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Effect of larval experience on learning mate preference in Bicyclus anynana

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Effect of larval experience on learning mate preference in *Bicyclus anynana*

An Honors Thesis submitted in partial fulfillment of the requirements of Honors Studies in Biological Sciences

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Abstract:

Phenotypic traits are shaped by the interaction of an organism's genes and the environment they experience. The plasticity of phenotypic traits is particularly responsive to developmental environments which can shape adult traits, such as expressed behaviors like mate choice and the ability to learn preferences. The Lepidoptera species *Bicyclus anynana* has been shown to learn mate preference based on social interaction following emergence from the chrysalis, however if and how the social complexity of the larval experience affects this ability to learn is less understood. Here I test the effects of isolation during the larval period on a female's ability to learn mate preference. I created 2 larval rearing environments, isolated and gregarious, used a split family design, and ran behavioral assays to determine the relative strength of preference learning in individuals reared in isolated and gregarious conditions. Using a split family design allowed me to control for between-family genetic effects. I found that isolation did not inhibit a female's ability to learn mate preference and gregariousness had no effect on relative strength of learned mate preference. These results suggest that social complexity experienced during development does play a role in the plasticity of mate choice learning in female *Bicyclus anynana.*

Introduction:

Phenotypic plasticity, the ability for a genotype to change expressed phenotypes in response to a unique environment, has been widely studied to gain a better understanding of how organisms can adapt in dynamic conditions (Pfennig, 2021). This ability to alter phenotype, whether it be morphology, physiology, or behavior, has been thought to be a driving force in natural and sexual selection. A change in environment can disrupt or alter development or homeostasis of an organism, so many species have evolved different adaptive mechanisms or traits to counteract this change (Whitman, 2009). For example, it has been shown that plants have plastic changes in seed longevity, leaf lifespan, and temperature responses of metabolic processes as a result of rising carbon dioxide levels in our atmosphere (Nicotra *et al*., 2010). This phenomenon has been found across many taxa and has intrigued scientists for decades, however there is still so much that is unknown about the mechanisms and effects of phenotypic plasticity.

Environmental factors typically vary throughout one's lifespan, but it has been shown that there are sensitive windows during development where the phenotype is particularly responsive (Fawcett & Frankenhuis, 2015). Zebrafish, for example, that lack social interaction during the larval period have increased expression of avoidance behaviors, like strength of swim movements and distance at which they react to conspecifics (Groneberg et al., 2020). This novelty in developmental processes -and subsequent phenotypic effects- that arise from the genotype interacting with the environment during development is classified as developmental plasticity (Moczek *et al*., 2011). Social interactions and observations of individuals in early life environments can contribute to plastic changes in phenotype, such as mating behavior, foraging techniques, and communicative signals (Leris & Reader, 2016). Moreover, activity-dependent social interactions, such as physical touch, have been shown to affect neural development, stress reactivity, and body size (Snell-Rood, 2020). Other investigations of developmental plasticity have revealed its effects on an individual's propensity for certain behaviors. More specifically, the ability to learn is a behavior known to be influenced by juvenile experiences. For example, in rats (*Rattus norvegicus*), the absence of maternal care and siblings during the pre weaning period negatively affects social learning in adulthood (Lévy *et al*., 2003). This plasticity caused by the developmental environment is also known to play a role in mate preferences and mate choice learning.

Many studies have assessed the mechanisms of mate choice and learning mate preferences across diverse animal taxa. *Drosophila melanogaster* fruit fly males can learn to avoid females of related species as well as recently mated females (Dukas, 2005). Birds use sexual imprinting to recognize their parents and gain a sexual preference for mates with similar traits (Iwrin & Price, 1999), while guppies are known to copy the mate preferences of their conspecifics (Dugatkin & Godin, 1992). The ability to learn sexual preference is an important element of reproduction, being that it can ultimately affect sexual selection and speciation. Mate choice can influence heritable traits and heterozygosity, both of which are important for the overall fitness of a population (Tregenza & Wedell, 2000). Because females usually invest more than males to produce viable offspring, their mating preferences are typically more prevalent and influential in nature (Tregenza & Wedell, 2000). Unquestionably, male characteristics can influence female mate selection, but a number of other extrinsic and intrinsic factors have been shown to shape the process of mate selection for females. For instance, chooiness is expected to decline with age and low quality conditions because of the costs associated with having strong preferences (Kelly, 2018). Moreover, the examples above of sexual imprinting and mate choice copying show how the social environment and learning can play an important role in female mate choice. Social learning generally allows for plastic changes in mate choice; there are documented cases where adult and juvenile social experiences, respectively, can lead to plastic changes in learned mate preference. For example, in wolf spiders, early experiences with courtship song or displays can increase selectivity for mates and courtship interactions during adulthood can shape future mating success (Herbets & Sullivan-Beckers, 2010). Despite this wealth of research showing the effects of social interactions during development having an influence on adult mate choices, less is known if and how the degree of socialness experienced in the larval periods for species that undergo metamorphosis influences mate choice learning in adulthood. Here I investigate this topic using the butterfly *Bicyclus anynana.*

