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Effect of larval rearing environment on female Bicyclus anynana mate choice

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Effect of larval rearing environment on female *Bicyclus anynana* mate choice

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Abstract

Around eighty percent of animals go through metamorphosis or drastic phase changes at some

point in their life. We know that juvenile interactions can influence adult behavior and mate

choice in species that don't go through metamorphosis, but we know very little about how social

interactions during early life stages of animals who go through metamorphosis affect mate

choice. To fill this knowledge gap, I used the butterfly *Bicyclus anynana* to assess whether

female butterflies raised in complete isolation from the beginning of their lives exhibit the same

mate preference as butterflies raised in normal social conditions. To test this, I used a split family

design, and raised sibling butterflies in social groups or isolation. Once adults, both isolated and

socially raised females were given the choice of mating with two male butterflies with different

manipulated wing patterns, 2 dorsal forewing spots and 4 dorsal forewing spots. I found that

female choice of mate based on the number of spots was not different from random. Female

butterflies raised in social and isolated conditions mated with both 2 and 4 spot males non-

preferentially. These results suggest that social rearing environment during pre-metamorphosis

does not play a role on mate choice later in life for adult female butterflies.

Introduction

Many animals display sexual selection when they decide who they will court and

ultimately reproduce with (Andersson & Iwasa, 1996). Mate choice is the process by which an

individual picks a mate, which is often based on characteristics such as song, body size,

ornamentation, and odor (Andersson & Iwasa, 1996). Mate choice can be seen when female

Common Grackle birds preferentially mate with male birds who have a larger repertoire of songs

(Searcy, 1992). Another example of mate choice is that female *Ameiva plei* lizards preferentially

mate with larger males (Censky, 1997). Mate choice can be observed in almost every species and can stem from different things such as genes or environment.

Mate choice in many species may be either socially learned or innate and due to genetics. In *Heliconious* butterflies, an area of 200 genes was identified that might contain genes which may be involved in visual and mate preference (Rossi et al., 2020). Mate choice can also be socially learned by observing an adult butterfly of the opposite sex (Westerman, Hodgins-Davis, Dinwiddie, & Monteiro, 2012). In *Bicyclus anynana*, female butterflies learn from social interactions which will then guide their decisions when selecting a mate. They can even experience a change of a pre-existing bias due to learning in a social event (Westerman et al., 2012). Social learning can lead to preferences for mate selection and even the development of behavior later in life (Verzijden et al., 2012). It was found that male adult *Heliconious* butterflies' mate choice was influenced by previous social experience with a female butterfly in contrast to that of a naïve male butterfly (Rather, Herzog, Ernst, & Westerman, 2022). Innate preferences are preferences that are genetically determined (Rossi et al., 2020). However, when we talk about innate preferences, we are usually only speaking about the preferences that are not influenced by experiences individuals have as adults. There has been a lot of research done that relates to innate preference (Spence & Smith, 2008)(Plath et al., 2019), but studies often ignore potential effects of juvenile experience in species that undergo metamorphosis. For example, a certain *Heliconious melpomene* butterfly subspecies, H. *m. malleti* has been shown to have a stronger innate mating preference than H. *m. rosina* (Rather et al., 2022). In reality, we don't know if these preferences are really innate, or if they are influenced in some way by larval experiences.

When it comes to species that go through metamorphosis, innate preference is considered to mean preferences that occur with lack of adult experiences. Larval experiences and environment are not taken into account when discussing or researching innate preferences. Around 80% of animals, including butterflies, go through metamorphosis or drastic phase changes at some point in their life (Wilson & Krause, 2012). We know that juvenile interactions can influence adult behavior and mate choice in many species that don't go through metamorphosis (Bastian, Sponberg, Suomi, & Higley, 2003)(Van den Berg, Van Ree, Spruijt, & Kitchen, 2000)(Van Den Berg et al., 1999). Rats that were isolated during their juvenile stage experienced negative effects on their social exploration as well as a decrease in opioid receptors (Van den Berg et al., 2000). Female guppies have been found to change their choice in male mates later during life, based off of the variation of male traits they were exposed to as a juvenile (Macario, Croft, Endler, & Darden, 2017). All of these experiments show the drastic changes that juvenile experiences can make on adult life.

