CHOOSINESS AS A COMPONENT OF LIFE HISTORY STRATEGIES IN CABBAGE WHITE BUTTERFLIES

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Sarah Jaumann

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Dr. Emilie Snell-Rood

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ABSTRACT

Many animals are choosy when selecting resources such as mates, food, or sites to lay eggs. For animals that lay eggs and do not subsequently care for their young, choosing the best sites for their eggs can greatly increase the survival and health of their offspring. Given these benefits, it is surprising that there is variation in choosiness; not all animals are choosy when laying eggs. Behaviors can be costly if they require energy and exhibit trade-offs with other traits that also require energy. I applied this idea to choosiness during oviposition, testing the hypothesis that animals are not choosy when being choosy is costly. In cabbage white butterflies, I demonstrated variation in choosiness and a trade-off between choosiness and fecundity, suggesting that being choosy is costly. If energetic costs determine degree of choosiness, then manipulating energy from food should lead to variation in choosiness. I manipulated food availability directly by varying nutrition and indirectly by varying butterfly density and thus potential competition for food. Density did not affect choosiness or other traits, but nutrition did. Poor adult nutrition led to lower levels of choosiness and lower fecundity but no changes in other traits. Thus, poor nutrition may decrease investment in multiple traits, including choosiness, rather than causing adaptive shifts in life history with increased investment in some traits. My results suggest that choosiness is energetically costly, but only direct cues about energy availability affect choosiness. These findings have implications for the health of butterflies and other pollinators.

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INTRODUCTION

Choosiness is a behavioral trait that is beneficial for animals in many contexts. Choosiness when selecting a mate can secure direct or genetic benefits, increasing survival of the individual and its offspring (Albo et al., 2013; Bussiere et al., 2005; Houle and Kondrashov, 2002; Kokko et al., 2003). Selectively choosing high-quality food items when food is plentiful can increase energy intake and reduce total foraging time (Kamil et al., 2012). In the context of habitat choice, being choosy can allow animals to reside in areas close to essential resources and away from predators and competitors (Pruitt et al., 2011; Quilodran et al., 2014). Many birds choose nest sites that are protected from competitors and predators (Barros et al., 2016; Buehler et al., 2017; Buxton and Sperry, 2017; Kondo et al., 2017), increasing the chances that their offspring survive. Similarly, insects deciding where to lay eggs secure fitness gains if they choose sites that support offspring growth, are high in nutrients, and are located away from predators or competitors (Chen et al., 2004; Sweeney and Quiring, 1998). In general, being choosy can have large pay-offs for animals in a wide range of contexts.

Given the many potential benefits of being choosy, it is surprising that there is a large amount of variation in choosiness. Not all animals are choosy in every situation, and some are choosier in some situations than others. Females that are nutritionally stressed are often less choosy (Dakin and Montgomerie, 2014; Hebets et al., 2008; Hunt et al., 2005) and occasionally more choosy (Fisher and Rosenthal, 2006) when selecting a mate. Foragers close to satiation may accept food types that they would otherwise reject

(Richards, 1983). Hungry animals can be less selective about which habitats they choose (Pruitt et al., 2011) and may be more likely to choose areas that are less protected from predators (Rowe et al., 1996). A common theme among these examples is that there seem to be changes in the costs of being choosy, leading to higher or lower levels of choosiness. Such costs could arise from the demands of sensory processing (Laughlin et al., 1998; Niven and Laughlin, 2008), cognition (Jaumann et al., 2013; Snell-Rood et al., 2013), or increased locomotion (Byers et al., 2005; Johnston, 1991) required for making selective decisions. Females that are nutritionally stressed are less choosy about who they mate with, indicating that there are energetic requirements of choosiness arising from those costs that are not met when the individual is food-deprived. This phenomenon is commonly referred to as "state dependence" or "condition dependence", as the degree of choosiness depends on the internal physical state, or condition, of the animal.

One consequence of these types of costs are life history trade-offs among fitness traits such as choosiness or fecundity. Trade-offs rely on the idea that energy is allocated across multiple body functions, but energetic resources are limited, resulting in life history trade-offs among body functions and fitness traits. When animals are nutritionally stressed and energy is even more limiting, trade-offs may be even more pronounced because the costs of investing in one trait (in terms of less investment in other traits) are even greater. Since choosiness is beneficial but may also be energetically costly, and because it could interact with other life history traits such as fecundity through, for example, predation or food availability, it may play into an animal's life history strategy. Others have proposed the idea that choosiness may be part of an animal's life history strategy (Hunt et al., 2005; Resetarits, 1996). If choosiness is part of a life history strategy, then variation in choosiness among individuals could arise if different individuals adopt different life history strategies and patterns of resource allocation as a result of environmental conditions.

Several lines of evidence suggest that choosiness may factor into life history evolution. Trade-offs between other types of cognitive processes, such as learning, and traditional life history traits like fecundity or egg size have been documented (Snell-Rood et al., 2011, 2013). In addition, these trade-offs have been traced to the costs associated with metabolically costly brains (Snell-Rood et al., 2009), which would likely apply to choosy decision-making as well as other forms of cognition. Although these ideas have been applied to mate choice (Hunt et al., 2005), they are lacking in other contexts where choosiness might be beneficial, so it unclear whether choosiness generally interacts with other life history traits, or only in the context of mate choice. Resetarits (1996) suggested that oviposition site selection in insects can be considered a life history trait, but few studies have followed up on this idea. Examining choosiness during oviposition in the context of life history would be informative given that oviposition site selection can be considered an indirect form of parental investment in insects and other taxa. In these insects, it functions similarly to parental care, which has long been considered a life history trait (Klug and Bonsall, 2010).

In this dissertation, I test the overarching hypothesis that variation in choosiness during oviposition can be explained in part by costs that are part of an animal’s life history. To test this hypothesis, I must first demonstrate that variation in choosiness during oviposition exists, and that it exhibits trade-offs with other life history traits. I address these predictions in Chapter 1 of my dissertation. Next, if choosiness is costly and potentially part of an animal's life history strategy, then I would expect variation in life history strategy to lead to variation in choosiness. There are various ways to induce variation in life history and energy allocation (Zera and Harshman, 2001), but I chose to manipulate the environment in order to induce shifts in life history due to ease of manipulation.

Food acquisition is an environmental factor known to affect life history strategy (Van Noordwijk and de Jong, 1986), so I manipulated food acquisition in larvae and adult butterflies to induce shifts in life history. In insects, food acquisition is directly related to resource nutrition (Boggs, 1988; Scriber and Slansky, 1981) and indirectly related to intraspecific competition resulting from high conspecific densities (Gibbs et al., 2004; Kivela and Valimaki, 2008). For competition, density *per se* may be the cue that triggers changes in life history, even if the underlying explanation is limited food availability. For example, mechanical stimuli from crowded conspecific conditions induce the morphological changes that result in the development of locusts or grasshoppers (Rogers et al., 2003), which are the same species but have very different life histories. Therefore, I manipulated nutrition and conspecific density separately in two different experiments, which are presented in Chapters 2 (density) and 3 (nutrition). In each experiment, I measured choosiness using a behavioral assay. In addition, I also measured more traditional fitness traits such as fecundity and egg size. My goal was to document shifts in life history traits, including choosiness, and trade-offs between choosiness and other traits, which would indicate that choosiness during oviposition interacts with other traits within a life-history framework.

CHAPTER 1: TRADE-OFFS BETWEEN FECUNDITY AND CHOOSINESS IN OVIPOSITING BUTTERFLIES[1]

ABSTRACT

Being choosy can allow animals to find and identify the best resources or safest locations to rear offspring. Despite these benefits, individuals vary in the degree to which they are choosy. One explanation is that choosiness represents a costly form of offspring investment and is part of a suite of life history trade-offs. We examined trade-offs between choosiness and fecundity in the cabbage white butterfly, *Pieris rapae*. To test the prediction that choosiness is negatively correlated with fecundity, we presented female cabbage whites with an array of host plant leaves that varied in nutrient content and conspecific density. Butterflies preferentially laid eggs on leaves with higher nutrient content and fewer conspecific models. In addition, butterflies that were choosier with regard to plant nutrition also tended to be choosy in terms of conspecific density. Choosy females were less fecund, with fecundity measured as the sum of eggs laid over three days and remaining mature eggs in their abdomen. These results are consistent with the idea that life history trade-offs include investment in costly behavioural traits that require time and energy, such as choosiness or cognition.

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[1] Jaumann, S., & Snell-Rood, E. C. (2017). Trade-offs between fecundity and choosiness in ovipositing butterflies. *Animal Behaviour, 123*, 433-440.

INTRODUCTION

Animals choose high-quality resources by attending to a range of cues, such as resource size (Ryan & Keddy-Hector, 1992), proximity to cover (Lima & Dill, 1990), or nutritional content (Bukovinszky et al., 2013; Schwarz, Durisko, & Dukas, 2014; Taylor, Schalk, & Jeanne, 2010; Yang, Walther, & Weng, 2015; Zweifel-Schielly, Leuenberger, Kreuzer, & Suter, 2012). Animals can also use social cues to avoid areas with high competition (Doligez, Danchin, Clobert, & Gustafsson, 1999; Reiskind & Wilson, 2004). Given that choosing the best resources can increase fitness of an individual or its offspring, it is unclear why all individuals aren't highly choosy. Animals are often choosy with respect to resources (e.g. Sims & Quayle, 1998) and mates (Bateson, 1983). On the other hand, some animals are not choosy in these same contexts (e.g. Feinsinger, Beach, Linhart, Busby, & Murray, 1987; Jennions & Petrie, 1997). There is even considerable individual variation within a species in the same context (Doak, Kareiva, & Kingsolver, 2006). One explanation for this variation in choosiness is that being choosy is costly in terms of energy or time. This potential cost could be part of a suite of life history trade-offs that may ultimately restrict choosiness in some individuals and promote variation in choosiness across individuals (Janz, 2002; Wiklund & Persson, 1983). The existence and robustness of such trade-offs could have implications for the range of traits generally considered to be important in life history evolution.

Life history theory proposes that individuals or species might adopt a variety of different reproductive strategies that reflect the finite energy or time available for reproduction and maintenance. For example, utilizing energy for one aspect of reproduction leaves less available energy for other functions, resulting in trade-offs (Roff, 1992). In the context of choosiness, energy is expended by the cognitive and sensory machinery required to process and store information about available choices (DeWitt, Sih, & Wilson, 1998; Isler & van Schaik, 2009; Laughlin, van Steveninck, & Anderson, 1998; Niven & Laughlin, 2008), and by the locomotor system as it is engaged while the animal is collecting that information (Byers, Wiseman, Jones, & Roffe, 2005; Johnston, 1991; Tucker, 1970). Choosiness may therefore exhibit trade-offs with components of fitness such as offspring number or survival. If time is more limiting than energy, a similar trade-off might emerge. There may be an opportunity cost associated with identifying and choosing the best options, rather than accepting the first resource an animal encounters (Stephens & Krebs, 1986). Time spent identifying the best resources could instead be spent rearing more offspring or laying more eggs, distributing them across a wider variety of sites. This idea recalls the speed-accuracy trade-off in the context of foraging (Chittka, Skorupski, & Raine, 2009). Individuals that favour accuracy may be able to find the best resources, at a cost of time (Chittka, Dyer, Bock, & Dornhaus, 2003) and potentially the total number of resources they can visit and offspring they produce. Conversely, individuals that favour speed might save time by being less accurate, thus maximizing the total number of resources they exploit and offspring they produce, even if some of those resources are unsuitable (Burns, 2005). Regardless of whether energy or time is more limiting, choosiness during nest site selection or egg-laying could be considered a form of costly reproductive investment that might exhibit trade-offs, especially in animals that have non-overlapping generations such as insects. Parasitoids, for example, seem to be egg-limited with regard to fecundity. Ovipositing females with small egg loads are more selective because the cost of being choosy decreases with decreasing egg load (Driessen & Hemerik, 1992; Heimpel & Rosenheim, 1998; Heimpel, Rosenheim, & Mangel, 1996; Rosenheim, Heimpel, & Mangel, 2000). Despite potential costs, choosiness during oviposition can enhance offspring survival in some insects, especially those that require specific resources for survival during the larval stage (Doak et al., 2006; Gripenberg, Mayhew, Parnell, & Roslin, 2010). Thus, choosiness should represent an important component of a life history strategy.

Here, we used the cabbage white butterfly *Pieris rapae* to test the hypothesis that choosiness exhibits trade-offs with fecundity. Life history trade-offs between egg size and egg number have already been established in butterflies and other insects (Fischer & Fiedler, 2001b; Fox & Czesak, 2000; Garcia-Barros, 2000; Seko, Miyatake, Fujioka, & Nakasuji, 2006). Trade-offs between adult choosiness and fecundity may stem from resources allocated either prior to adulthood (such as the majority of their protein), or those allocated during adulthood (Karlsson & Van Dyck, 2009). For instance, adult-acquired carbohydrates contribute to egg production and may exhibit trade-offs with traits involved in choosiness, such as brain metabolism or flight. In this study, we offered females different oviposition sites of varying quality. Although choosiness with regard to mate choice has also been studied in butterflies (Kemp, 2007), it is difficult to create differences in the quality of different males, and it is equally difficult to quantify male quality. Therefore, we focus solely on choosiness during oviposition in this study. As larvae, cabbage whites feed on host plants in the family Brassicaceae. The nutrient content of a host and presence of conspecifics have particularly high impacts on larval survival. Thus, we focus on these two host-plant characteristics. With regard to plant nutrition, adult females differentiate between highly fertilized and poorly fertilized plants (Chen, Lin, Wang, Yeh, & Hwang, 2004), potentially using plant colour or transpiration rate as cues (Myers, 1985). They also use social cues to avoid laying eggs near conspecifics, presumably to avoid competition experienced by offspring (Sato, Yano, Takabayashi, & Ohsaki, 1999), which can be extremely costly (Gibbs, Lace, Jones, & Moore, 2004; Kivela & Valimaki, 2008). Individual lab-reared female butterflies in this study were presented with an oviposition assay containing host plant leaves that varied in nutritional status and the presence of conspecifics. We predicted that if there is a trade-off between choosiness and fecundity, females that were choosier with respect to host plant nutritional status and conspecific presence would have lower fecundity.

METHODS

The focal butterflies in our study were thirty-three female cabbage whites reared in the lab from wild-caught mothers. We assessed choosiness in these focal butterflies using a behavioural assay in which females were allowed to choose where to lay eggs. We investigated trade-offs of choosiness by looking at potential relationships between choosiness in the context of nutrition and density, and between choosiness in each context and estimated fecundity.

Butterfly Collection and Husbandry

Focal female cabbage whites used in the assay were reared in the lab from six mothers collected in gardens on the University of Minnesota St. Paul campus. We kept track of the mother of each focal butterfly to determine if there was any signal of genetic effects on choosiness. Mothers were individually placed in 61x61x61 cm Bug-Dorm cages with a damp washcloth and cup of water covered with tulle to maintain humidity, a small sponge soaked with 10% honey solution to provide a source of carbohydrates (changed daily), and a leaf of organic cabbage to promote oviposition. Once eggs were laid on the organic cabbage leaves, they were transferred to an incubator and larvae were reared in the lab on artificial diet modified from established methods (Troetschler, Malone, Bucago, & Johnston, 1985; see Snell-Rood, Espeset, Boser, White, & Smykalski (2014) and Supplementary Material in the Appendix). Emerged adult focal butterflies were individually marked with a pen and relocated to 61x61x61 clear vinyl and mesh cages in the greenhouse with the same amenities given to their wild mothers. The focal females also had access to males, and 4-12 butterflies with a sex ratio of approximately 1:1 were maintained in each mating cage. Females remained in the mating cages for three days, a sufficient period of time for mating to occur so that females would be motivated to lay eggs in the behavioural assay.

Host Plant Preparation

Host plants for the behavioural oviposition assay were grown under natural light in greenhouse facilities at the University of Minnesota, with an extended photoperiod provided by overhead lights after September, when day length began to decrease. As detailed below, we manipulated host plants to present focal butterflies with different options with regard to nutritional status and conspecific density.