Bicyclus anynana, a gregarious species of butterfly, has been a model organism for biological research and used to examine different aspects of phenotypic plasticity in a variety of contexts. For example, they have been used in studies looking at wing pattern development- as well as- the relationship between eyespots and predation (McMillan *et al*., 2002; Lyytinen *et al*., 2004). Moreover, this species is largely known for its seasonal polyphenism, where two forms, each having distinctive morphological and behavioral traits, are induced by different environmental factors experienced during development (Kooi & Brakefield, 1999). This species is also often used in studies of behavioral plasticity. For example, both male and female *B. anynana* have been shown to change their mating behaviors based on the number of individuals found in their environment (Westerman *et al*., 2014). Furthermore, female *B. anynana* choose their mating partners based on the UV reflective eye spots found on the dorsal wings of the males, however, social interactions following emergence can determine which male wing pattern a female finds more attractive, suggesting that mate preference is learned (Robertson & Monteiro, 2005; Westerman *et al*., 2012). Social context can also shape their neural development; preliminary data shows that female *B. anynana* raised in isolation developed faster and have larger optic lobes, but smaller central brains compared to females reared in gregarious conditions (Allen, 2019). I will be examining if this developmental plasticity caused by the complexity of the social environment during a female's larval stage also impacts its ability to learn mate preference that is expressed later in adulthood.

To test the hypothesis that isolation during the larval period can affect the relative strength of learned preference, I used a split family design and mate choice learning assays on siblings reared in gregarious and isolated conditions respectively. Studying the effects of isolation on a female's ability to learn mate preference will allow us to further explore how social-ecological factors affect behavioral plasticity and the mechanisms underlying mate choice learning.

Methods:

Study animal and husbandry

Bicyclus anynana, commonly known as the "Squishing Bush-Brown," is a small gregarious species of butterfly native to the woodlands and savannah of eastern Africa. This species can express two seasonal forms which are induced by the rearing environment and have distinctive morphological and behavioral traits (Kooi & Brakefield, 1999). The first laboratory colony was established in Leiden, The Netherlands in 1988 from females collected in Malawi. In 2006, another colony was created at Yale University in New Haven, Connecticut from eggs collected from a colony in Rochester, New York where its individuals originated from the colony in Leiden. The colony used in this study was established in Fayetteville, Arkansas in 2017 from eggs collected in Singapore, which was created from eggs from Yale University. All butterflies were reared in mesh cages in a greenhouse maintained at approximately 27º C and 60-80% relative humidity, and 13 h:11 h light:dark photoperiod to induce wet season phenotype (Kooi & Brakefield, 1999). All larvae were fed young corn plants and adult butterflies were provided with sliced bananas and wet cotton.

Split family design

To cultivate adults raised in both isolated and gregarious conditions, two families were made weekly over the course of the experiment (29 families so far) through the selection of a virgin male (5 days old) and female (3 days old) from the breeding colony. One of the two families created was chosen for egg collection each week. The newly emerged individuals were placed in a mesh cage (30.38 cm x 30.48 cm x 62.23 cm) containing one young corn plant for the female to lay her eggs on and sliced banana and wet cotton as a source of food and water. The female was dusted with PF-33 clownfish orange fluorescent powder (Risk Reactor) to facilitate detection of copulation. The pair was left in the cage for a week so that copulation and oviposition could occur. The eggs and first nstar hatchlings were then collected and moved to the isolated and gregarious rearing conditions, respectively.

As new hatchlings were around other first nstar caterpillars in the Family Cage, they were automatically sorted to the gregarious rearing condition and the eggs were used for isolated conditions. Gregarious rearing conditions consisted of 2 corn plants placed within a mesh sleeve (74.93 cm x 100.33 cm) and 15 individuals from one family (Figure 1B). Isolated individuals were reared in their own mesh sleeve with one corn plant (Figure 1A). Corn plants were replaced as needed, so caterpillars were fed ad libitum. Developing individuals were left in their respective mesh sleeves until pupation. After pupation, the pupae were removed from their sleeve and sorted by sex and rearing condition and placed into a small mesh cage until they emerged.