There has also been some research on how different experiences of an organism in pre-metamorphosis can affect the organism in a later life stage (Wong & Kölliker, 2014). For example, *Manduca sexta* larvae that received shock conditioning paired with a certain odor avoid this odor later as adults (Blackiston, Casey, & Weiss, 2008). *Lobesia botrana* caterpillars who are fed a certain type of grape show preference as adults for ovipositing on the plant of the grape they were exposed to as a caterpillar (Moreau, Rahme, Benrey, & Thiery, 2008). These two studies suggest that larval experience can influence future adult preferences, however it remains unclear if larval experience can influence adult mating decisions.

Organisms raised in isolation rather than raised socially can display different characteristics as well. In honeybees, it was found that socially isolated larvae reduced the bees' resilience to infection later in adulthood. This showed that social and nutritional deficits could affect honeybees during later stages of life (Walton, Toth, & Dolezal, 2021). It has been found that isolated females have larger optic lobes, smaller central brain size, and develop faster than those raised socially (Allen, 2019). It has also been suggested that social interaction could affect the amount of energy allotted to brain development (Allen, 2019). It is possible that the brains of butterflies raised in isolation form differently than those raised socially, especially since preliminary data suggests that these butterflies' brains' have a different morphology (Allen, 2019). Since butterflies raised in isolation have larger optic lobes, they might be more attracted to the males with 4 dorsal forewing spots because they reflect more UV light and are flashier. They may base more of their mate preference on these visual cues than butterflies that do not have enlarged optic lobes, and this could possibly lead to a difference in innate preference. In humans, social interaction is needed for certain social and interpersonal skills to develop (Walton et al., 2021)(Meltzoff & Kulh, 2016; Takahashi et al., 2014), something similar to this may occur during a caterpillars larval stage that may affect their choices later in life. So, while butterflies are developing, if they don't have social involvement, their brain may develop differently or have genes triggered/not triggered at different times. There has been work done on the brains of bees' that show how complex their brain is, especially in the processing and stimulus of visual cues (Paulk, Dacks, Phillips-Portillo, Fellous, & Gronenberg, 2009). There are so many different parts of the brain, such as the medulla, lobula, and central brain, that are sensitive to different visual cues and are maintained by a delicate balance of pathways and neurons (Paulk et al., 2009). Since bee and butterfly brains are broadly similarly structured (Montgomery, Merrill, &

Ott, 2016), this would lead me to hypothesize that if a butterfly is raised in isolation, it may process visual cues differently and exhibit different mate preferences from females reared in social environments.

We know very little about how the early stages of animals who go through metamorphosis affect mate choice. In the butterfly *Bicyclus anynana*, most of an individual's life, 26-27 days, is spent in the caterpillar stage (Oostra et al., 2011), compared to the 3-7 days spent as a pupa, (Kooi & Brakefield, 1999) and the 7-21 days as adults (Westerman & Monteiro, 2016). Both males and females in the experimental species I used express mate choice for a different number of traits, but one of these being dorsal forewing spot number. Socially reared female *B. anynana* have been known to show a bias against increased number of spots on males (Westerman et al., 2012). They also select mates based on other male traits such as dorsal eyespot size and brightness (K. A. Robertson & Monteiro, 2005). Previous work has shown that social rearing environment in the caterpillar stage influences brain development and morphology in adult female butterflies (Allen, 2019). Similar chemosensory and visual genes are present in Lepidoptera caterpillar and butterfly heads, (Ernst & Westerman, 2021) so caterpillars may have the capacity to detect chemical and visual social cues that are relevant to adult decisions. It is possible that caterpillar social environment also influences adult behavior, especially since we know that butterflies reared in isolation have different brain morphology than those reared socially.

To test this hypothesis, we reared female *B. anynana* in complete isolation and females socially in sibling groups of 15. We then tested to see if these butterflies exhibited similar choices when choosing between males with 2 or 4 dorsal forewing spots.

Methods

2.A Study organism:

In this experiment we used *Bicyclus anynana*, a butterfly species native to Africa. This species

lives in a subtropical environment that experiences extreme wet and dry seasons. Due to this, *B.*

anynana has two different seasonal forms: wet season, and dry season (Brakefield, 1997). They

express phenotypic plasticity and differ not only in morphology, but in behavior as well. Dry

season female butterflies will wait until the end of the dry season to lay their eggs, while wet

season females will lay their eggs right after mating (Prudic, Jeon, Cao, & Monteiro, 2011). Wet

season forms of female *Bicyclus anynana* have very bright ventral spots, while dry season

females have very dull ventral spots (Prudic, Stoehr, Wasik, & Monteiro, 2014). There are more

visible dorsal spots in female wet season *Bicyclus anynana* than in the dry season. In the

different seasons, there is a difference in sexual selection as well. Both sexes use visual cues, and

in the different seasons there is a difference in which sex is the choosey sex. Males are choosier

in the dry season form, while females are the more choosey sex in the wet season form (Prudic et

al., 2011). Wet season butterflies develop faster and reproduce more than dry season butterflies

(Brakefield, 1997; Dongmo, Bonebrake, Hanna, & Fomena, 2018). For this experiment we chose

to use wet season *B. anynana* because it is in this season where the female is the more choosey

sex (Prudic et al., 2011).