Fertilizer was used to create high and low nutrition host-plant options because cabbage whites can sense differences in fertilization levels (Myers, 1985) and prefer relatively more fertilized plants (Chen et al. 2004). To produce a difference in fertilization level, cabbages (*Brassica oleraceae* var. Earliana) were grown from seed in Sunshine® Professional Growing Mix (Mix #15/LC15) and randomly assigned to high or low-fertilizer treatments. Five grams of Osmocote Classic 14-14-14 N:P:K slow release fertilizer was applied twice to each high-fertilizer cabbage plant, approximately 3 weeks apart. Two grams of fertilizer was concurrently applied to low-fertilizer plants. Two batches of cabbages were grown in the early and late summer for a consistent supply of mature leaves with no signs of senescence. To verify a difference between the two fertilizer treatments, we quantified nitrogen content from plants in both batches. Three leaves from different plants in each treatment group were pooled and analysed for nitrogen content at the University of Minnesota Research Analytical Lab using the Dumas method (Matejovic, 1995). The nitrogen analysis indicated an average of 1.5605% nitrogen for the high-fertilizer cabbages and 1.3475% nitrogen for the low-fertilizer cabbages across two batches of plants. Thus, the fertilizer treatment resulted in an average difference of 0.213% nitrogen between the subset of high and low-fertilizer cabbages tested in the two batches of plants.

To create host-plants with different levels of conspecific density, dead model female cabbage whites were pinned in oviposition posture onto the cabbage leaves. Dead, pinned conspecifics are commonly used as social cues in insects, including butterflies (Jones, Ryan, & Chittka, 2015; Otis et al., 2006). Butterflies used as models were purchased from Carolina Biological Supply as eggs and reared to adulthood in the lab on artificial diet to ensure homogeneity among models and exclude the possibility that the population tested in the assays would have prior experience with these conspecifics. Model females were frozen immediately upon emergence and remained frozen until they were pinned in oviposition posture. One pin through the thorax secured the model to the pin, and two crossed pins were used to hold down the abdomen in a curled posture mimicking that of an ovipositing female butterfly. To ensure that the proper position was maintained over time, models were allowed to dry for at least three days before use in an assay. At that time they were pinned to leaves by piercing a leaf with the pin and securing the pin to the leaf with tape. We replaced a model in the assay with a fresh model if the head, thorax, or any of the wings fell off.

To prepare each experimental cage for the behavioural assay, six leaves were harvested from six different 2-4 month-old plants to equalize plant variation across butterflies. We visually size- and age-matched leaves within each cage and discarded leaves after three days of use in each run of the assay. New leaves from the same plants were used in subsequent assays. The six cabbage leaves were arranged randomly in each cage, nestled within water-filled floral stem tubes refilled daily to keep the leaves fresh. Each tube was propped up in a small ceramic pot to mimic the position of leaves on a full cabbage plant. To provide different options with regard to nutritional status, half of the leaves were from high-fertilizer plants and the other half were from low-fertilizer plants. For different options in the context of conspecific density, three models were pinned on each of two leaves, one model was pinned on each of two leaves, and the remaining two leaves remained empty. The six leaves therefore represented the six factorial combinations of nutritional status and conspecific density (Figure 1).

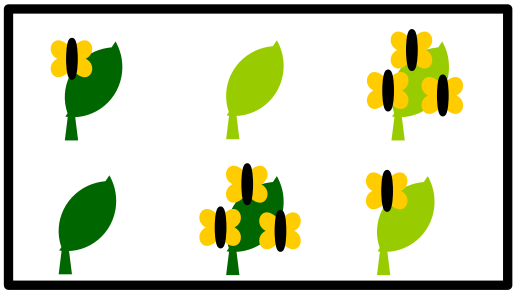

Figure 1. Diagram of behavioural assay. Choosiness in focal females was tested in a behavioural assay where females were exposed to the factorial combinations of nutritional status (low fertilization: light leaves, high fertilization: dark leaves) and conspecific density (number of model butterflies: zero, one, or three) on six host plant leaves.

Behavioural Assay

Mated focal female butterflies were moved from mating cages to experimental cages where we assessed their egg-laying choosiness with a behavioural assay. In addition to the host plant leaves described previously, each experimental cage contained a damp washcloth and cup of water covered with tulle. A sponge soaked with honey solution was provided and refreshed daily. Focal butterflies were individually placed inside these experimental cages and allowed to oviposit. At least five females were simultaneously, but separately, tested during one run of the assay. Every day for three consecutive days, the number of eggs laid on each leaf was recorded at approximately the same time of day. Eggs were discarded after counting. The six leaves in each cage were transferred to another cage each day and arranged in a new random order such that no butterfly ever experienced the same set of six leaves twice. The same sets of leaves collected on the first day were used across the three days of the assay as they did not wilt within that period of time. Additionally, the location of the honey solution-soaked sponge was rotated between the four corners of the cage every day to avoid location bias.

Abdomen Dissections

Once a focal female butterfly completed three days in the assay, she was frozen in a sealed container at - 20°C and later dissected in order to quantify the number of eggs remaining in the abdomen. Abdomens were removed from the body and kept in 1x PBS buffer for the duration of the dissections. Eggs were removed through a ventral slice in each abdomen, and all mature eggs were counted. Mature eggs were those with a fully developed chorion, identified by their full yolk, yellowish colour, and striated shell. Dissections were performed under a Leica M165 C dissecting microscope at 12.5x magnification. The number of eggs dissected out of the abdomen of each individual, or its *egg load*, was added to the number of eggs it laid in the assay as a measure of fecundity. Thus, our measure of fecundity was the total number of eggs laid in the assay plus the egg load, or number of eggs dissected out of the abdomen. We chose this measure of fecundity because *P. rapae* butterflies only produce eggs for about a week as adults

(Scott, 1992) and we sacrificed them at six days old. Thus, whatever eggs they had already laid and developed within their abdomens by that point was a relatively good estimate of lifetime fecundity, had we allowed them to continue living. Twenty-nine out of the original thirty-three focal butterflies we tested were used for fecundity analyses because we were unable to perform dissections for four individuals.

Statistical Analysis

The results of the behavioural assay and dissections were analysed using the lme4 package (Bates, Maechler, Bolker, & Walker, 2014) in Rstudio version 3.0.2 (R Core Team, 2013), with generalized linear mixed effects models (GLMMs) and number of eggs laid per leaf as the response variable. We tested thirty-three female butterflies, each over three days (three repetitions of the assay), and we counted eggs for each of the six leaves for a total of 594 observations. Predictors included conspecific density and nutritional status as fixed effects and day of assay (one, two, or three), individual, and mother as random effects. All models we considered included nutritional status and density as fixed effects and individual as a random effect. The largest model we considered included all of these effects and interactions between density and nutritional status, between density and day, and between nutritional status and day... To select the best models, we used the Akaike Information Criterion (AIC), corrected for small sample size (AICc using the bbmle package in R (Bolker & R Development Core Team, 2014)). Once AICc identified the terms that comprised the best model, we ran GLMMs with poisson distributions and Tukey’s pairwise contrasts (multcomp package in R (Hothorn, Bretz, & Westfall, 2008)) to identify significant differences within and between only the fixed effects.

To better understand the relationship between choosiness in different contexts and the relationship between choosiness and fecundity, an average *nutritional choosiness score* and an average *density choosiness score* were calculated. The nutritional choosiness score was computed by first calculating the proportion of eggs laid on less-fertilized leaves and the proportion of eggs laid on highly-fertilized leaves for each focal butterfly for each day in the assay. We then took the absolute value of the difference between these two proportions. Finally, we averaged theses values for each of the three days together for each butterfly. This average was the final nutritional choosiness score. The density choosiness score was calculated in a similar manner. First we calculated the proportion of eggs laid on leaves with zero models, the proportion of eggs laid on leaves with one model, and the proportion of eggs laid on leaves with three models for each focal butterfly for each day in the assay. We then computed the difference between the proportion of eggs laid on leaves with three models and leaves with one model, the difference between the proportion of eggs laid on leaves with three models and leaves with no models, and the difference between the proportion of eggs laid on leaves with one model and leaves with no models. We averaged together the absolute values of these three differences for one day in the assay for each butterfly. Our final density choosiness score for one focal butterfly was the average of these values across the three days of the assay. In each context, a higher score indicates more discrimination between different leaves and thus more choosiness. We did not have reason to specify causality between variables, so we used a Pearson's correlation to test for a relationship between nutritional choosiness scores and density choosiness scores, and between each type of choosiness score and fecundity. We also wanted to see if the tendency to be choosy was due to differences in egg load, where individuals with a higher egg load may be less choosy, dumping their eggs on whatever plants they encounter (Papaj, 2000). Thus, we determined whether the butterflies who laid fewer eggs during the assay were also the choosier ones by using a Pearson's correlation to test for a relationship between the proportion of eggs laid (eggs laid in assay/total fecundity) and choosiness scores in each context.

RESULTS

Choosiness

The results show that focal butterflies laid more eggs on highly-fertilized leaves with fewer models, indicating choosiness in the context of both nutrition and conspecific density. Model selection using AICc determined that the best model, Model 1, included the fixed effects of nutrition, density, and the interaction between nutrition and density. Model 1 also included the random effects of individual, day, and mother (Table 1). The three next best models identified by the AICc analysis, Models 2, 3, and 4, had dAICc values of 2.1, 2.1, and 4.1, respectively. All three models included all the factors in Model 1. Model 2 included the additional interaction of day and density. Model 3 included the additional interaction of day and nutrition. Model 4 was the largest model we considered, with all of the terms in Model 1 plus interactions between day and nutrition and day and density (Table 1). Models 2, 3, and 4 did not differ markedly from Model 1 (Table 2), and the model with the next lowest dAICc value after Model 4 had a dAICc value of 13.9 (Table 1). Therefore, we focus on Model 1 here, but a list of all models considered in the analysis can be found in the Supplementary Material in the Appendix (Table 3).

Table 1. List of the top five models identified by AICc. All models tested included nutritional and density choosiness as fixed, additive effects. Only the model with the lowest dAICc value was discussed in this study because the next two best models showed results that were essentially the same, and the model with the next highest dAICc value had a dAICc value of 13.9. All of the top models included nutritional and density choosiness and the interaction between those two factors as fixed effects, and individual and day as random effects.

Model	Random effects	Interactions	dAICc
1	day, mother, individual	density x nutrition	0.0
2	day, mother, individual	density x nutrition day x density	2.1
3	day, mother, individual	density x nutrition day x nutrition	2.1
4	day, mother, individual	density x nutrition day x nutrition day x density	4.1
5	day, individual	density x nutrition	13.9

Table 2. Statistical results for fixed effects in Models 1, 2, 3, and 4. All models include nutrition as a fixed effect with levels of high (H) and low (L) fertilizer. All models also include density as a fixed effect with three levels: no conspecifics (0), one conspecific (1), or three conspecifics (3). Model 1 was the best model according to AICc, so contrasts are shown for the interaction between nutrition and density for Model 1 (Figure 4). Models 2, 3, and 4 also had low dAICc values, but estimates and standard errors for the main effects did not differ markedly from Model 1, so the contrasts for the interaction effect are not shown.

Model	Effect/Contrast	Estimate	Standard Error	p-value
1	intercept	2.19971	0.23494	< 0.001
1	nutrition H-L	-0.20603	0.04229	< 0.001
1	density 0-1	-0.19135	0.04212	< 0.001
1	density 0-3	-0.86429	0.05203	< 0.001
1	density 1-3	-0.67293	0.05363	< 0.001
1	nutrition H/density 1 x nutrition H/density 0	-0.19135	0.04212	< 0.001
1	nutrition H/density 3 x nutrition H/density 0	-0.86430	0.05203	< 0.001
1	nutrition L/density 0 x nutrition H/density 0	-0.20604	0.04229	< 0.001
1	nutrition L/density 1 x nutrition H/density 0	-0.65380	0.04843	< 0.001
1	nutrition L/density 3 x nutrition H/density 0	-0.94354	0.05352	< 0.001
1	nutrition H/density 3 x nutrition H/density 1	-0.67295	0.05363	< 0.001
1	nutrition L/density 0 x nutrition H/density 1	-0.01469	0.04425	0.99945
1	nutrition L/density 1 x nutrition H/density 1	-0.46245	0.05015	< 0.001
1	nutrition L/density 3 x nutrition H/density 1	-0.75219	0.05508	< 0.001
1	nutrition L/density 0 x nutrition H/density 3	0.65826	0.05377	< 0.001
1	nutrition L/density 1 x nutrition H/density 3	0.21049	0.05872	0.00448
1	nutrition L/density 3 x	-0.07925	0.06298	0.80399

	nutrition H/density 3			
1	nutrition L/density 1 x nutrition L/density 0	-0.44777	0.05029	< 0.001
1	nutrition L/density 3 x nutrition L/density 0	-0.73751	0.05521	< 0.001
1	nutrition L/density 3 x nutrition L/density 1	-0.28974	0.06004	< 0.001
2	intercept	2.19970	0.23493	< 0.001
2	nutrition H-L	-0.20604	0.04229	< 0.001
2	density 0-1	-0.19135	0.04212	< 0.001
2	density 0-3	-0.86430	0.05203	< 0.001
2	density 1-3	-0.67294	0.05363	< 0.001
3	intercept	2.19970	0.23493	< 0.001
3	nutrition H-L	-0.20604	0.04229	< 0.001
3	density 0-1	-0.19135	0.04212	< 0.001
3	density 0-3	-0.86430	0.05203	< 0.001
3	density 1-3	-0.67294	0.05363	< 0.001
4	intercept	2.19971	0.23494	< 0.001
4	nutrition H-L	-0.20604	0.04229	< 0.001
4	density 0-1	-0.19135	0.04212	< 0.001
4	density 0-3	-0.86430	0.05203	< 0.001
4	density 1-3	-0.67294	0.05363	< 0.001

Focal butterflies laid fewer total eggs over time, but in the best model there were no interactions between day and nutritional or density choosiness. Mother explained the highest percentage of random effects variance, about 14.7%. Nutritional choosiness scores ranged from an average of 0.30 for some mothers to 0.53 for other mothers and density choosiness scores ranged from an average of 0.26 for some mothers to 0.34 for other mothers, with higher scores indicating more choosiness. When considering the fixed effects, focal butterflies laid significantly more eggs on highly fertilized leaves than less fertilized leaves, suggesting that they are choosy when deciding between plants that vary in nutrition, (z = -4.87, p < 0.001; Figure 2).

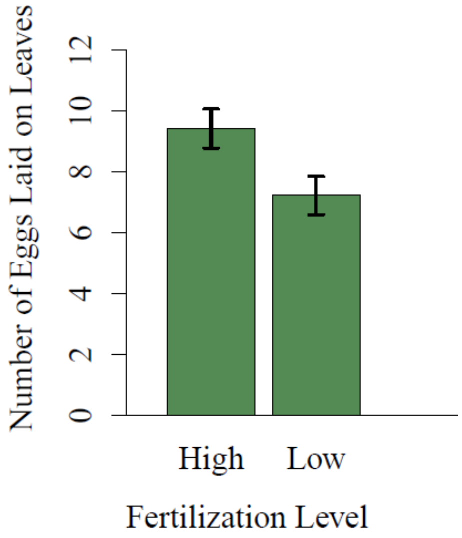

Figure 2. Butterflies prefer fertilized host plants. In the behavioural oviposition assay, focal butterflies laid significantly more eggs on leaves from highly fertilized plants than leaves from poorly fertilized plants ($z = -4.87$, $p < 0.001$). Bars denote ± 1 SE. N = 33 butterflies.

The analysis of fixed effects also revealed that in general, focal butterflies preferred to lay eggs on leaves with fewer models (Figure 3). Indeed, butterflies were apparently sensitive to small differences in the number of models on the leaves in the assay. They laid significantly more eggs on leaves with no models than on leaves with one model ($z = -4.54$, $p < 0.001$), and significantly more eggs on leaves with one model than on leaves with three models ($z = -12.55$, $p < 0.001$). They also laid significantly more eggs on leaves with no models than on leaves with three models ($z = -16.61$, $p < 0.001$). Although the interaction between nutritional choosiness and density choosiness was significant, the pattern of egg-laying on leaves with different densities was similar for both highly fertilized leaves and poorly fertilized leaves, with females generally laying more eggs on highly fertilized leaves (Figure 4). All Tukey pairwise contrasts were significant except for contrasts between highly and poorly fertilized leaves with three models, and between poorly fertilized leaves with no models and highly fertilized leaves with one model (Table 2).