There were some issues with egg hatching and pupae emergence, so not all families that were created were able to be used in this experiment.

Mate choice learning assay

Social interactions following emergence have been shown to influence which male wing pattern a female finds more attractive in *B. anynana*, suggesting that mate preference is learned (Westerman et al., 2012). To test the effect of rearing condition on a female's ability to learn mate preference, individual newly emerged females (from gregarious and isolated conditions) were placed into a cage with a 3-day old 4-spot virgin male for approximately 3 hours to be given the chance to learn sexual preference. On the 3rd day after emergence, she was painted with an orange fluorescent powder and placed into a mesh cage with a 2-spot male and 4-spot male. These males were matched in age (3-day old) and size. The males were checked everyday until one had the fluorescent powder on its abdomen (indicating copulation and female choice), and his phenotype was recorded.

Manipulation of male wing pattern

Two male phenotypes were created, where eyespots were painted on the dorsal forewings with a Fish Vision white UV reflective paint, similarly to (Westerman et al., 2012). The day before mate choice trials or training occurred, the paint was placed on top of the existing spots for the 2-spot males. For the 4-spot males, 2 spots were placed between the existing spots. Thus all males had the same amount of paint on their wings, though different numbers of UV reflective spots.

Statistical analyses

All statistical analyses were done in R (ver 4.1.2, “Bird Hippie” within Rstudio). Three chi squared analyses were done on the data collected: gregarious vs isolated mate choice and expected results vs observed for both conditions.

Ethical Statement

Experiments were done at the Biology Greenhouse at the University of Arkansas, and butterflies were reared and maintained according to USDA APHIS permit p526p-20-00417. Caterpillars were provided with corn ad libitum. The butterflies were fed mashed banana and provided with wet cotton ad libitum. All animals were either frozen for future analyses or maintained in the breeding colony until natural death after use in experiments.

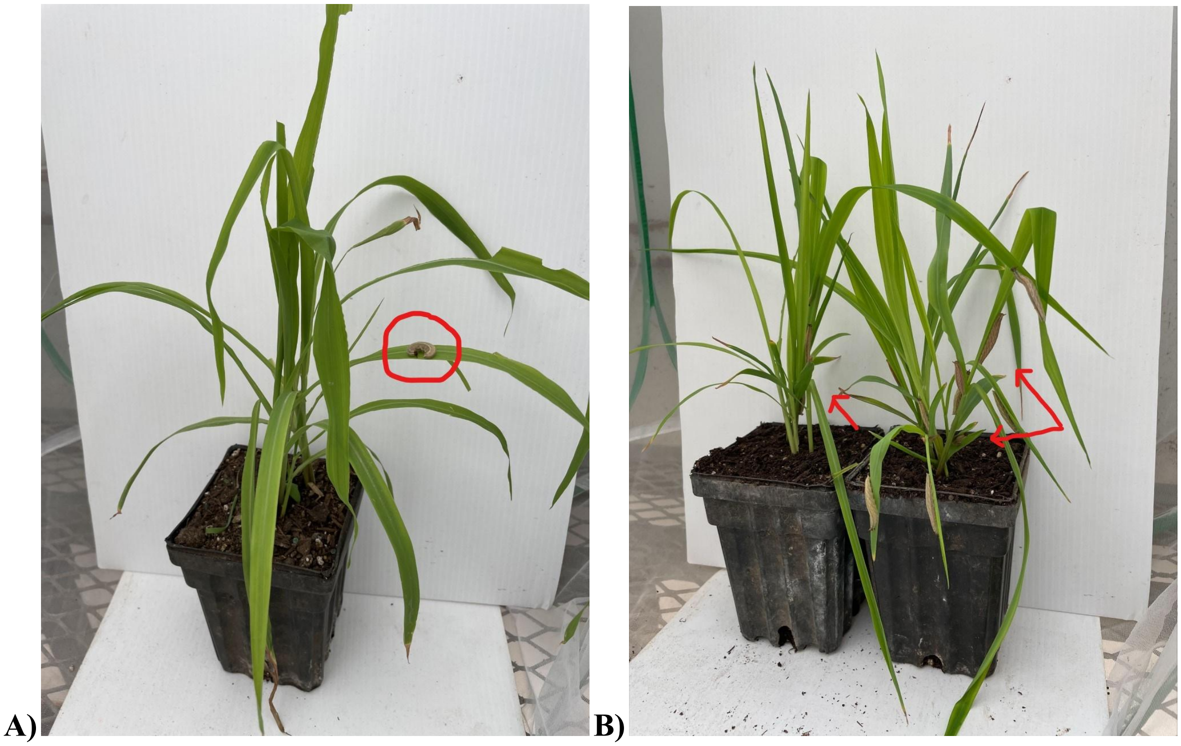

Figure 1: Example of larval rearing conditions. A) isolated rearing condition, B) gregarious rearing condition. Photos taken by Taryn Tibbs.