The population we used was reared in our greenhouse at the University of Arkansas,

Fayetteville, AR, USA in mesh cages (100cm x 160cm). Our greenhouse has a constant

temperature of 27°C and a relative humidity of 60-80%. There was a light to dark period of

13:11. Our population was established from a shipment of about 1000 *B. anynana* eggs in March

2017 from Singapore, which was serially transferred from the original population in Leiden via

Buffalo, NY, USA, New Haven, CT, USA, and Singapore. In 1988, the very first population of

B. anynana was established in Leiden University, Netherlands from 80 pregnant females

collected in Malawi, East Africa (Brakefield et al., 2001). High levels of heterozygosity have

been maintained due to the high number of adults produced in each generation (Brakefield et al.,

2001; Van Oosterhout, Zijlstra, Van Heuven, & Brakefield, 2000).

2.B Social and Isolated Rearing Environment

Using our colony containing hundreds of individuals, we bred and selected virgin males

and female pairs to begin two new families each week. The two pairs were given banana on top

of wet cotton, which served as the food and water source and were then placed into a mesh cage

(39.88cm x 39.88cm x 59.94cm) for breeding. A host plant (corn, *Zea mays*) was placed into the

cage for eggs to be laid upon. After six days, the larvae and eggs were collected and organized

into rearing conditions. Three eggs were placed onto 5 individual plants in respective isolated

sleeves, and 15 eggs were put in a separate sleeve to be reared in a social environment, as

described in more detail in the two treatments below. The sleeve with the isolated caterpillars did

not come into any contact with other butterflies or caterpillars. These sleeves are made of mesh

which allow in necessary elements such as light and air but keep out other organisms that could

interfere with the experiment. The parent butterflies were frozen for further measurements or

research. Once the first larvae emerge from an egg in an isolation sleeve, the other 2 eggs were

removed from the cage so that the larvae could develop in complete isolation, from larvae to

adult. Extreme care was taken to label each family by name and date so that they wouldn't be

confused with another family. The caterpillars received new corn plants every few days, and

those corn plants were watered as needed. The pupae were sexed using a dissecting microscope,

and the socially reared males were placed in the colony cage while females were kept for behavioral assays. The female pupae were then divided into emergence cages based on their treatment (social or isolated). We continued rearing this way until we had completed 5 tests per treatment. After each female emerged from its pupa, we waited 3 days before conducting the mate choice experiment.

Figure 1: Caterpillar rearing conditions:(A) Isolated (B) Social
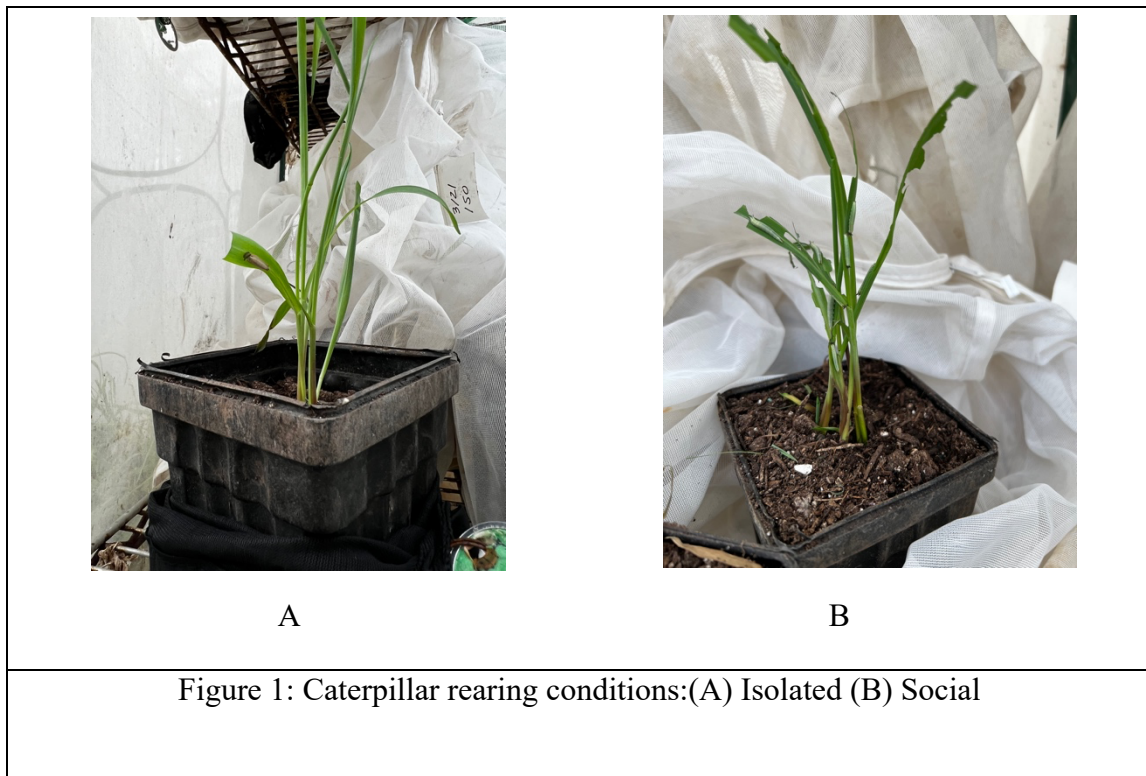
2.C Treatment 1- social female choice