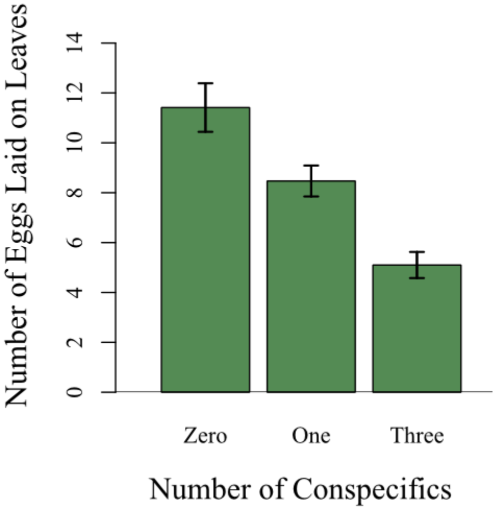

Figure 3. Butterflies avoid conspecifics on host plants. Results from the behavioural oviposition assay showed that focal butterflies laid significantly fewer eggs on leaves with three conspecific models compared to leaves with one model ($z = -12.55$, $p < 0.001$) and significantly fewer eggs on leaves with one model than they laid on leaves with no models ($z = -4.54$, $p < 0.001$). They also laid significantly fewer eggs on leaves with three models than on leaves with no models ($z = -16.61$, $p < 0.001$). Bars denote ± 1 SE. N = 33 butterflies.

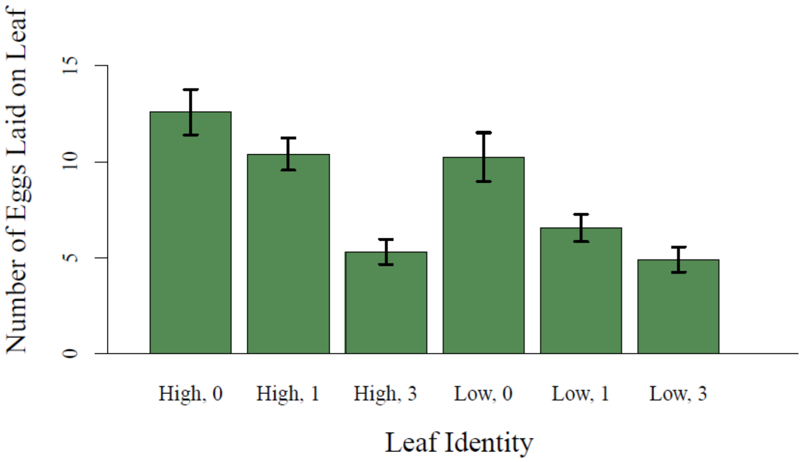

Figure 4. Focal butterflies exhibit density choosiness differently based on nutrition. Female butterflies were given six host leaves on which to lay eggs. Half were highly fertilized (High) and half were poorly fertilized (Low). Leaves had densities of zero (0), one (1), or three (3) conspecific models. Oviposition patterns were similar for highly fertilized leaves and poorly fertilized leaves, although females generally laid more eggs on the highly fertilized leaves compared to their poorly fertilized counterparts. For both sets of leaves, butterflies laid the most eggs on leaves with no models, followed by leaves with one model. They laid the fewest number of eggs on leaves with three models. All Tukey pairwise contrasts were significant except between highly and poorly fertilized leaves with three models, and between poorly fertilized leaves with no models and highly fertilized leaves with one model (Table 2). Bars denote ± 1 SE. N = 33.

Across the thirty-three focal butterflies we tested, there was a significant positive correlation between density and nutritional choosiness scores ($r = 0.365$, $p = 0.037$; Figure 5). Butterflies that differentiated more between the two fertilizer levels also differentiated more between leaves with different numbers of conspecific models. Thus, butterflies that tended to be choosier with regard to nutrition were also choosier with regard to conspecific density and vice versa.

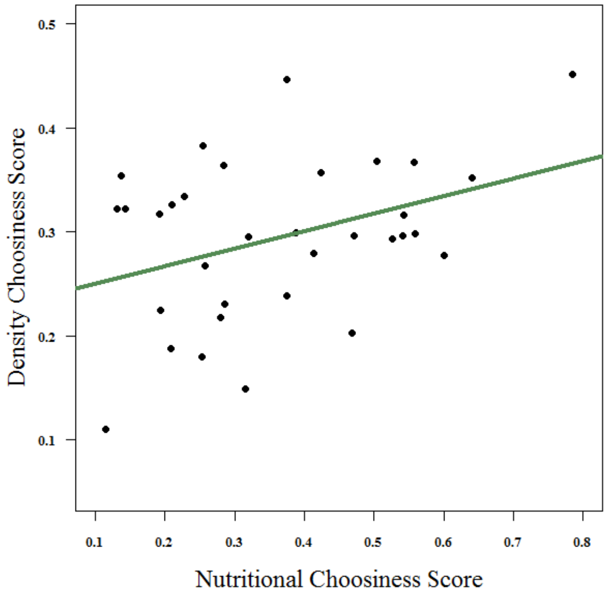

Figure 5. Choosy butterflies tend to be choosy in multiple contexts. There was a significant positive correlation between choosiness with regard to nutrition and choosiness with regard to conspecific density during oviposition ($r = 0.365$, $p = 0.037$). Ovipositing butterflies that were choosier in one context were also choosier in the other context. N = 33.

Fecundity Trade-offs

We were interested in whether focal butterflies that were choosier were less fecund. As predicted, there was a significant negative correlation between fecundity (sum of eggs laid in the assay and eggs dissected out of the abdomen) and density choosiness scores (*r* = -0.495, *p* = 0.00629; Figure 6a), and a marginally significant correlation between fecundity and nutritional choosiness scores in the same direction (*r* = -0.334, *p* = 0.0762; Figure 6b). There was no detectable relationship between the proportion of total eggs laid (eggs laid in assay/total fecundity) and choosiness scores in either context (density: *r* = -0.068, *p* = 0.726; nutrition: *r* = -0.226, *p* = 0.237).

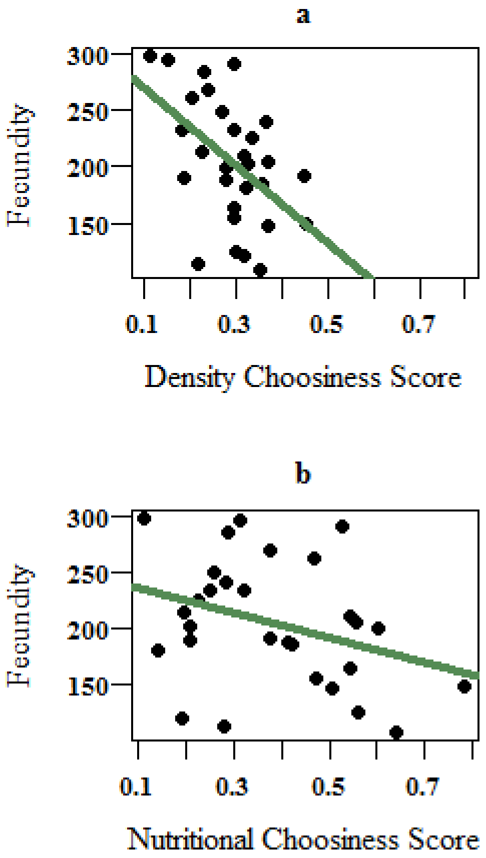

Figure 6. Choosiness is associated with a fecundity trade-off in butterflies. Focal cabbage white butterflies were tested in a behavioural oviposition assay where they were allowed to lay eggs on leaves that differed in nutritional status and conspecific density. There was a significant negative correlation between fecundity and choosiness with regard to density during egg-laying, *r* = -0.495, *p* = 0.00629 (a) and a marginally significant

correlation between fecundity and choosiness with regard to nutrition, $r = -0.334$, $p = 0.0762$ (b). Since we did not have information about the direction of causality for each pair of variables, we performed Pearson's r correlations instead of linear regressions. N = 29 for both correlations.

DISCUSSION

Cabbage white females are choosy with regard to plant nutrition and conspecific density when deciding where to lay eggs. Consistent with previous findings, we found that cabbage whites prefer to oviposit on highly fertilized plants over poorly fertilized plants (Chen et al., 2004, Figure 2), despite differences in plant nitrogen levels as small as 0.213% nitrogen. Females may be choosing plants with a higher nitrogen content because increases in nitrogen, even increases less than 1%, positively affect the performance of caterpillars (Hwang, Liu, & Shen, 2008). Previous studies showed that *P. rapae* females avoid plants with conspecific larval cues (Sato et al., 1999). In our study, cabbage whites also preferred to lay eggs on leaves with no adult conspecific models (Figure 3), consistent with the idea that they generally avoid hosts with conspecific cues. High larval density can have significant detrimental effects on fitness in butterflies, including lower body size and pupal mass, longer development time, and decreased survival for densities as low as five larvae per whole plant (Gibbs et al., 2004; Kivela & Valimaki, 2008). Therefore, it likely benefits females to avoid laying eggs near conspecifics if such behaviour reduces future conspecific competition experienced by their offspring.

Our results suggest that cabbage whites that are choosy in terms of nutrition are also choosy with respect to conspecific density. We found a significant positive correlation between nutritional choosiness and density choosiness (Figure 5). Butterflies that were choosy in one context were also choosy in the other context, indicating that they may be investing in choosiness in general, rather than being choosy in only one context. Although previous work found a trade-off between choosiness in multiple contexts (Ohsaki & Sato, 1994), generalized strategies where individuals pay attention to multiple cues in the same context have also been observed. For example, females in a variety of taxa attend to multiple cues during male courtship (Candolin, 2003).

Butterflies that were choosier in the context of nutrition and conspecific density were also less fecund (Figure 6), which could explain why some butterflies were not choosy in either context. These results suggest that choosiness exhibits life history trade-offs due to energy or time constraints. Life history theory predicts trade-offs between parental investment (including choosiness) and other life history traits such as fecundity or survival (Roff, 1992). Here, butterflies that were choosier had fewer eggs and butterflies that were less choosy had more eggs. Thus, individuals may be adopting different but potentially equally successful reproductive strategies where they either lay many eggs with little regard for the suitability of where they are laid, or they lay fewer eggs but lay them in the best locations.

A trade-off between fecundity and choosiness could stem from limited energy or time. Energetic costs might include the cognitive and sensory demands of using information (DeWitt et al., 1998; Laughlin et al., 1998) or the energetically expensive flight inherent in sampling across long periods of time, either because the butterfly must sample many host plants to get enough information or because hosts are spaced far apart (Dudley, 1991; Heinrich, 1975). Such costs could drive a trade-off between choosiness and fecundity when time or energetic resources are limited and would have major effects on traits directly related to fitness such as egg size and egg number. For instance, previous work in insects demonstrated that learning ability, which is similar to choosiness in its cognitive nature, can exhibit trade-offs with longevity (Burger, Kolss, Pont, & Kawecki, 2008; Jaumann, Scudelari, & Naug, 2013), egg number (Mery & Kawecki, 2004; Snell-Rood, Davidowitz, & Papaj, 2011), or larval competitive ability (Mery & Kawecki, 2003). Given such trade-offs, choosiness may not be the best strategy for all individuals.

Opportunity costs, rather than energetic costs, could drive a trade-off between choosiness and fecundity in butterflies. Ovipositing cabbage whites are often time-limited rather than egg-limited (Root & Kareiva, 1984). Thus, choosiness may incur time costs during oviposition, and a negative relationship between choosiness and fecundity in our study could be due to differences in egg load among females. Individuals that were choosier about where to lay eggs may have been choosy because they had fewer eggs and were therefore less time-limited (Courtney, Chen, & Gardner, 1989; Courtney & Hard, 1990; Jones, 1977b; Odendaal & Rausher, 1990). On the other hand, an alternative strategy could be that butterflies with large egg loads could save time and maximize the number of eggs they lay by ovipositing on anything that resembles a suitable host plant, even if some eggs are erroneously laid on low-quality or incorrect plants (Berenbaum, 1981; Davis & Cipollini, 2014; Straatman, 1962). In this case, the butterfly can afford to be less choosy and it might actually be better for her to spread her eggs across many plants (Root & Kareiva, 1984). However, several lines of evidence suggest that egg load alone was likely not driving the negative correlation between choosiness and fecundity. First, we might expect a change in choosiness across days if egg load was driving the observed oviposition patterns (Javoiš & Tammaru, 2006; Mangel & Roitberg, 1989). Yet no interactions between day and choosiness were present in the best model identified by AICc (Table 1), indicating that butterflies were likely relatively consistent in how choosy they were across the three days they were tested, despite a decline in egg load with age. Second, there was no detectable relationship between choosiness and proportion of eggs laid during the assay (out of the total eggs produced), a measure of general readiness to lay eggs that might be related to egg load (Courtney, Kibota, & Singleton, 1990). A butterfly laying eggs based on egg load may be more or less choosy if it decides to lay a larger proportion of its available eggs, but we did not detect that relationship here. Finally, our measure of fecundity included both eggs dissected out of the abdomen and the total number of eggs laid in the assay. We can therefore conclude that choosiness is negatively correlated with the eggs produced by females, not just the number of eggs they chose to lay. Taken together, these findings suggest that the negative correlations between fecundity and choosiness are likely not solely due to differences in egg load. However, we cannot completely rule out the effects of egg load, given that host quality can affect both egg load and oviposition behaviour (Papaj, 2000).

Here we suggest that trade-offs may underlie variation in choosiness, but it is still unclear what factors might induce, for example, a choosy low-fecundity strategy over another strategy. At the proximate level, genetic variation or maternal effects may influence the development of one strategy or another. Indeed, with regard to where our focal butterflies laid their eggs, almost 15% of the random effects variance stemmed from maternal identity. At the ultimate level, environmental factors such as nutrition (Boggs, 2009; Colasurdo, Gélinas, & Despland, 2009), competition, (Einum & Fleming, 1999), resource quality (Hunt, Brooks, & Jennions, 2005; Rollinson & Hutchings, 2013) or resource variability or unpredictability (Bocedi, Heinonen, & Travis, 2012; Krebs, Kacelnik, & Taylor, 1978; Ratikainen, Sodal, Kazem, & Wright, 2012) may select for different life history strategies. The evolution of choosy behaviour may have feedbacks in the evolution of other life history traits. For example, if individuals make better decisions by being choosy and finding more nutritious hosts, they might be able to afford to lay smaller eggs (Braby, 1994; Swanson et al., 2016). On the other hand, choosy butterflies might lay larger eggs if being choosy results in less time available for egg-laying and there is a trade-off between egg size and number (Fischer & Fiedler, 2001b; Garcia-Barros, 2000; Seko et al., 2006). Alternatively, if an individual invests more in choosing a host plant for its offspring, it might pay to also allocate more energy to egg size or other forms of investment (Snell-Rood, Burger, Hutton, & Moczek, 2016). Future work might focus on measuring egg size to tease apart these possibilities.

This work suggests that choosiness, or the *carefulness* of a decision, factors into an animal's life history strategy. This view fits into a broad view of what constitutes a life history trait (Badyaev & Qvarnstrom, 2002; Snell-Rood, 2012). Choosiness in reproductive decisions is part of the suite of life history traits that exhibit trade-offs with one another (Janz, 2002; Wiklund & Persson, 1983). Not only are physiological traits such as egg size (Fox & Czesak, 2000), brain size (Sol, 2009), or muscle mass (Zera & Denno, 1997) components of life history evolution, but behavioural traits such as learning (Mery & Kawecki, 2004; Snell-Rood et al., 2011) or choosiness during mate choice (Hunt et al., 2005) or oviposition may also show trade-offs with reproductive traits such as egg number.

ACKNOWLEDGEMENTS

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CHAPTER 2: BUTTERFLIES DO NOT ALTER CHOOSINESS IN RESPONSE TO VARIATION IN DENSITY[2]

ABSTRACT

High conspecific densities are associated with increased levels of intraspecific competition and a variety of negative effects on performance. However, changes in life history strategy could compensate for some of these effects. For instance, females in crowded conditions often have fewer total offspring, but they may invest more in each one. Such investment could include the production of larger offspring, more time spent engaging in parental care, or more choosy decisions about where offspring are placed. For animals that have a relatively immobile juvenile stage, the costs of competition can be particularly high. Females may be able to avoid such costs by investing more in individual reproductive decisions, rearing young or laying eggs in locations away from other females. We tested the hypothesis that conspecific density cues during juvenile and adult life stages lead to changes in life history strategy, including both reproduction and oviposition choices. We predicted that high-density cues during the larval and adult stages of female *Pieris rapae* butterflies lead to lower fecundity but higher conspecific avoidance during oviposition, compared to similar low-density cues. We used a 2x2 factorial design to examine the effects of low and high conspecific density during the larval and adult stages of butterflies on avoidance behavior and fecundity. We found that past information about conspecific density did not matter; all butterflies exhibited similar levels of fecundity and a low level of conspecific avoidance during oviposition regardless of their previous experience as larvae and adults. These results suggest that *P. rapae*

females use a fixed, rather than flexible, conspecific avoidance strategy when making oviposition decisions, and past information about conspecific density has no effect on life history and current reproductive investment. We speculate that this may be partially because past conspecific density *per se* is not a reliable cue for predicting current density and levels of competition, and thus it does not affect the development of life history strategies in this system.

