m3) BioQuip mesh cages were placed roughly in the center of the field and evenly spaced to minimize shading and inconsistencies in terrestrial cues between cages.



Cohort	Group Size	Sample size	$\bar{x}$; [95% CI]
Lineage Indoor	1	16	92.06; [47.40, 136.71]
Lineage Indoor	2	16	95.22; [42.47, 147.98]
Lineage Indoor	4	16	123.01; [96.89, 149.14]
Lineage Indoor	8	32	113.20; [87.15, 139.25]
Lineage Outdoor	1	16	92.00; [23.15, 160.85]
Lineage Outdoor	2	20	141.51; [97.23, 185.80]
Lineage Outdoor	4	16	177.88; [117.11, 238.65]
Lineage Outdoor	8	32	148.77; [89.89, 207.64]
Wild Migrants	1	13	133.24; [104.75, 161.73]
Wild Migrants	2	12	150.74; [131.59, 169.88]
Wild Migrants	4	16	182.29; [167.37, 197.20]
Wild Migrants	8	16	185.35; [173.58, 197.11]
Wild Reared	1	16	134.32; [85.20, 183.44]
Wild Reared	2	16	143.60; [95.14, 192.07]
Wild Reared	4	16	153.05; [119.48, 186.62]
Wild Reared	8	32	153.41; [130.01, 176.80]

Table B1. Average flight heading and 95% confidence interval, in degrees, of every individual from the same experimental group size within each of the four cohorts. Across all four cohorts, there was a general trend of improvement in orientation accuracy towards the ideal 180°-225° heading as group sizes increased.