Females used in this treatment were selected from a family group they were reared in of fifteen other caterpillars, as seen in Figure 1-B. As soon as the females emerge from the chrysalis, they were placed in a 39.88cm x 39.88cm x 59.94cm mesh cage and provided food and water. On the second day post emergence, the mate choice assay was conducted. At this time, the female for testing was dusted with orange rodent-tracking fluorescent powder and placed into a separate mesh cage with two 3-day old male butterflies, one painted to have 2 dorsal forewing

UV reflective eyespot pupils and one painted to have 4 dorsal forewing UV reflective eyespot

pupils, as described below and seen in Figure 2. The male butterflies were of similar size and had

no wing or body damage. Although it has been found that females do not prefer a certain size of

male butterflies (Breuker & Brakefield, 2002), we controlled for size anyways.

Figure 2: Male butterflies painted for mate choice assays. 2 spotted male on the left, 4 spotted male on the right.

Male butterfly wing pattern manipulation was performed in a very precise manner. I

painted the male butterflies to have either 2 or 4 dorsal wing spots. Male butterflies were

carefully transferred and pinned onto a wooden board, and the small spots were painted with UV

paint (Fish vision white) (Westerman et al., 2012). The butterflies were left to dry for five

minutes then were carefully taken off the wooden board and put back into their mesh cage. After

24 hours, these two male butterflies were put into the mesh cage with the female butterfly. After

another 24 hours, the male butterflies were checked for copulation by looking for UV powder

transfer on their claspers. If neither had mated after 24 hours, I waited another 24 hours, and if

neither had mated at this point, the trial with the family was stopped. After this, the female and

male butterflies used in this experiment were frozen and labeled with family, date, treatment, and

mate chosen.

2.D Treatment 2- isolated female choice

Females in this experiment were raised in complete isolation, as seen in Figure 1-A. After emergence from the chrysalis, the isolated female was placed into a 39.88cm x 39.88cm x 59.94cm mesh cage and provided food and water. When the female was two days post-emergence, it was dusted with the orange fluorescent tracking powder to indicate copulation. The female was then placed with two male butterflies (same as described in treatment 1). The same mating procedure as above was followed.