Results:

My current sample size is 12 data points, six females from the isolated conditions and six from the gregarious conditions. I did not find an effect on larval social experience on mate preference learning, though there is a trend towards isolated females mating more with 4 spot males (trainer phenotype) (N=12, Fisher's exact test χ^2 = 1.7778 and p=0.1818, Figure 2). I did not find statistical significance between either condition and their expected frequency for random mating (Isolation: Fisher's exact test χ^2= 1.778 and p=0.1818, Figure 3) (Gregarious: Fisher's exact test χ^2=0, p=1.0, Figure 4). I did not find statistical significance between either condition and their expected frequency for innate mate preference (Isolation: Fisher's exact test χ^2=3.375 and p=0.0606, Figure 5) (Gregarious: Fisher's exact test χ^2=0, p=1.0, Figure 6).

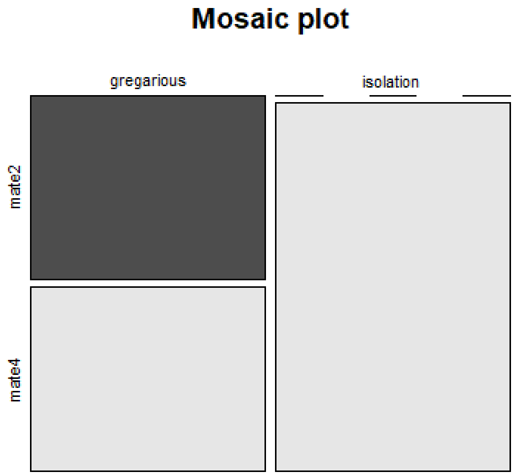

FIGURE 2: Mosaic plot of isolated vs gregarious conditions. Fisher's exact test N=12, χ^2= 1.7778, and p=0.1818.

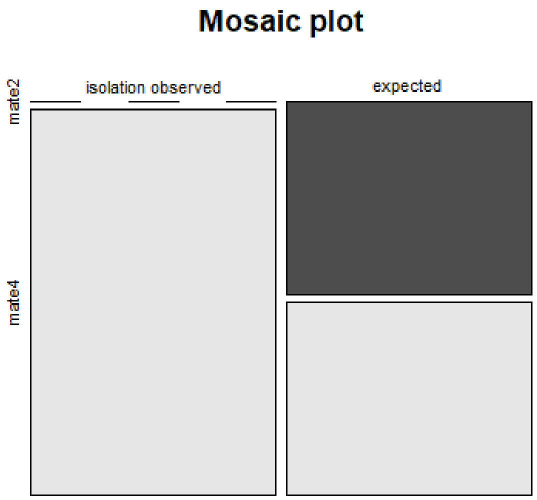

FIGURE 3: Mosaic plot of isolated conditions vs expected frequency. Fisher's exact test N=6, χ^2= 1.7778 and p=0.1818.

FIGURE 4: Mosaic plot of gregarious conditions vs expected frequency. Fisher’s exact test N=6, χ^2=0, and p=1.0.

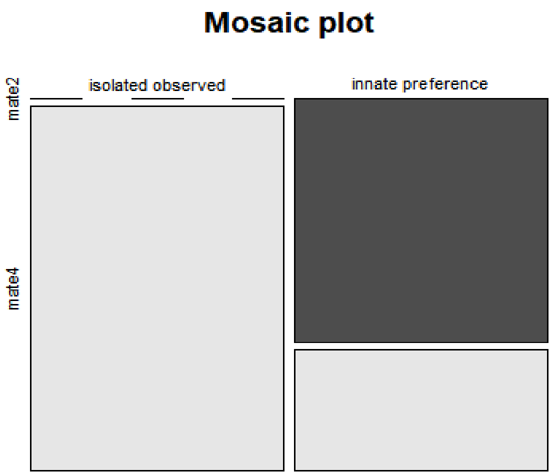

FIGURE 5: Mosaic plot of isolated conditions vs innate preference. Fisher's exact test N=6, χ^2=3.375, and p=0.0606

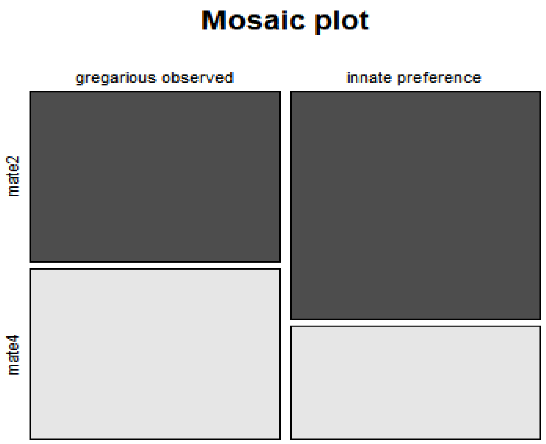

FIGURE 6: Mosaic plot of gregarious conditions vs innate preference. Fisher's exact test N=6, χ^2=0, and p=1.