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[2] Sarah Jaumann, Emilie C. Snell-Rood; Butterflies Do Not Alter Conspecific Avoidance in Response to Variation in Density. *Integr Comp Biol* 2017 icx034. doi: 10.1093/icb/icx034

INTRODUCTION

High conspecific densities can have negative fitness consequences if the presence of conspecifics interferes with an individual’s ability to survive and produce surviving offspring. For instance, crowding is associated with smaller size, lower fecundity, reduced offspring viability, and higher rates of offspring starvation in various invertebrate and vertebrate taxa (Peters & Barbosa, 1977; Tella et al., 2001; C. G. Wiklund & Andersson, 1994). High densities can have especially detrimental effects on species with juvenile stages that are less mobile or otherwise relatively unable to disperse. The larvae of many holometabolous insects, for example, are slow-moving and confined to the immediate area in which they hatch (Jones, 1977a). High larval densities in these insects can result in negative fitness consequences such as lower body size and pupal mass, longer development time, and decreased survival (Alto, Muturi, & Lampman, 2012; Gibbs, Lace, Jones, & Moore, 2004; Yoshioka et al., 2012). These consequences are often attributed to the effects of intraspecific competition (Gibbs et al., 2004; Kivela & Valimaki, 2008).

Although many species seem to experience negative effects of offspring competition under high-density conditions (Thompson, Marshall, & Monro, 2015), other species cope with high juvenile densities by adjusting their development and life history strategy (Moran, 1992). For example, salamanders and locusts develop into distinct morphs with different feeding, dispersal, and reproductive habits depending on early exposure to varying densities of conspecifics (Rogers et al., 2003). Larval density cues can also alter resource allocation or the speed of development, leading to changes in life history traits and, ultimately, adult morphology and behavior (Kasumovic & Brooks, 2011; Kasumovic, Hall, Try, & Brooks, 2011). For example, some invertebrates allocate more energetic resources to melanization and immune function (Barnes and Siva-Jothy, 2000; Niemela, Vainikka, Lahdenpera, & Kortet, 2012) or to organs used to obtain food (Duarte, Re, Flores, & Queiroga, 2014) when exposed to conspecific cues during development. Similarly, crickets and grasshoppers can speed up development in response to high conspecific density, developing into smaller adults that adopt different but potentially adaptive reproductive strategies compared to conspecifics reared at low densities (Applebaum & Heifetz, 1999).

It is unclear why some organisms seem to lack such adaptive responses to high juvenile densities. It is possible that some of these species do respond in potentially adaptive ways, but that their responses are overlooked when fitness is not assessed in the context of life history theory. For example, high-density conditions often lead to reduced fecundity in insects (Peters & Barbosa, 1977). At first glance, this seems like a cost of living in high-density conditions. However, it may actually be part of an adaptive life history strategy whereby females produce fewer offspring but invest more in each one (Stearns, 1976). Such life history flexibility may depend upon prior exposure to high conspecific density during either development or adulthood, when altered energy acquisition (Joshi & Mueller, 1988) or allocation (Marchand & Boisclair, 1998) could lead to physiological changes. These changes manifest as suites of life history traits that together comprise potentially adaptive strategies under high-density conditions, for example fast growth and dispersal capability (Applebaum & Heifetz, 1999). Low fecundity with high investment in individual offspring may be one such strategy. Investment in individual offspring arguably includes traits like egg size as well as behavioral traits such as choosiness during egg-laying (Resetarits, 1996), so female butterflies that are less fecund could compensate by producing larger eggs (Garcia-Barros, 2000) or by being choosier about where they lay them (Jaumann & Snell-Rood, 2017).

Little attention has been devoted to the idea that the choosiness of egg-laying or rearing decisions could be a behavioral component of a life history strategy adapted to crowded conditions. Some species that experience high densities and associated competition can adaptively avoid conspecifics when deciding where to rear or deposit offspring. For example, females of many species disperse and actively seek out sites with lower conspecific densities (Henaux, Bregnballe, & Lebreton, 2007) or exhibit behavioral avoidance of conspecifics when looking for sites to rear young or lay eggs (Jaumann & Snell-Rood, 2017; Quilodran, Estades, & Vasquez, 2014; Stein & Blaustein, 2015). Some frogs and insects, for example, pay attention to conspecific cues to avoid laying eggs on or in resources that may already have offspring from other females (Prokopy & Roitberg, 2001; Stein & Blaustein, 2015). Such avoidance behavior can directly prevent negative fitness effects of high conspecific density in these females’ offspring (Stein & Blaustein, 2015; Sweeney & Quiring, 1998). Avoidance behavior may be less important for species that occur at lower densities and may actually be attracted to conspecifics (Miller et al., 2013; Raitanen et al., 2014).

Here, we test the hypothesis that high-density cues lead to changes in reproductive investment, in terms of fecundity and choosiness. Shifts in energy acquisition and allocation in response to environmental cues such as conspecific density can occur throughout an organism's life, affecting its life history strategy and investment in reproductive behavior (Muller, Kull, & Muller, 2016; Swanger & Zuk, 2015). Yet there is limited information across vertebrates and invertebrates about the effects of density cues across life stages on reproductive investment, even though the timing of information could affect the range of a potential plastic response (Snell-Rood et al., 2015). Exposure to density cues during the juvenile or adult stage could have differential effects on adult fecundity or conspecific avoidance during egg-laying. To address this hypothesis, we designed an experiment with low and high conspecific density treatments during the larval and adult stages of *Pieris rapae* cabbage white butterflies, an extremely abundant and highly mobile species. Females lay eggs singly, spreading eggs across many plants (Root and Kareiva, 1984), so each egg can be considered a separate oviposition event (Nylin and Janz, 1993). High larval densities can massively reduce performance in butterflies (Gibbs et al., 2004; Kivela & Valimaki, 2008), so conspecific avoidance strategies during oviposition in high-density environments should be strongly favored. Indeed, cabbage white females do generally avoid conspecific cues (Sato, Yano, Takabayashi, & Ohsaki, 1999), similar to other butterfly species regularly subjected to larval competition (Rausher, 1979; Rothschild & Schoonhoven, 1977; Shapiro, 1981; Wiklund & Ahrberg, 1978; Williams & Gilbert, 1981). We test the prediction that females reared in high densities during the larval and adult stages adopt a reproductive investment strategy of producing fewer offspring but behaviorally investing more in each one. We expect that these females will be less fecund but more likely to avoid laying eggs near conspecifics than females reared alone. To test this prediction, we created the factorial combinations of life stage and density level by housing female *P. rapae* larvae and adults alone or in groups of five, resulting in four different treatments. We then assayed choosiness during oviposition and measured fecundity in individual female butterflies.

METHODS

Butterfly Collection and Husbandry

At least fifty wild, gravid female cabbage white butterflies were collected from gardens at the University of Minnesota St. Paul campus over the course of a season, from May to October 2014. *P. rapae* requires approximately a month to mature from egg to adult, so offspring were tested approximately one month after their wild mothers were collected and their eggs were obtained (or one month plus a few extra weeks if females were stored in the refrigerator; see details below). All butterflies used in our study were lab-reared first generation offspring of these wild mothers. Eggs were collected from mothers over the course of the season and reared in the laboratory under identical conditions. Wild mothers were not required for further analysis and were discarded after eggs were collected. To collect eggs, we housed wild mothers in 61x61x61 cm clear vinyl and mesh “Bug-Dorm” cages in a greenhouse, ensuring that there were no more than ten females to a cage. Each cage was supplied with a damp washcloth, cup of water covered with tulle, and non-host plant watered daily to provide humidity. A small sponge soaked with 10% honey solution provided carbohydrates. An organic cabbage leaf was placed in each cage to promote oviposition. The washcloth, cup of water, and sponge were refreshed daily. Cabbage leaves were also replaced daily. If eggs were observed on a leaf after 24 hours in a cage, it was stored in a climate chamber set at a 14-hour photoperiod, 23°C, and 70% relative humidity until the eggs hatched. To control for larval nutritional environment, we transferred larvae from the cabbage to 147.87-mlplastic cups containing artificial agar-based diet modified from established methods (Jaumann and Snell-Rood, 2017; Troetschler, Malone, Bucago, & Johnston, 1985). Because first-instar larvae are delicate, we waited until they were second instars to transfer them to the diet (7-10 days post laying). The diet contained wheat germ (5% by weight), casein (3%), sucrose (2.4%), Wesson salt mix (0.9%), torula yeast (1.2%), cholesterol (0.36%), linseed oil (0.47%), vitamin mix (1%), sorbic acid (0.15%), ascorbic acid (0.3%), cellulose (1%), and cabbage powder (1.5%). Small amounts of methyl paraben and streptomycin were added to prevent the growth of fungi or bacteria in the diet. Most of the ingredients were purchased from Frontier Agricultural Sciences- Entomology Division. The streptomycin was purchased from Sigma-Aldrich.

Larvae were allowed to pupate and emerge as adults in the diet cups. Every day, newly emerged females were individually marked with a Sharpie pen on their hindwings and either transferred directly to mating cages in the greenhouse or stored in a 6°C refrigerator until there were open mating cages available in the greenhouse, because mating generally stimulates egg development (Papaj, 2000). The refrigerator slows development, so adult females were less than a day old or the developmental equivalent when transferred to mating cages. Stored females were fed outside the refrigerator once every five days with 10% honey solution until satiation and allowed to digest for 30 minutes before being placed back in the refrigerator. Females were not used if they were stored in the refrigerator longer than 3 weeks. Once females were transferred to mating cages, they were kept there for three days. After three consecutive 24 hours in the mating cages, the butterflies were immediately moved to experimental cages where they were subjected to a behavioral assay measuring egg-laying behavior in response to conspecific cues. Butterflies in mating and experimental cages were cared for in a manner identical to wild females although the plants inside the cages differed. Larval density was manipulated in the diet cups, and adult density was manipulated in the mating cages (see below). Starting in late August, overhead lights were used in the greenhouse to maintain a 15-hour photoperiod to preserve normal butterfly behavior.

Density Manipulations

Females were provided with conspecific density cues during both the larval and adult stages. During the larval stage, caterpillars were reared in either a low-density treatment of one caterpillar per 147.87-ml diet cup or a high-density treatment of five caterpillars per cup. Previous work in butterflies shows negative effects of density at five larvae per plant (Gibbs et al., 2004). A density of five caterpillars per cup is likely comparable in terms of nutrition, but encourages social interaction more because the larvae are confined to a smaller space. Five is also the maximum number of pupae per cup that minimizes disease and allows for enough space for individuals to eclose without physical deformities (personal observation). Cups were checked daily; most cups never ran out of food, but if caterpillars consumed most of the food in their cup, they were transferred together to a new diet cup.

Adult females were exposed to either a low-density treatment of one female and one male per mating cage or a high-density treatment of five total females and five total males per mating cage. Thus, we kept a consistent sex ratio of 1:1 across treatments, to try to minimize potential differences in male spermatophores transferred to females during mating. Males were randomized across cages. We surveyed mating cages twice daily and immediately replaced any dead butterflies to maintain density at a constant level. We accounted for possible variation in whether or not a female mated (or sterility of males) by only including females that laid at least 10 eggs per day (which is generally associated with a fertile mating; see Behavioral Assay section below). We assessed the oviposition behavior of individual females in each of the four factorial combinations of larval and adult density treatments. All females were tested alone to avoid confounds involved with testing multiple females together, such as social cues unable to be controlled for. Only one out of five individuals from each larval and adult high-density treatment was included in analysis to avoid pseudo-replication.

Host Plant Preparation

Cabbage host plants (*Brassica oleraceae* var. Earliana) were grown under natural light in greenhouse facilities at the University of Minnesota St. Paul campus. All plants experienced identical growing conditions to minimize variation. Plants were grown from seed in Sungro Sunshine Professional Growing Mix soil, Mix #8/LC8 (SKU: 5292601), with 70-80% sphagnum. Five grams of Osmocote Classic 14-14-14 N:P:K slow release fertilizer was applied twice to each cabbage plant, approximately 4 weeks apart. Batches of cabbages were grown in the early and late summer for a continuous supply of fresh, mature leaves.

We conducted behavioral assays from June to December 2014, with supplemental greenhouse lighting to maintain a 15-hour photoperiod during the later months. For each run of the behavioral assay, we placed two cabbage leaves in each of ten to fifteen experimental cages. To spread potential host plant variation across cages, the leaves within and between cages were all clipped from different plants. We visually size and age matched leaf pairs within each cage. Within each cage, the two cabbage leaves were placed side-by-side, with approximately 25 cm of empty space between them. Each leaf was wedged into a water-filled floral stem tube refilled daily to keep the leaf fresh. Each tube was propped up in a small ceramic pot to mimic the position of leaves on a full cabbage plant. The honey solution-soaked sponge was placed in front of and equidistant from the two ceramic pots to prevent location bias.

We provided females with conspecific cues during oviposition to evaluate their response to social cues subsequent to experiencing low or high conspecific density conditions during the larval and adult stages. The conspecific cue we used was dead, pinned “model” conspecific butterflies on one of the two leaves in each experimental cage. Using dead, pinned conspecifics is an established way of providing social cues in insects, including butterflies (Jones, Ryan, & Chittka, 2015; Baude et al., 2011; Dawson and Chittka, 2012; Kawaguchi et al., 2006, 2007; Otis et al., 2006). These models primarily provided a visual conspecific cue to the butterflies. Chemical cues are certainly important and may even induce stronger preferences than visual cues. Yet visual cues alone have been found to be sufficient when they were explicitly teased apart from chemical cues (e.g. Sommerlandt et al., 2014). Butterflies, more than many other insects, rely extensively on vision and use visual cues to make behavioral decisions, especially at close range (Hern et al., 1996). For example, males are attracted to the visual, rather than chemical, conspecific cues associated with conspecifics when searching for puddles (Otis et al., 2006). Male *P. rapae* often try to mate with dead conspecific females (Obara and Hidaka, 1968; personal observation), and attraction to dead females seems to be solely based on visual cues (Obara and Hidaka, 1968). Females also use visual cues to choose host plants during oviposition (Renwick and Chew, 1994). At short distances, such as those inside our experimental cages, vision is especially important in identifying appropriate host plants (Hern et al., 1996). Pierid butterflies in particular are able to use visual conspecific cues without associated chemical cues when deciding where to lay eggs. *Pieris brassicae*, a species closely related to *P. rapae,* strongly avoided leaves with yellow conspecific model eggs that mimicked their natural egg color but lacked chemical cues. However, they did not show avoidance if the models were green (Rothschild and Schoonhoven, 1977), indicating that they were paying attention to the specific color of conspecific eggs. Here, we were able to more precisely manipulate visual cues compared to chemical cues, so we decided to focus on visual cues. We also know, from a previous study, that cabbage whites tend to avoid laying eggs on leaves with these models (Jaumann & Snell-Rood, 2017). The cabbage white butterflies that we used as models were purchased from Carolina Biological Supply as eggs and reared to adults on artificial diet in the lab, to limit variation among models and ensure that all experimental butterflies were equally naïve with regard to models. Model females were immediately frozen upon emergence and stored in the freezer until they were pinned for use as models. To provide a cue relevant for oviposition, female models were pinned in oviposition posture by securing them to a base via a pin through the thorax and curling the abdomen down with two crossed pins. We allowed models to dry for at least one week to ensure that they remained permanently in this posture. Three of these models were then attached to one of the two leaves in each experimental cage before a butterfly was introduced to the cage. Females thus had a choice between an empty leaf and a leaf with three conspecifics during the behavioral assay. To attach a model to a leaf, we removed the crossed pins and attached the remaining pin through the thorax to the leaf by piercing the leaf and securing the pin to the back of the leaf with tape. Tape was similarly attached to the empty leaf in the cage to control for the presence of tape and space available for oviposition. A model was discarded and replaced with a fresh model if its head, abdomen, or any wings fell off. Models were stored in a shaded area in the lab when not in use to prevent wing color from fading.