2.E Statistical Analysis

All statistical analyses were performed using R and R studio, Version 1.3.1093. We compared choice of females raised socially vs raised in isolation for males with 2 or 4 spots using Fisher's exact test for small sample sizes. To assess whether females exhibit non-random preferences, we used Fisher's exact test with $\alpha= 0.05$ to determine whether the females (both isolated and socially raised) had preferences statistically different from random.
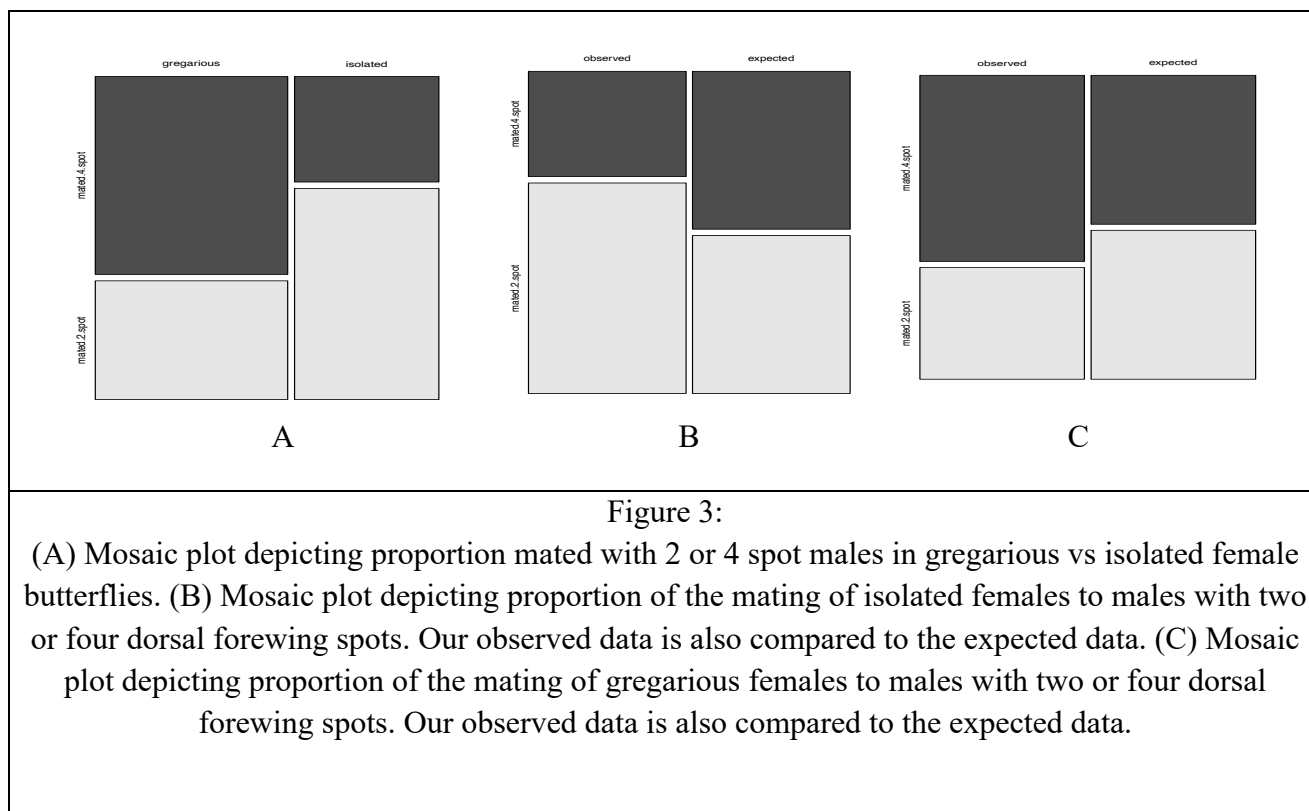
2.G Ethical Note

This study was conducted with the animals treated as humanely as possible. All butterflies were reared in accordance with laboratory conditions specified by U.S Department of Agriculture permit P526P-20-00417. Caterpillars were raised in mesh bags and provided ample food and water. Pupae and adult butterflies lived in cylindrical mesh cages. Both caterpillars, pupae, and adult butterflies were in a walk-in chamber that is maintained at wet season conditions. Butterflies I used were frozen, and all butterflies I did not use were put in colony

cages and let to live natural lives. They were always fed corn as caterpillars and fed bananas *ad libitum* as adults.

Results

We found that social rearing environment does not play a role in mate choice later in life (N = 14, p value = 0.5921, Odds Ratio= 3.043639) (A in Figure 3). In addition, we found that isolated female butterflies mated randomly when choosing between 2 and 4 spot males (4 mated with 2 spot, 2 mated 4 spots, N = 6, p value = 1, Odds Ratio = 0.5302858) (B in Figure 3), and that social butterflies also mated randomly when choosing between 2 and 4 spot males (3 mated with 2 spot, 5 mated with 4 spot, N = 8, p value = 1, Odds Ratio = 1.613716) (C in figure 3). Out of

Figure 3:
(A) Mosaic plot depicting proportion mated with 2 or 4 spot males in gregarious vs isolated female butterflies. (B) Mosaic plot depicting proportion of the mating of isolated females to males with two or four dorsal forewing spots. Our observed data is also compared to the expected data. (C) Mosaic plot depicting proportion of the mating of gregarious females to males with two or four dorsal forewing spots. Our observed data is also compared to the expected data.

my twelve assays, there were 4 which contained butterflies from the same families, but all other assays came from separate families.