Discussion:

Isolation during larval rearing probably did not inhibit female *B. anynana* ability to learn mate preference. There was no effect of gregariousness on the relative strength of learned mate preference. There is a trend of isolated females mating more with 4 spot males. Preliminary data shows that isolated females develop faster and have proportionally larger optic lobes but proportionally smaller central brains than females raised in gregarious conditions (Allen, 2019). These findings suggest that the complexity of the larval social environment is possibly a factor in the developmental plasticity of mate choice learning, which supports our hypothesis predicting that isolation would have an effect on the strength of learned mate preference. However, the results of my study may be due to a small sample size, and a larger sample may result in isolated individuals learning at a similar rate in comparison to individuals from gregarious conditions.

Studies examining learning in animals reared in isolation have produced conflicting results. For example, rats that lack social interaction during development had reduced social learning skills in comparison to gregarious individuals (Lévy *et al*., 2003). However, another study found that rats reared in isolation show an improvement in place and reversal learning (Wongwitdecha & Marsden, 1996). These effects of isolation show that some forms of learning do not depend on social experience. A similar study to my own showed that female *B. anynana* do not learn to detect and choose against kin in mate choice based on larval social experience (Robertson, 2018). Moreover, its known that females do not need experience with males for sex recognition or to assess male quality via visual cues (Westerman *et al*., 2012). Similarly, female paper wasps *Polistes fuscatus* who lack experience with males use male ornaments to discriminate between high and low quality males (Tibbetts, 2019). Although, experience is important for nestmate recognition in paper wasps; they learn ol factory signatures during a sensitive period after emergence from pupation to discriminate between nestmates and non-nestmates (Singer & Espelie, 1992). Therefore, the role of social experience in accurately learning and or assessing a signal may be dependent on what information the signal conveys. Further research should explore if and how isolation influences learning abilities of different signals.

My results suggest that juvenile isolation did not inhibit the ability to learn mate preference in female *B. anynana*. This may highlight the importance of being able to learn mate preference for this species. Wing pattern preference learning bias can be compared to birdsong learning, where individuals often learn traits of conspecifics because they are more readily available to them in their social environment compared to traits of heterospecifics (Irwin & Prince, 1999). By the same token, the ability to learn mate preference is thought to facilitate interspecific discrimination between sympatric species with similar morphologies, and in turn lead to reproductive isolation (Westerman *et al*., 2012). For example, Magurran and Ramnarine (2003) introduced two allopatric species of guppies and showed they were able to learn to distinguish conspecifics from heterospecifics in a few days. They suggest that their results provide evidence that learned mate preferences are likely to contribute to the process of speciation. Furthermore, the ability to learn mate preference may be important in species that express seasonal polyphenisms because the ability to learn can allow for successful mate recognition or mate selection, rather than having a genetically determined mate preference (Westerman *et al*., 2012). If this is true for *Bicyclus anynana*, it might help explain the prevalence of the ability to learn mate choice, regardless of the social complexity experienced during development.

This study focused on learning in females and how it is affected by juvenile isolation. However, it is known that male and females have differences in behaviors, including the ability to learn mate preferences. In *B. anynana*, males are known to readily learn preferences for mates with reduced ornamentation (number of eye spots), in contrast to females who learned preference for enhanced ornamentation (Westerman *et al*., 2014). Similarly, previous studies have shown that *B. anynana* has distinct behavioral differences between the seasonal forms (Kooi & Brakefield, 1999; Prudic *et al.*, 2011). Behavioral plasticity found in my study should point to further research on mate preference learning between seasonal forms in *B. anynana* as well as in males.

Conclusion:

I found that isolation possibly does not inhibit a female's ability to learn mate preference and gregariousness had no effect on relative strength of learned mate preference. These results suggest that social complexity experienced during development does play a role in the plasticity of mate choice learning in female *B. anynana.* Further experimentation should explore how isolation affects the learning abilities of other signals and the learning abilities of male *B. anynana* and its seasonal forms.

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