Behavioral Assay

We simultaneously tested ten to fifteen butterflies per run of the assay. For each run, one mated female cabbage white was placed inside each experimental cage and allowed to oviposit on the two leaves inside. We recorded the number of eggs laid on each leaf every 24 hours at approximately the same time of day for three days. We only included females in our dataset if they laid at least ten eggs during each of the three days of the assay. Laying fewer eggs is an indication that the female either did not mate or mated with an infertile male. Due to this stringent criterion, our final sample size was smaller than the total number of individuals we reared and tested in the behavioral assay. However, this criterion likely minimized individual variation in motivation to lay eggs due to different numbers of fertilized eggs, which could result from variation in male quality or very small spermatophores. We calculated the proportion of eggs laid on the leaf with models each day, and we used the average proportion across the three days of the assay, or the *choosiness score*, as the response variable in our analyses. The higher the score, the more a butterfly tends to lay eggs on leaves with conspecifics and the lower the score, the more they tend to avoid laying eggs on these leaves. Every day, eggs were removed from the leaves after counting, and the leaf pairs in each cage were rotated to another cage so that no butterfly ever experienced a pair of leaves more than once. The same pairs of leaves were used across the three days of each run of the assay because they remained turgid for that amount of time. However, leaves typically began to wilt after approximately five days. We therefore discarded all leaves after each assay, so new leaves from the same original plants were used in each run of the assay. Any chemical cues associated with the removed eggs were randomized with respect to treatment during re-use.

Abdomen Dissections

Egg load alone can affect how choosy insects are during oviposition (Doak, Kareiva, & Kingsolver, 2006). To verify that egg load did not significantly affect our data, we quantified the number of eggs remaining in the butterflies' abdomens after the oviposition assay. Butterflies that completed the assay were frozen in a sealed container and stored at - 20°C. To dissect out remaining eggs, abdomens were detached from the body and eggs were dissected out in 1x PBS buffer. Eggs were removed through a ventral slice in the abdomen. We counted all mature eggs, defined as eggs with a fully developed chorion- those that were fully yolked, with a yellowish color and striated shell. We used a Leica M165 C dissecting microscope at 12.5x magnification for all dissections. The number of eggs dissected out of the abdomen of each individual was added to the number of eggs it laid in the assay as a measure of fecundity. Female cabbage whites in the wild generally live and produce eggs for about a week as adults (Scott, 1992). We sacrificed females on the sixth day after adult emergence, so our measure of fecundity approximates the number of eggs our butterflies could have laid in that time.

Statistical Analysis

We distributed larvae and adult females randomly and equally across all four treatments. However, we ended up with variable amounts of individuals across treatments due to our stringent 10-egg-per-day criterion of inclusion of females in our dataset (low larval/low adult density: n = 11; low larval/high adult: n = 7; high larval/low adult: n = 15; high larval/high adult: n = 15). Thus, our final overall sample size of N = 48 female butterflies was also much less than the total number of individuals we initially reared and set up for testing in the behavioral assay. We analyzed our data in Rstudio version 3.0.2 using analyses of variance (R Core Team, 2013). Larval and adult density treatments were fixed effects in our linear models, and we looked for effects of these treatments, and their interactions, on choosiness score and our measure of fecundity. The data were log-transformed to meet parametric assumptions of normality and equal variance across treatments. In addition, we wanted to see if the mean of each of the four treatment groups was significantly different from 0.5. A mean of 0.5 is what we would expect if all butterflies in a treatment group laid eggs randomly, resulting in an approximately equal number of eggs laid on the leaf with and the leaf without models. For each treatment group, we calculated 95% Basic bootstrap confidence intervals with 1,000 replicates using the boot package in R (Canty & Ripley, 2105) to see if a score of 0.5 was significantly different from the average scores of each group. If the confidence intervals do not include 0.5, then the treatment means are likely to be significantly different from 0.5, or random egg-laying. We also conducted a power analysis using the pwr package in R (Champely, 2015) to assess the probability of detecting an effect with our current dataset and the sample size we would need to get a power of 0.8, both of which evaluate the likelihood real effects of our treatments. Our data are archived in the DRYAD repository.

RESULTS

There were no significant effects of larval ($F(1,45) = 0.0077$, $p = 0.9304$) or adult ($F(1,45) = 0.1709$, $p = 0.6813$) treatment on choosiness score when the larval × adult interaction was not included in the ANOVA (Figure 7). This was also true with the interaction included (larval: $F(1,44) = 0.0076$, $p = 0.9311$, adult: $F(1,44) = 0.1673$, $p = 0.6845$), and the interaction was also not significant ($F(1,44) = 0.0615$, $p = 0.8053$). In addition, a model containing only the intercept was not significantly different from the model with the larval and adult treatment effects (*F*(45,47) = 0.0893, *p* = 0.9147), further indicating that the treatments likely had no effects.

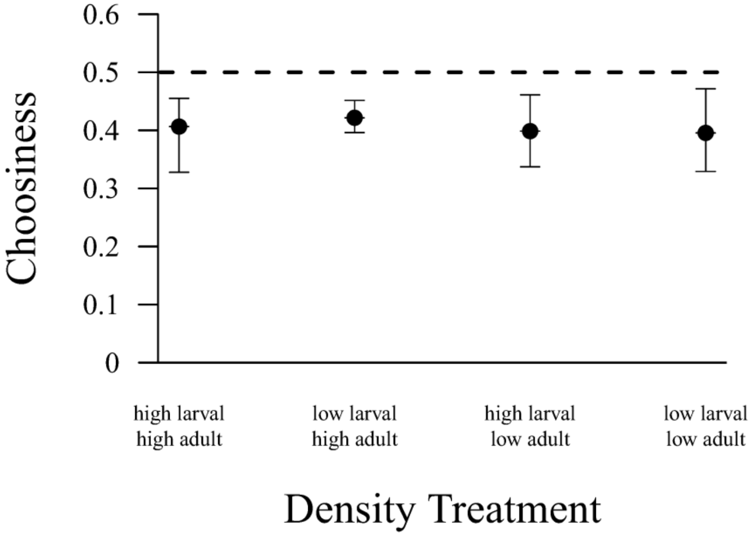

Figure 7. Choosiness score, as measured by the average proportion of eggs laid on the leaf with conspecific models, of females exposed to low or high density conditions during the larval and adult stage. Low-density larval and adult conditions consisted of a single female, and high-density larval and adult conditions consisted of one female plus four other individuals (total of five individuals). Choosiness scores close to 0.5 (dashed line) indicate random egg-laying, and are therefore associated with females that were less choosy. Scores closer to zero are associated with higher choosiness. There were no significant differences among the four treatments. Bars show 95% bootstraped confidence intervals.

Our 95% bootstrap confidence intervals suggest that for choosiness score, each of the four treatment means was significantly different from 0.5, or random egg-laying, since 0.5 was outside the 95% confidence intervals for all four treatments (low larval/low adult: (0.3278, 0.4550), low larval/high adult: (0.3961, 0.4515), high larval/low adult: (0.3372, 0.4611), high larval/high adult: (0.3292, 0.4716), Figure 15). Thus, butterflies in all treatment groups were likely avoiding conspecifics at a low level. The mean of all four groups was around 0.4, where values closer to 0 indicate that butterflies avoid the leaf with models and lay a smaller proportion of their eggs on that leaf.

There were no significant effects of larval ($F(1,45) = 0.1218$, $p = 0.7287$) or adult ($F(1,45) = 0.1415$, $p = 0.7085$) treatment on our measure of fecundity (Figure 8). Again, this was true when the interaction was included in the model (larval: $F(1,44) = 0.1213$, $p = 0.7293$, adult: $F(1,44) = 0.1410$, $p = 0.7091$). The interaction was also not significant ($F(1,44) = 0.8113$, $p = 0.3726$).

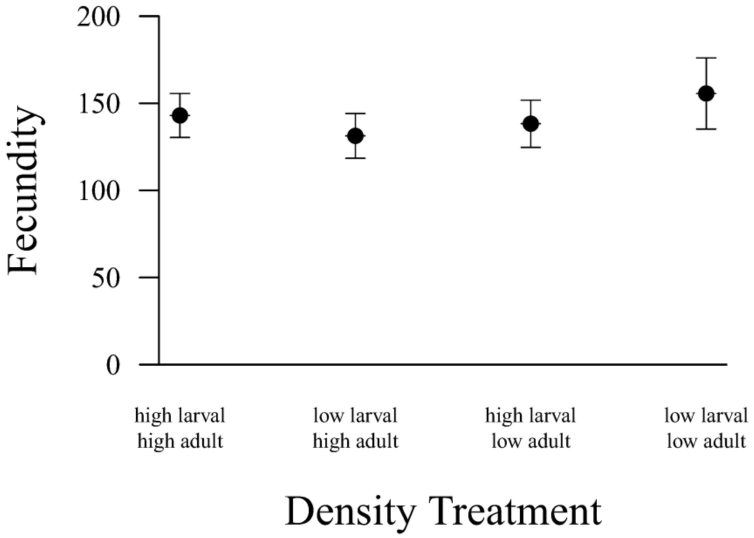

Figure 8. Average fecundity of females exposed to low or high density conditions during the larval and adult stage. Low-density larval and adult conditions consisted of a single female, and high-density larval and adult conditions consisted of one female plus four other individuals (total of five individuals). Fecundity included the number of eggs laid in the behavioral assay and the number of eggs in the abdomen. There were no significant differences among the four treatments. Bars denote standard error.

The results of a power analysis on the model including the interaction between larval and adult density showed a probability of detection of 0.089 with a significance level of 0.05. For a power of 0.8 with the small effect size we obtained (0.014), the analysis indicated that we would need a sample size of approximately 780 butterflies.

DISCUSSION

We expected females in the high larval/high adult density treatment to invest relatively more in avoiding laying eggs near conspecifics, and consequently have lower fecundity. However, there were no significant differences in fecundity (Figure 8) or choosiness score among treatments (Figure 7), suggesting that cabbage white butterflies do not vary in terms of fecundity or change the extent to which they avoid conspecifics during oviposition, regardless of their previous density experiences. Rather, all treatments had very similar average fecundities and choosiness scores. All choosiness score means were approximately 0.4, indicating that butterflies consistently laid about 60% of their eggs on leaves without conspecific cues in our study. Butterflies reared under different densities all tended to avoid laying eggs near conspecifics at this baseline level of avoidance. This baseline was significantly different from random egg-laying across leaves with and without conspecifics (choosiness score of 0.5).

Several different explanations may account for the lack of any density effects and the fixed level of conspecific avoidance that we observed. The most likely possibility is that a consistent, low level of conspecific avoidance during oviposition may be a sufficient strategy for dealing with natural variation in density in this species, particularly if cues such as conspecific density are not suitable predictors for future levels of competition. There may be too much temporal and spatial variation in conspecific density in natural environments for it to reliably predict future density at any given time. A host plant patch for a cabbage white could be anything from a few roadside weeds to a huge crop field where hundreds of individuals congregate. Additionally, adults tend to be highly mobile and do not visit all host plants within a patch (Root & Kareiva, 1984). Thus, females probably visit many patches that vary in larval density within their adult lifetimes, so past experience with conspecific density during any life stage may not be informative about current levels of competition. Unpredictable forces such as larval predation (Molleman, Remmel, & Sam, 2016) or pesticide application by humans (El-Fakharany & Hendawy, 2014; Schlaepfer, Runge, & Sherman, 2002) may add further noise. If no cue reliably predicts future conspecific density, a fixed strategy may be more adaptive than a plastic one (Reed, Waples, Schindler, Hard, & Kinnison, 2010). Thus, females might be more likely to spread eggs across different host plants with a low level of conspecific avoidance, regardless of previous experiences with conspecifics. This pattern is essentially what we observed in our study (Figure 7), with average choosiness scores of the different treatments clustering around 0.4, a low level of conspecific avoidance. Alternatively, it is possible that food availability, rather than the number of conspecific larvae *per se*, is a reliable cue that triggers differences in adult reproductive traits (Bauerfeind & Fischer, 2005). Food availability often cues dispersal (Bowler & Benton, 2005) and could similarly cue choosy oviposition behavior in adults. It may even interact with conspecific density to affect behavior. However, we specifically did not manipulate food availability, providing adults and larvae with unlimited food so that no individuals in the high-density treatments were food-deprived. Our aim here was twofold: 1) to reduce variation in mortality across our high-density treatments so that all individuals were consistently exposed to four other conspecifics and 2) to reduce differences in food acquisition between high and low-density treatments that could affect oviposition behavior and confound our results. However, we may not have seen effects on behavior if food availability is actually the more relevant cue (Gibbs and Breuker, 2006; Gibbs et al., 2004; Saastamoinen et al., 2010), or even if an abiotic cue like temperature, which could affect oviposition (Papaj, 2000) and co-vary with butterfly density, mediates conspecific avoidance.

Several other alternatives might explain our results, though they seem less likely than the idea that conspecific density is an unreliable cue. Butterflies may be able to cope with the conspecific densities they experience through other means, in which case mothers' oviposition decisions may have little impact. Negative effects of high conspecific density are certainly well-documented at the larval stage in butterflies (Gibbs et al., 2004; Kivela & Valimaki, 2008). Some caterpillars may be able to avoid these effects by leaving the plant on which they hatched and moving to a different host plant, though this is not always possible, especially if host plant density is low, given that many larvae are relatively immobile (Jones, 1977a). Individuals that do survive high-density larval conditions may be able to compensate for negative effects on fitness during later life stages (Hyeun-Ji & Johansson, 2016; Metcalfe & Monaghan, 2001), or by investing available resources in other traits that we did not focus on in this study. For example, butterflies and other insects allocate resources differently during and after metamorphosis depending on the nutritional quality of the food they consume as larvae (Boggs & Freeman, 2005; May, Doroszuk, & Zwaan, 2015). Females can also resorb eggs as adults if food resources are scarce (Bell & Bohm, 1975). In general, females with fewer eggs may have stronger wings (Guerra, 2011) or larger eggs (Garcia-Barros, 2000). However, it is notable that variation in density across life stages did not affect female reproductive characteristics such as egg size and fecundity in the butterfly *Bicyclus anynana* (Bauerfeind & Fischer, 2005). If cabbage white butterflies are already capable of coping with high larval densities, to some extent, by altering resource allocation, we might not see strong effects of high larval density on all measures of adult reproductive investment. Indeed, variation in conspecific density across life stages did not have detectable effects on cabbage white conspecific avoidance or fecundity in our study.

It is also possible that cabbage whites *do* respond to high densities with flexible conspecific avoidance behavior during oviposition, but that our experiment was unable to detect this response. This again seems unlikely given our small effect size and the result that we would need 780 butterflies to detect a significant effect with a power of 0.8. However, it was surprising that we did not uncover stronger conspecific avoidance behavior during oviposition, even if fixed rather than flexible. Previous work in *P. rapae* found evidence of strong conspecific avoidance, equivalent to choosiness scores of ~0.15, in response to conspecific cues on cabbage leaves (Schoonhoven et al., 1990). The closely related congener *Pieris brassicae* also exhibits stronger avoidance, equivalent to choosiness scores of ~0.15 - 0.2, in response to conspecific cues (Rothschild and Schoonhoven, 1977). Both of these values are lower than our choosiness scores of 0.4, indicating weaker conspecific avoidance in our study. This may be due to differences in the types of conspecific cues that were used. Previous studies in Pierids measured oviposition avoidance behavior in response to chemical cues associated with conspecific eggs laid on the leaves, rather than the primarily visual cue of adult conspecifics used in our study. At short distances, such as those inside our experimental cages, vision is important in identifying appropriate host plants. Pierid butterflies in particular are able to use visual conspecific cues alone when deciding where to lay eggs (Rothschild and Schoonhoven, 1977; Traynier, 1986). However, chemicals associated with conspecific eggs, are also important in oviposition and do influence preferences. Conspecific eggs act as deterrents in *P. rapae*, even after they have been brushed off of host plant leaves (Schoonhoven et al., 1990). Thus, using fresh leaves every day of the behavioral assay would have been ideal in our study. Due to logistical constraints, we were unable to grow enough plants to use new leaves for each individual every day, so we re-used leaves that had previously had eggs. It is possible that chemicals from the previously laid eggs affected oviposition. However, since individuals in all treatments experienced the same conditions in the behavioral assay, and leaves were randomized with respect to re-use, there was not any systematic bias that would have differentially affected any one of the treatments. If chemical cues did affect our results, it would have only been the absolute, not relative, level of conspecific avoidance (which we measured to be about 0.4). Since eggs tend to induce avoidance in *P. rapae*, in the context of our experiment the effect of previously laid eggs would be to mitigate preferences between the leaf with models and the empty leaf. Therefore, if anything our finding of moderate conspecific avoidance is conservative; if we were able to use chemical cues, our data may have revealed stronger conspecific avoidance during oviposition. Nevertheless, post hoc analyses of the data using only leaves set up on the first day (thus, without any previous eggs) showed no qualitative differences from analyses with the full dataset.