Discussion

After performing this experiment, I found that there is no significant difference between any of the groups. Neither gregarious nor isolated female butterflies mate preferentially with males that have a certain number of spots. There is also no significant difference between the observed and expected number of 2 vs 4 spot males mated for gregarious or isolated females.

However, the sample size for this experiment was not as large as we wanted it to be. The COVID pandemic delayed the start of my experiment by a year, and there were other circumstances, such as a new heater, and loss of corn during winter months that caused us to lose some caterpillar families. Due to these unforeseen circumstances, we did not reach the sample size that we originally wanted. We started 22 families and lost 4, and the final sample size we reached was 14 females (8 gregarious and 6 isolated). However, if these results hold true at a larger sample size, this outcome would suggest that, in these conditions, social rearing environment does not later effect mate choice in *B. anynana*. This is contrary to my hypothesis that isolated butterflies would preferentially mate with males with a larger number of dorsal forewing spots due to the isolation and differential brain allocation (Allen, 2019).

There has been a lot of research done to show how social isolation and stress at the juvenile stage can affect morphology or behavior later in life. Rats that are socially isolated as juveniles show a decrease in an important defensive burying tactic as adults (Arakawa, 2007). Rats who are socially isolated as a juvenile are also shown to reduce social motivation when the rats became adults (Van Den Berg et al., 1999). Honeybees who experience nutritional and social stress early in life experience reduced resilience to a common bee virus (Walton et al., 2021). Paper wasps that experience social isolation can't learn or remember other wasps, and they also can't distinguish between wasp faces in images (Tibbetts, Desjardins, Kou, & Wellman, 2019). It

has also been found in animals such as *B. anynana*, that social rearing environment does play a role in neuro-morphological differences, such as the allocation of development energy to visual processing (Allen, 2019). This suggests that rearing environment does have morphological effects on the butterfly *B. anynana*. It is known that social environment, especially isolation in juvenile stages, influences many types of adult behaviors. However, we see no evidence of the social isolation affecting the female butterfly's mate choice in this experiment. We know that isolated female butterflies and social female butterflies have neuro-morphological differences, but these morphological differences do not appear to influence mate choice.

However, there have been some experiments that show that learning is a stronger force than innate preference, and it has been shown that neuro-morphological differences do not necessarily have behavioral effects. Many experiments have been done to show that many species learn preferences for mates and do not possess or use innate preference. It was found that female butterflies that are raised socially mate randomly and do not innately avoid sibling butterflies (D. N. Robertson, Sullivan, & Westerman, 2020). Male Enallagma damselflies raised in isolation have no preference for either morph when mating, but have a learned preference for a certain morph if previously exposed to that type of female (Fincke, Fargevieille, & Schultz, 2007). Male butterflies do not have an innate preference for dorsal hindwing spot number, but males can exhibit learned preferences (Westerman, Chirathivat, Schyling, & Monteiro, 2014). These suggest that although rearing environment can have neuro-morphological effects, it might not necessarily have effects on mate choice later in life, as well as how powerful learning experiences can be.

It is important to realize that these butterfly experiments did not test all of the factors that might play a role in a caterpillars development. In the wild, caterpillars are socially exposed to

other caterpillars, adult butterflies, and other species as they are growing and maturing. All of these variables could play an important role in their brain development as well as modifying their innate behaviors. While I was able to manipulate caterpillar rearing environment, I was not able to expose them to adult butterflies. The social groups were raised with 15 sibling caterpillars, but they were not exposed to any adult butterflies. Being exposed to adults and other environment factors may prove to be important for development. For example, naïve jays are found to receive a long term fitness benefit from being able to witness certain behaviors in adults (Griesser & Suzuki, 2017). Being reared with adults could be an important part of maturation in the wild. It would be interesting to see if exposing caterpillars to adults influences their mating choices as adults.

Conclusion

This experiment showed that when caterpillars are raised either socially or in isolation, adult female butterflies mate randomly and do not have a preference for the amount of dorsal forewing spots on male butterflies. I was expecting the social and isolated groups mate choices to be different; however, this was not the case. To build off of this experiment I would like to continue these mate assays so that I could get a bigger sample size. Also, in the future we should raise the social groups of caterpillars around butterflies, since we don't know if this has any effect on the caterpillars behavioral development.

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