We also may have failed to detect strong conspecific avoidance or treatment effects if the butterflies in our experiment lacked the motivation to be choosy with only two oviposition sites to choose from. We wanted to simplify the experiment and eliminate as many confounds as possible by providing each butterfly with two host options per day. Two-choice tests are common in oviposition assays in butterflies and moths (e.g. Rothschild and Schoonhoven, 1977; Bossart, 2003; Cahenzli, Wenk, & Erhardt, 2015; Groeters, Tabashnik, Finson, & Johnson, 1992; Nylin, Bergström, & Janz, 2000; Tabashnik, Wheelock, Rainbolt, & Watt, 1981). However, with so few options, being less choosy by accepting all available sites may have been a better strategy for the butterflies. We attempted to avoid this issue by providing each female with six options over the span of three days. It is possible that butterflies do not integrate or remember information about individual host plants over multiple days, although there is evidence to suggest that they can remember the color of flowers rewarded with sucrose solution for the same period of time (Kandori & Ohsaki, 1996). Regardless, if few host options contributed to low choosiness during oviposition, such a phenomenon would still be consistent with the idea that current conditions, and not past experience, is largely what determines conspecific avoidance behavior in these butterflies.

Our density treatments may have been too extreme or not extreme enough. The latter is unlikely. Because our adult butterflies were housed in relatively small cages, our adult high-density treatments had higher densities than those experienced naturally by butterflies in the population we tested. For our larval high-density treatments, a density of 5 caterpillars per cup facilitates more social contact than the larvae would likely encounter outside of the laboratory, due to the small space of a 147.87-ml cup. We avoided larval densities higher than 5 caterpillars per cup because we did not want to confound our behavior measurements by inducing differences in survival or fitness in the larval stage. The density we chose was the maximum possible before caterpillars become diseased at high rates and adults have wing deformities upon emergence due to lack of space (personal observations).

Broadly, our results show that fecundity and conspecific avoidance behavior in cabbage whites does not depend on conspecific density experienced during larval and adult life stages. Rather, butterflies adopted a relatively fixed strategy of moderate avoidance behavior during oviposition, regardless of the level of larval or adult conspecific density they had previously experienced. The level of conspecific density prior to oviposition may not be a reliable cue for predicting the level of competition a female's offspring will face, and thus it may not lead to changes in life history traits. A "rule of thumb" of consistently low conspecific avoidance may be sufficient for dealing with the range of conspecific density this species naturally encounters. However, *P. rapae* is somewhat unique in that it is an especially widespread species (Scott, 1992), so it could be informative to explore the behavior of a less dense species that might adopt a different strategy when making decisions during oviposition. For example, *Pieris napi* is closely related to *P. rapae* and *P. brassicae* but is much less common in North America (Scott, 1992) and likely experiences lower conspecific densities. *P. napi* actually seems to show conspecific attraction under some conditions (Raitanen et al., 2014), unlike its congeners, so it is a promising candidate for species comparisons of oviposition behavior as a function of the social environment.

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CHAPTER 3: ADULT NUTRITIONAL STRESS DECREASES CHOOSINESS AND FECUNDITY

ABSTRACT

Despite the benefits of being choosy in contexts such as mate choice, foraging, and oviposition, not all animals are choosy. One potential explanation for this somewhat surprising observation is that behavioral traits can be energetically costly. To determine if there are energetic costs to being choosy, we consider choosiness in the context of life history. If choosiness requires energy, then it should exhibit trade-offs with other life history traits that also require energy. Nutritional stress further limits the energy available for all body functions, potentially amplifying these trade-offs. Thus, the nutritional state of an animal might determine how choosy it is. Poor nutrition during either development or adulthood could affect life history, so we tested two alternative hypotheses about the effects of nutritional stress across life stages on choosiness and other life history traits: 1) poor nutrition leads to adaptive shifts in life history traits, and 2) poor nutrition causes non-adaptive energetic stress that has global negative effects on a range of traits. Cabbage white butterflies (*P. rapae*) were reared in a 2x2 factorial experiment with low or high nutrition during the larval and adult stage. We then measured a range of adult life history traits, including choosiness during oviposition as a behavioral trait. We assessed choosiness by allowing females to decide between host plants with and without conspecific models, since females cabbage whites tend to avoid conspecific cues during oviposition. Females that were nutritionally stressed as adults were less choosy and less fecund, while females nutritionally stressed as larvae invested more in thorax muscle.

There were no other significant effects on these traits or the other life history traits we measured. These results most closely supported the adaptive shift hypothesis, since nutritional stress led to reduced choosiness and fecundity but increased investment in thorax muscle. Further experiments that measure additional life history traits would help confirm our findings. Nevertheless, our results show that nutritional stress affects behavioral traits and other, more commonly measured life history traits such as fecundity, simultaneously. In addition, the life stage during which nutritional stress occurs is important, since adult but not larval stress affected life history. In some cases, adults may be able to compensate for poor early nutrition. These findings could help inform management policies in terms of which plants are suitable nectar resources for pollinators.

INTRODUCTION

Animals are choosy about the decisions that they make in a variety of contexts, and being choosy often leads to substantial pay-offs in terms of self and offspring survival. During foraging, selectively choosing food items of high quality can increase caloric intake and potentially reduce total time spent foraging (Kamil et al., 2012). Especially in poor-quality environments, mates that provide genetic or direct benefits can improve an animal's chances of surviving and producing high-quality offspring (Albo et al., 2013; Bussiere et al., 2005; Houle and Kondrashov, 2002; Kokko et al., 2003). Sites for rearing young or laying eggs that are associated with nutritious resources (Chen et al., 2004; Kondo et al., 2017; Poelman et al., 2013) and protection from competitors and predators (Barros et al., 2016; Buehler et al., 2017; Buxton and Sperry, 2017; Quilodran et al., 2014; Stein and Blaustein, 2015; Sweeney and Quiring, 1998) can considerably increase offspring survival.

These potentially large payoffs of being choosy are seemingly incompatible with the observation that animals are not always choosy. There is variation in choosiness across individuals, and even within individuals. In the context of mate choice, older females in populations with female-skewed sex ratios are less choosy about which male they select as mates (Tinghitella et al., 2013). Nutritionally stressed females are sometimes more choosy when choosing mates (Fisher and Rosenthal, 2006), but often they are less choosy (Dakin and Montgomerie, 2014; Hebets et al., 2008; Hunt et al., 2005). Foraging animals may choose food items that they usually reject when they are either close to satiation (Richards, 1983) or hungry (Kislalioglu and Gibson, 1976). Animals can also be less choosy about which habitats they choose when they are hungry (Pruitt et al., 2011), for example selecting locations that are more vulnerable to predation (Rowe et al., 1996). Overall, these examples demonstrate that being choosy may come with costs, whether these costs are failing to mate or using up limited energy during decision-making that could be used for other functions.

One possible explanation for why some individuals are not choosy is that while choosy decision-making can lead to higher fitness, it may also be costly in terms of time or energy. The magnitude of these costs may determine the extent to which animals are choosy. For example, searching for and identifying quality resources can be costly in units of time or energy. Time costs could arise, for example, if reproductive output is more limited by time than by the energy required to produce offspring. Some insects produce many eggs but only live for a short time as adults, so they are more limited by time than by the number of eggs they can produce (Doak et al., 2006; Root and Kareiva, 1984). Thus, these insects face speed-accuracy trade-offs when searching for places to lay eggs (Goubault et al., 2004). Energetic costs may stem from the sensory processing involved in identifying high-quality resources (Laughlin et al., 1998; Niven and Laughlin, 2008), the cognitive power required to learn cues associated with them (Jaumann et al., 2013; Snell-Rood et al., 2013), or the physical demands of searching (Byers et al., 2005; Johnston, 1991; Tucker, 1970). While time costs are less relevant for some organisms, all require energy, allocating acquired resources across body functions to survive and reproduce.

One way to determine if traits, including behavioral traits such as choosiness, are energetically costly is to study them in the context of a life history framework. Life history theory posits that the energy available to an animal is finite, and allocating a limited amount of energy across all the functions that require energy leads to trade-offs between some sets of traits (Zera and Harshman, 2001). Thus, behavioral traits such as choosiness should exhibit these trade-offs if they require energy that other body functions also compete for. When nutrients are scarce and even fewer resources are available to support all body functions, these trade-offs may be amplified (Boggs, 2009). The effects of poor nutrition on more direct life history traits have been well-studied, but behavioral traits such as choosy decision-making, which may have less direct effects on fitness, have been studied less in the context of life history. For example, when young animals develop on nutritionally diluted or imbalanced diets, allocation to growth and storage generally decreases while allocation to locomotion or somatic maintenance, as measured by lifespan, may increase (Colman et al., 2009; Connolly, 1966; Lyn et al., 2011; McCay et al., 1989; Russell et al., 1987; Scriber and Slansky, 1981; Warne and Crespi, 2015). Yet others have suggested that behavioral traits may also compete for limited energy in a life history framework (Hunt et al., 2005; Resetarits, 1996; Snell-Rood et al., 2011). If this suggestion is true, then manipulating nutrition and available energy within a life history framework could provide insight into why some animals are choosy and others are not. For example, nutritional stress could lead to increased investment in the most important traits at the expense of others, which could manifest as an increase or decrease in choosiness depending on how important choosiness is to the animal. Alternatively, choosiness may decrease along with other measures of performance if nutritional stress that limits energy availability is simply non-adaptive (Boggs, 2009). Either way, the extent to which an animal is choosy should change when available energy decreases if choosiness is energetically costly.

Poor nutrition during the juvenile or adult stage could shift energy allocation to traits such as choosiness, but it is unclear which stage is more important because nutritional stress during different life stages can be expressed in different ways. For instance, poor nutrition during the juvenile stage can have immediate effects or effects that manifest in adults. Lower nutrition during development can decrease growth and storage in juveniles (De Block and Stoks, 2005; Scriber and Slansky, 1981; Yu et al., 2015) and reproductive performance in adults (May et al., 2015). These effects can be interrelated if impaired growth during the juvenile stage alters allocation to reproduction in adults (Brough and Dixon, 1990; Wong and Kolliker, 2014). However, these same adults may invest more in dispersal capability instead, through allocation of energy to muscles (Karlsson and Johansson, 2008). Poor nutrition during the adult stage often impairs reproductive performance (Dias and Macedo, 2011; Djerdali et al., 2008; Koskela et al., 1998; Scott and Fore, 1995), similar to poor nutrition during the juvenile stage, but it may have slightly different implications for fitness. Development would be unaffected by adult nutritional stress, but poor nutrition during adulthood could have stronger negative effects on reproduction if plasticity in resource allocation in response to nutritional stress is more limited after development (Snell-Rood et al., 2015). When there is nutritional stress during both stages, nutritional state may have different effects at different life stages. In some cases, nutritional congruence or incongruence, or whether nutritional state is matched or mismatched across life stages, determines adult reproductive performance, including fecundity, egg size, and overall reproductive investment (Bauerfeind and Fischer, 2005b). In other cases, variation in nutrition during different life stages affects different life history traits. For example, nutrition during the juvenile stage may impact survival most strongly, while nutrition during the adult stage has larger effects on reproduction (Boggs and Freeman, 2005; Boggs and Ross, 1993). These patterns may depend on whether the nutritional composition of food consumed during the juvenile stage remains constant or changes in the adult stage (Boggs, 2009).

We utilize a life history framework to investigate investment reproductive behavior under nutritional stress. Our general hypothesis is that variation in nutrition across life stages affects choosy reproductive behavior in adults, similar to other forms of reproductive investment studied in the context of life history. Using the cabbage white butterfly *Pieris rapae*, we designed a 2x2 factorial experiment to test for effects of low and high nutrition during the larval and adult stages on adult reproductive investment. We tested for effects of each treatment on measures of reproductive investment such as egg number, egg size, and choosiness during oviposition, the latter representing a measure of behavioral reproductive investment. To assess choosiness, we allowed females to choose between oviposition sites with and without conspecific cues, the latter being the more favorable option in this species (Jaumann and Snell-Rood, 2017; Rothschild and Schoonhoven, 1977). Poor nutrition could affect adult reproductive investment in two ways. One hypothesis is that it adaptively shifts life history strategy and energy allocation, resulting in trade-offs. If variation in nutrition leads to a change in energy allocation across traits, we predict that poor nutrition will lead to decreased investment in some but not all reproductive traits. Specifically, in the low-nutrition environments we might expect increased investment in individual offspring at the expense of number of offspring produced (Gliwicz and Guisande, 1992). In the context of our experiment, this prediction would translate to an increase in egg size and choosiness, both forms of investment in individual offspring, with a correlated decrease in fecundity. An alternative hypothesis is that poor nutrition causes non-adaptive energetic stress, leading to reduced investment in all reproductive traits. If this is true, we predict reduced performance in all three measures of reproductive investment in individuals that experience poor nutrition during one or both life stages. However, decreased investment in all three reproductive traits could still be part of a shift in energy allocation if energy is generally allocated away from reproduction to other body functions, such as dispersal, in poor environments. To account for this possibility, we also measured thorax protein, a metric for investment in flight muscle, since dispersal-reproduction trade-offs commonly occur in Pierids and other insects (Karlsson and Johansson, 2008; Stjernholm et al., 2005), and such trade-offs are influenced by food availability (Zera and Denno, 1997). If poor nutrition causes non-adaptive energetic stress, we also expect decreased investment in flight muscle in low-nutrition treatments.

METHODS

We utilized a 2x2 factorial design to test for effects of nutrition during the larval and adult life stages on oviposition behavior and other life history traits. Experimental female butterflies were direct descendants of wild-caught mothers. Each individual was randomly assigned to a low or high larval nutrition treatment and a low or high adult nutrition treatment. Using a behavioral assay, we assessed choosiness during oviposition by quantifying avoidance of dead, pinned conspecific models, since conspecific cues are known to be oviposition deterrents in cabbage whites (Jaumann and Snell-Rood, 2017; Rothschild and Schoonhoven, 1977). We subsequently measured fecundity, egg size, and thorax protein to see how they interacted with oviposition behavior across the four treatments.

Butterfly collection and husbandry

Our experimental subjects were the lab-reared offspring of wild butterflies. Wild cabbage white female butterflies were collected from the University of Minnesota Saint Paul campus between May and August 2015. Immediately after collection, butterflies were placed in 60x60x60 cm clear vinyl and mesh BugDorm-2 cages (Item # BD2120) in a greenhouse facility on the University of Minnesota Saint Paul campus. Each cage had no more than fifteen wild females and an approximately equal number of wild-caught males to encourage mating and egg development. Each cage was furnished with a wet washcloth and non-host plant to maintain appropriate levels of humidity. Sponges soaked with 10% honey solution served as a food source. One or two organic cabbage leaves, which are *P. rapae* host plants, were used to collect eggs from the wild females. Cages were maintained daily by watering the non-host plant and moistening the washcloth, replacing the sponges, collecting cabbage leaves with any eggs laid, and replacing used leaves with fresh leaves. Leaves with eggs were transferred to incubators kept on a 14-hour photoperiod, at 23°C and 70% relative humidity.

Larval nutrition manipulation

In this experiment, we created two different larval diets that corresponded to low and high larval nutrition by manipulating the protein in the diet. Our agar-based artificial diet was adapted from a recipe used in previous work in *P. rapae* (Troetschler et al., 1985) and subsequently modified (Jaumann and Snell-Rood, 2017). Since most of the protein butterflies obtain is acquired as larvae (Boggs, 1987; Boggs, 2003), to create the low and high nutritional treatments for larvae we varied the amounts of casein and torula yeast, the major protein-containing ingredients in the diet. We also manipulated cellulose, a filler, to keep the total dry weight of the diet constant. All other ingredients remained constant. We specifically chose these two diets because they were the only pair out of 4 diets in a pilot study in cabbage whites that showed moderate differences in both female forewing length and body mass upon adult emergence (Chapter 3 Supplementary Material in the Appendix). See the Chapter 3 Supplementary Material for details on the exact composition of each of the two diets (Table 4). After the diet was prepared, it was poured into 4-oz disposable cups. To make sure that the treatments had meaningful differences in protein, we measured the % nitrogen (%N) in the diet using 5 replicate cups of diet for the low and high nutrition treatments. Percent nitrogen was measured using the Dumas method (Matejovic, 1995) at the University of Minnesota Research Analytical Lab. A t-test showed that %N was significantly different between the two diets

($t_8 = 13.3928$, $p = 1.143e^{-5}$), with a mean of 2.7021 %N for the low diet and a mean of 3.1548 %N for the high diet.

Larvae were transferred from cabbage leaves to artificial diet when they were second instars (7-10 days after eggs were laid) and large enough to handle without being harmed. Larvae were reared three per cup and kept in the same incubators as the first-instar larvae. They remained in the cups until they pupated and were removed upon adult emergence. Emerged adults were kept in a 6°C refrigerator until mating in preparation for the behavioral assay. All adult butterflies were individually labelled on both hindwings with a fine-tipped permanent marker and stored alive in the refrigerator in glassine envelopes. They were kept in the refrigerator for no longer than two weeks. Every five days, females in cold storage were removed from the fridge and hand-fed with 10% honey solution. For mating, females were transferred from the refrigerator to BugDorm mesh and vinyl insect cages in the greenhouse that were devoid of cabbage leaves. Mating cages consisted of five to seven experimental female butterflies with an approximately equal number of experimental males. Each cage contained females from both larval nutrition treatments, and they all had access to males from both larval nutrition treatments. We introduced males into the mating cages one day before females to allow for development. Females were housed in mating cages with the males for three days to promote mating, which stimulates egg development and motivation to lay eggs. After three days, each female was individually transferred to an experimental cage for the behavioral assay.

Adult nutrition manipulation

Butterflies mostly consume carbohydrates from nectar as adults (Boggs, 1987). The honey-soaked sponges we supplied in cages approximated the nutritional content of nectar (White Jr, 1975), as butterfly-pollinated flowers are enriched in amino acids along with sugar (Erhardt and Baker, 1990). To create high and low nutrition treatments for the adult butterflies, half of the butterflies from each larval nutrition treatment had ad lib access to one honey sponge per individual every day in the behavioral assay while the other half did not. The second half had ad lib access to a water sponge instead, and access to a honey sponge from approximately 11am-1pm daily, two hours during the active part of their day. The honey or water sponge was always placed in a corner of the cage and rotated to a different corner daily to prevent location bias (Figure 9). Since butterflies from each larval nutrition treatment experienced either ad lib or restricted access to honey as adults, the result was a 2x2 factorial experimental design. After the third day in the experimental cage, all females were frozen in glassine envelopes, sealed in containers, and stored in a -20°C freezer until they were further processed to measure life history traits.

Host plant preparation for behavioral assay

Host plants for the behavioral assay were grown from seed in greenhouse facilities on the University of Minnesota Saint Paul campus. Cabbage was used as a host plant (*Brassica oleraceae* var. Earliana). Plants were grown in Sungro® Professional Growing Mix (Mix #15/LC15) soil. Five grams of Osmocote Classic 14-14-14 N:P:K slow release fertilizer was applied twice to each plant, two weeks apart, with the first application one month after seeds were planted. Aphids are a common greenhouse pest in the University of Minnesota facilities that can cause substantial host plant stress. To minimize variation arising from greenhouse pest herbivory and ensure that the plants were in good condition, 1/3 tbsp of OHP Marathon 1% granular systemic insecticide was applied to each plant. Unlike spray pesticides, this insecticide does not affect adults, and adult female butterflies readily oviposit on treated plants. Several batches of cabbages were planted over the course of the season to ensure that we always had access to leaves that were about the same size and age across the entire experiment. All batches of plants were grown in identical conditions, and individual plants used for the assay were at least one month old from the date of planting.

Individual cabbage leaves were used as host plants for the butterflies in the behavioral assay. For one experimental cage containing one female butterfly, leaves that were size and age matched were clipped from six different cabbage plants to prevent the possibility of small amounts of variation in the plants driving any behavioral patterns we observed. Each leaf was placed into a stem tube filled with water, which was propped up in a small ceramic flower pot. The pots were arranged in two rows of three on top of a moist washcloth on the floor of the cage (Figure 9). Stem tubes were refilled with water daily to ensure that the leaves did not dry out during the three days of the behavioral assay.

We created different host plant options for the butterflies in order to assess how choosy they were about where they laid eggs. We attached adult conspecific models to some of the leaves in each experimental cage as one option, and other leaves remained empty. Previous work has consistently shown that cabbage whites avoid leaves with conspecific cues (Jaumann & Snell-Rood, in review; Jaumann and Snell-Rood, 2017; Rothschild and Schoonhoven, 1977; Schoonhoven et al., 1990), likely because they indicate future offspring competition (Sato et al., 1999). Using the models allowed us to examine the extent to which butterflies discriminated between two host options and chose the better option (i.e. the leaves without models) by comparing how many eggs they laid on leaves with and without models. We used pinned dead, conspecific adult butterflies as models, which is a standard way of providing social information to insects (Baude et al., 2011; Dawson and Chittka, 2012; Jones et al., 2015; Kawaguchi et al., 2006, 2007; Otis et al., 2006). Half of the leaves in the cage each had three models, and the other three leaves had no models (Figure 9). We randomized which leaves had the models every day of the assay.

Butterflies used as models originated from eggs ordered from the Carolina Biological Supply laboratory line to minimize variation across individuals. They were reared from eggs on standard artificial diet in the lab (Jaumann and Snell-Rood, 2017), immediately frozen in glassine envelopes on the day they emerged as adults, and stored in a -20°C freezer. Female butterflies only were selected as models. They were removed from the freezer and pinned onto the cabbage leaves in the characteristic curled-abdomen oviposition position using tape (Jaumann and Snell-Rood, 2017). Tape was attached to empty leaves to control for the presence of the tape and the space taken up by both the models and the tape. Models were used across one run of the behavioral assay and across multiple assays. However, if the wings, head, or abdomen of a model became damaged, either physically or through sun exposure, it was replaced with a new model.

Behavioral assay

We completed 19 identical runs (10-15 cages each) of the behavioral assay in order to obtain an appropriate sample size, since not all butterflies met our egg-laying criteria for inclusion in the dataset (see below). An individual female from either the low or high larval nutrition treatment was placed inside a cage the day following her three days in the mating cage and allowed to lay eggs on the six leaves in the experimental cage for three sunny days. At the end of each day (24 hours after the female was placed in the cage), we counted the number of eggs laid on each of the six leaves. *P. rapae* females lay eggs singly (Root and Kareiva, 1984), so each egg can be considered a separate oviposition event (Nylin and Janz, 1993). Thus, placement of individual eggs in prime locations is a form of investment in individual offspring in these butterflies. We then rotated the leaves and models so that the butterflies experienced a new set of leaves and models each day, and randomized the locations of the leaves each day. To get an overall measure of choosy oviposition behavior, we summed the eggs laid on all three leaves with models together and the eggs laid on all three empty leaves together each day for each individual. For each individual, we calculated the proportion of eggs laid on leaves with models each day, as this is a measure of how strongly butterflies avoided these models. We averaged the proportion of eggs laid on leaves with models across the three days of the assay for each butterfly. The absolute value of the difference between this proportion and a proportion of 0.5 was the “choosiness score”, the behavioral response variable. A proportion of 0.5 would mean that the butterfly was not choosy because she laid her eggs randomly- about half of her eggs on leaves with models and about half on empty leaves. Thus, values close to 0 would indicate little difference between the butterfly's egg-laying pattern and random egg-laying, meaning that she was not choosy. Values farther away from 0 would mean that the butterfly's choices were very different from random egg-laying, or more choosy. We only included females in our dataset that laid at least 10 eggs total every day for each of the three days, because fewer than 10 eggs a day indicates that the butterfly likely did not mate and is laying sterile eggs. There were 58 butterflies that met this criterion and which were subsequently used for analysis.

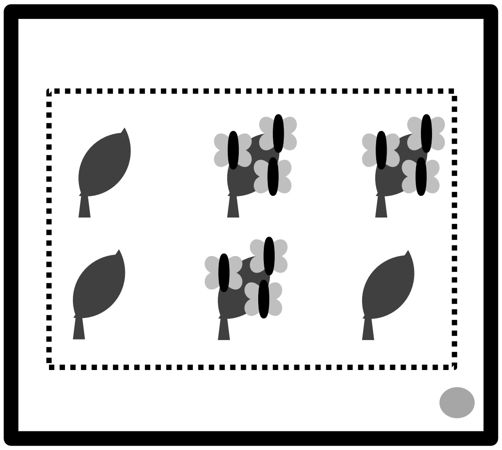

Figure 9. Diagram of one experimental cage. The solid black line denotes the sides of the cage, and the dashed line represents the moist washcloth underneath the host plant leaves. The leaves were inserted into water-filled stem tubes propped up in small ceramic pots to maintain freshness (tubes and pots not shown). The leaves were arranged in two rows of three, and half of them were randomly chosen to exhibit conspecific models. The female butterfly in the cage, which was from either a low or high larval nutrition treatment, had

access to a honey or water sponge (gray circle), corresponding to the high or low adult nutrition treatments respectively. The sponge was rotated to different corners of the cage daily to prevent location bias.

Measurement of additional life history traits

We were interested in the relationships between oviposition behavior and other life history traits, including fecundity, egg size, and thorax protein. To examine other reproductive traits besides oviposition behavior, we dissected the abdomens of the females that had been in the assay to measure fecundity and egg size. Abdomens were dissected in 1x PBS buffer under a Leica M165 C dissecting microscope at 12.5x magnification. The ventral abdomen was sliced longitudinally to reveal the ovarioles. For each female, we counted the total number of mature eggs, defined as conical-shaped eggs that possessed a fully developed chorion with a full yolk, yellowish color, and striated shell. Fecundity was measured as the number of mature eggs dissected out of a female plus the total number of eggs she laid in the assay. In the wild, adult cabbage whites tend to live about a week (Scott, 1992). Since all females in our experiment had an adult lifespan of six days (3 days in mating cages and 3 days in experimental cages), our measure of fecundity is over a lifespan that closely matches that of wild females. For all females that had enough mature eggs (i.e. at least three and ideally five) that were fully intact, we used the Leica software to save an image of three to five eggs removed from the ovarioles. All eggs in each image were on the same plane of focus. Using ImageJ 1.x software (Schneider, Rasband, & Eliceiri, 2012), we recorded average egg area as a measure of egg size, by measuring each mature egg in the image separately and averaging across all measured eggs.

We also measured thorax protein content as a proxy for investment in flight muscle and dispersal capability, which is known to exhibit trade-offs with traits related to reproductive investment (Stjernholm et al., 2005; Srygley and Chai, 1990; Snell-Rood and Steck, 2015). Thorax protein, a measure of investment in flight muscle, was measured using a Bradford assay (Snell-Rood et al., 2014). Each individual thorax was ground with a pestle in 400 µl of 2% sodium sulfate after the dry mass of the entire thorax was recorded. For each of two replicates per individual, 10 µl aliquots of sample was diluted by a factor of twenty in sodium sulfate (with the exception of a few samples with lower concentrations that required a four-fold dilution with 50 µl aliquots). To estimate the protein concentration of the samples, we created standard curves with Bovine Serum Albumin standards of known concentration. We were able to measure the protein of 8 individuals with one standard curve, so tests were completed in several batches. We used the cuvette function of a Thermo Scientific nanodrop (NanoDrop 2000c Spectrophotometer) to measure the absorbance of our standards and samples, and the average R-squared for the standard curves was 0.976. Thorax protein was expressed in mg protein/g thorax.

Statistical analysis

We first assessed effects of nutrition during both the larval and adult stage on the life history traits of choosiness, fecundity, egg size, and thorax protein. Since body size and fecundity are positively correlated in butterflies and other insects (Honek, 1993), body size was a covariate in all analyses that included fecundity. To estimate body size, we measured forewing length for each individual female, which is a good proxy for body size in butterflies, including cabbage whites (Boggs, 1986; Jones et al., 1982). The forewing was removed with forceps and was subsequently photographed next to a metric ruler. Wing length (base of wing attachment to wing apex) was calculated from each image with ImageJ 1.x, using the ruler for scale (Schneider, Rasband, & Eliceiri, 2012). For choosiness, fecundity, and egg size, we used R version 3.0.2 to conduct analyses of variance for unbalanced data (car package: Fox & Weisberg, 2011), with larval nutrition (low or high), adult nutrition (low or high), and their interaction as fixed effects (R Core Team, 2013). Fecundity was log-transformed for normality, though the raw data are shown in figures. Post-hoc analyses were performed using the contrast function in the lsmeans package in R to investigate choosiness in more detail (Lenth and Hervã, 2015).

To investigate the effects of nutrition on thorax protein, we used the lme4 package to construct a linear mixed model (Bates, Maechler, Bolker, & Walker, 2014), and we used the lmerTest package to estimate p-values for the fixed effects, using the Satterthwaite approximation for degrees of freedom (Kuznetsova, Brockhoff, & Christensen, 2014). To determine which terms to keep in the final model, we used the AICctab function in the bblme package (Bolker & R Development Core Team, 2014). We also compared this approach with backwards elimination of non-significant effects using the step function in the lmerTest package. We wanted to compare thorax protein among the sacrificed adults in the four treatments, similar to the other traits we measured.

However, it is possible that butterflies might re-allocate energy to or away from the thorax as adults, even within a day (Snell-Rood et al., 2013). For this reason, ten “reference” females from each larval treatment were sacrificed immediately after they emerged as adults, so that they were not exposed to adult treatments. These females were included in the model as a third level in the adult nutrition factor, in addition to the low and high levels. The thorax protein data was log-transformed for normality, but the raw data are shown in figures, with the two replicates for each individual averaged together into one observation per individual.

We were also interested to see if our nutrition treatments might act on life history through trade-offs between traits. Thus, we wanted to know if any pairwise combination of traits that we measured exhibited a positive or negative correlation. We performed separate Pearson’s r correlation tests for each possible pair of the four traits we measured (choosiness, fecundity, egg size, and thorax protein). To compare thorax protein to the other variables, we averaged together the two replicates for each individual, resulting in one observation per individual. For all correlations, the data were analyzed by 1) pooling all treatments together and then 2) looking at each of the four treatments separately. For correlations including fecundity, we used the ppcor package to calculate partial correlations, with body size as a covariate, although the covariates are not included in the figures (Kim, 2012).

We were unable to obtain fecundity data from two females because they were too dry to get accurate egg counts from abdomen dissections, so analyses including fecundity (but not egg size; see below) have a sample size of 56 female butterflies. Furthermore, because some female abdomens contained few intact eggs, we were only able to obtain egg size measurements for forty of the butterflies out of the full dataset, so that was the sample size for all analyses including egg size.

RESULTS

First, we assessed the effects of our nutrition treatments on life history traits, specifically choosiness, fecundity, egg size, and thorax protein. There were significant effects of treatment on choosiness and fecundity. Female butterflies that experienced low nutrition as adults were significantly less choosy during oviposition ($F_{1,54} = 6.430$, $p = 0.014$; Figure 10) and significantly less fecund ($F_{1,51}= 4.918$, $p = 0.031$; Figure 11) than adults that had ad lib access to honey solution. There was no significant effect of larval nutrition and no significant interaction for choosiness (larval: $F_{1,54} = 2.120$, $p = 0.151$; interaction: $F_{1,54} = 0.454$, $p = 0.504$) or fecundity (larval: $F_{1,51} = 0.002$, $p = 0.963$; interaction: $F_{1,51} = 0.416$, $p = 0.522$), though the effect of larval nutrition was approaching significance for choosiness. Females from the high larval/low adult nutrition group were less choosy than females from the other three groups. Because of this trend, we conducted post-hoc analyses to look at differences in choosiness among treatments and found that there was a significant difference between the high larval/low adult nutrition and low larval/high adult nutrition treatments only ($p = 0.016$). There were no significant main or interaction effects of treatment on egg size (adult: $F_{1,36} = 0.044$, $p = 0.835$; larval: $F_{1,36} = 0.465$, $p = 0.500$; interaction: $F_{1,36} = 0.005$, $p = 0.943$; Figure 12).

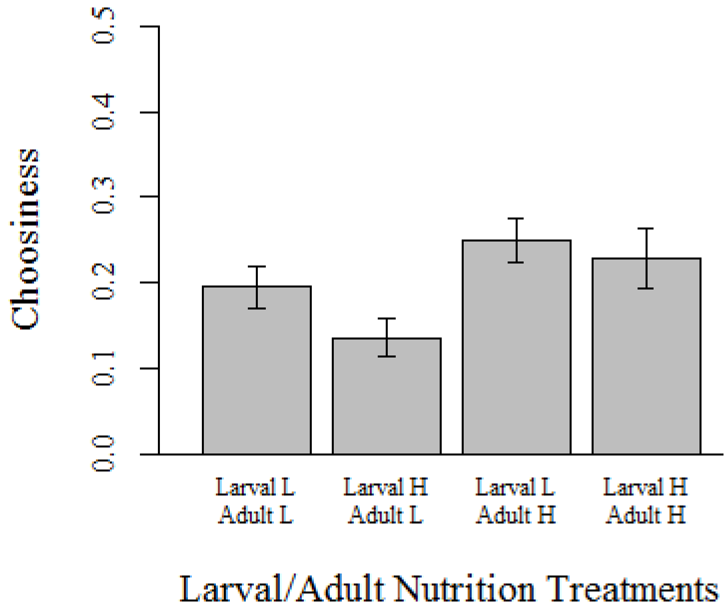

Figure 10. Effects of larval and adult nutrition on choosiness. L denotes a low nutrition treatment and H denotes a high nutrition treatment. Choosiness was calculated by taking the absolute value of the difference between the proportion of eggs laid on leaves with conspecific models and 0.5. A proportion of 0.5 would mean random egg-laying (half of eggs laid on leaves with models and half laid on leaves without models). Values closer to 0 and a proportion of 0.5 thus represent less choosy individuals, while values further away from 0 represent choosier individuals. There was a significant effect of adult nutrition on choosiness, but no significant effects of larval nutrition or the interaction between the two factors (adult: $F_{1,54} = 6.430$, $p = 0.014$; larval: $F_{1,54} = 2.120$, $p = 0.151$; interaction: $F_{1,54} = 0.454$, $p = 0.504$). Bars denote standard error.

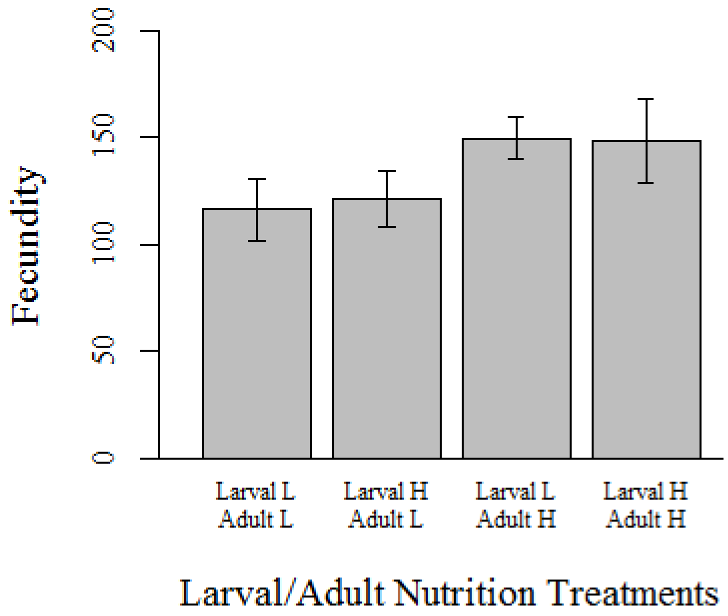

Figure 11. Effects of larval and adult nutrition on fecundity, measured as the total eggs laid in the assay plus the eggs dissected out of the abdomen after butterflies were sacrificed. L denotes a low nutrition treatment and H denotes a high nutrition treatment. There was a significant effect of adult nutrition on fecundity, but no significant effects of larval nutrition or the interaction between the two factors (adult: $F_{1,51}$= 4.918, p = 0.031; larval: $F_{1,51}$ = 0.002, p = 0.963; interaction: $F_{1,51}$ = 0.416, p = 0.522). Bars denote standard error.

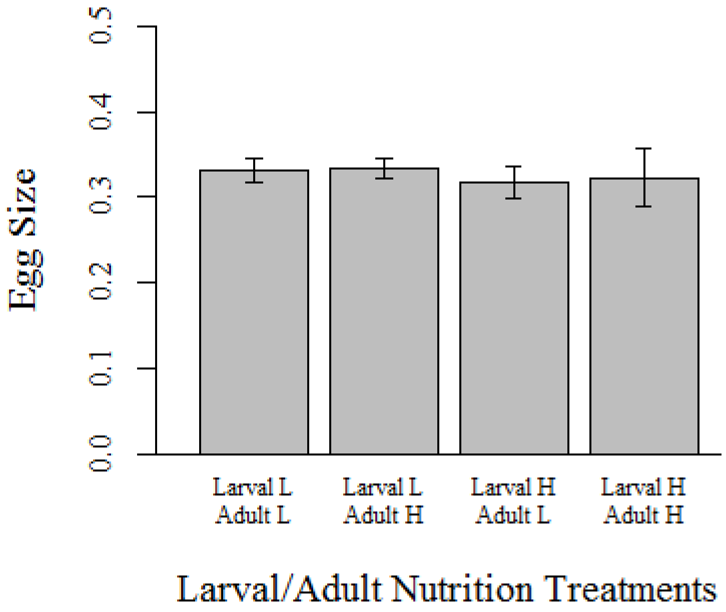

Figure 12. Effects of larval and adult nutrition on egg size, specifically average egg area calculated across at least three eggs dissected out of sacrificed female butterflies. L denotes a low nutrition treatment and H denotes a high nutrition treatment. There were no significant effects of adult nutrition, larval nutrition, or their interaction on egg size (adult: $F_{1,36} = 0.044$, $p = 0.835$; larval: $F_{1,36} = 0.465$, $p = 0.500$; interaction: $F_{1,36} = 0.005$, $p = 0.943$). Bars denote standard error.

We used linear mixed models to analyze the thorax protein data. We took two approaches to determine which terms to include in the final model: AICc and backwards stepwise elimination of non-significant effects. The largest model we considered included adult nutrition, larval nutrition, and the interaction between larval and adult nutrition as fixed effects, plus batch and individual as random effects. Our smallest model only contained larval and adult nutrition as fixed effects (see Supplementary Material in

Appendix, Table 5). The model with the lowest dAICc value included larval and adult nutrition as fixed effects and individual and batch as random effects. The model with the next lowest dAICc value was identical except it did not contain batch, and all subsequent models had dAICc values of 6.2 or higher. The final model suggested by backwards stepwise elimination of non-significant effects was identical to the second model identified by AICc (the model without batch). Differences between the model that included batch and the model that did not include batch were negligible, so here we focus on results for the model with the lowest dAICc value- the model including batch. The only difference is that the effect of larval nutrition (see below) was more significant in the alternative model that did not include batch, so the results we report here are conservative.

There were significant main effects of larval and adult nutrition on thorax protein, but there was no real effect of adult nutrition *per se*. The significant effect of adult nutrition on thorax protein was entirely driven by differences between the reference females and experimental females, not by nutrition ($F_{2,15.71} = 59.257$, $p < 0.001$; Figure 13). Reference females that were sacrificed immediately after emergence had very low levels of thorax protein compared to experimental females (reference females vs. low adult nutrition experimental females: $\beta = 1.387$, SE = 0.142, $p = 1.11e^{-7}$; reference females vs. high adult nutrition experimental females: $\beta = 1.433$, SE = 0.136, $p = 8.10e^{-7}$). Nutrition treatment did not matter between low and high adult nutrition experimental females ($\beta = 0.046$, SE = 0.098, $p = 0.645$). Thus, females did allocate additional resources to the thorax during their adult lifetimes, but not in a manner that differed when adult nutrition was low or high. However, there was a significant difference in thorax protein between low and high larval nutrition females, with low larval nutrition females having higher levels of protein compared to high larval nutrition females ($F_{1,74.31}$ = 3.847 , p = 0.054; Figure 13).

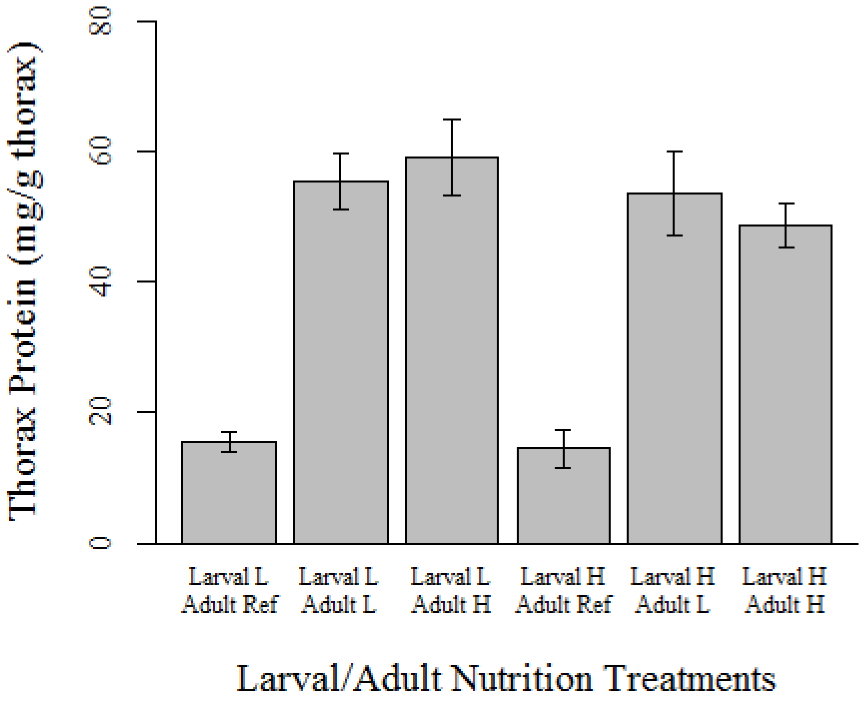

Figure 13. Effects of larval and adult nutrition on female butterfly thorax protein, a proxy for investment in flight muscle and dispersal capability. L denotes a low nutrition treatment and H denotes a high nutrition treatment. Ref indicates that the female was a “reference” female sacrificed immediately after emergence, from either the low or high larval nutrition treatments. These females were used as comparisons against experimental females, in case patterns of resource allocation to or from the thorax are altered across the adult lifetime of butterflies. There was a significant effect of adult treatment on thorax

protein ($F_{2,22.94} = 65.132$, $p = 3.485e^{-10}$), but the effect was entirely driven by differences between the reference and experimental butterflies, not adult nutrition. There were no significant effects of larval nutrition or the interaction between the two factors (larval: $F_{1,68.84} = 2.593$, $p = 0.112$; interaction: $F_{2,65.44} = 0.630$, $p = 0.536$). Bars denote standard error.

To test if there were any trade-offs between traits, we were interested in any trade-offs between life-history traits, we measured correlations of traits 1) across treatments and 2) induced by nutrition treatment. The correlation tests indicated a relationship between egg size and choosiness, but not between any of the other pairs of traits (Figure 14). In the pooled data there was a significant positive correlation between choosiness and egg size ($r = 0.379$, $p = 0.016$; Figure 14b). At the treatment level, the only treatment for which there was a significant negative correlation between choosiness and egg size was the high larval/high adult nutrition treatment ($r = 0.885$, $p = 0.046$), with the caveat that the sample size for that treatment was only 5 butterflies after imposing our dissection criteria on egg size. There was no significant relationship between choosiness and egg size for the other three treatments (low larval/low adult nutrition: $r = 0.580$, $p = 0.132$; high larval/low adult nutrition: $r = 0.439$, $p = 0.237$; low larval/high adult nutrition: $r = 0.144$, $p = 0.569$). There was no evidence of a relationship between choosiness and fecundity, either when pooling all four treatments together ($r = 0.014$, $p = 0.918$; Figure 14a) or looking at the four treatments separately (low larval/low adult nutrition: $r = -0.364$, $p = 0.176$; high larval/low adult nutrition: $r = 0.209$, $p = 0.522$; low larval/high adult nutrition: $r = -0.147$, $p = 0.539$; high larval/high adult nutrition: $r =$

0.184, *p* = 0.647). There was also no relationship between fecundity and egg size in the pooled data, though again the trend was negative (*r* = -0.142, *p* = 0.384; Figure 14c). The same was true when considering each treatment separately (low larval/low adult nutrition: *r* = -0.397, *p* = 0.333; high larval/low adult nutrition: *r* = -0.078, *p* = 0.847; low larval/high adult nutrition: *r* = -0.266, *p* = 0.286; high larval/high adult nutrition: *r* = -0.245, *p* = 0.721).

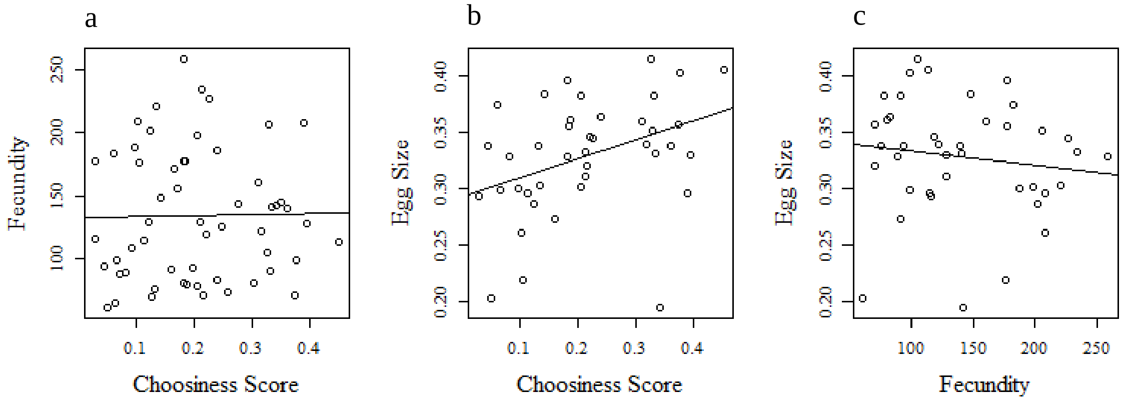

Figure 14. Relationships between pairs of reproductive life history traits, including (a) choosiness and fecundity, (b) choosiness and egg size, and (c) fecundity and egg size. To determine if trade-offs between life-history traits could produce shifts in life history strategy, Pearson’s r was used to test for correlations between reproductive traits. There was only a significant, negative correlation between choosiness and egg size, suggesting a possible trade-off between the two traits (*r* = -0.439, *p* = 0.00461).

Finally, there were no relationships between thorax protein and any measure of reproductive investment in the data pooled across treatments (thorax protein and choosiness: *r* = 0.105, *p* = 0.435, Figure 15a; thorax protein and fecundity: *r* = 0.010, *p* = 0.945, Figure 15b; thorax protein and egg size: *r* = 0.195, *p* = 0.229, Figure 15c). The same was true for all pairs of variables when examining potential relationships separately by treatment, including thorax protein and choosiness (low larval/low adult nutrition: *r* = 0.024, *p* = 0.932; high larval/low adult nutrition: *r* = 0.030, *p* = 0.923; low larval/high adult nutrition: *r* = 0.202, *p* = 0.394; high larval/high adult nutrition: *r* = -0.272, *p* = 0.446), thorax protein and fecundity (low larval/low adult nutrition: *r* = -0.111, *p* = 0.699; high larval/low adult nutrition: *r* = -0.494, *p* = 0.089; low larval/high adult nutrition: *r* = 0.342, *p* = 0.134; high larval/high adult nutrition: *r* = 0.095, *p* = 0.815), and thorax protein and egg size (low larval/low adult nutrition: *r* = 0.220, *p* = 0.600; high larval/low adult nutrition: *r* = 0.101, *p* = 0.797; low larval/high adult nutrition: *r* = 0.282, *p* = 0.256; high larval/high adult nutrition: *r* = -0.185, *p* = 0.766).

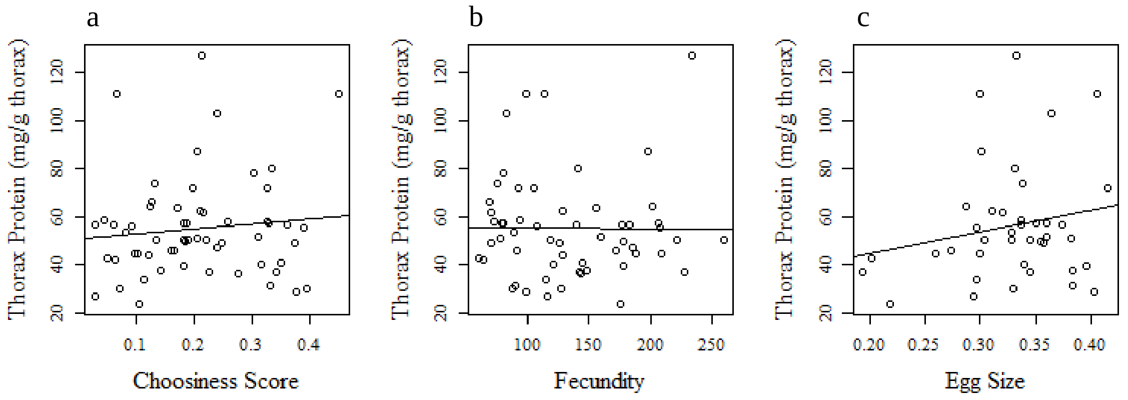

Figure 15. Relationships between reproductive life history traits and thorax protein, a proxy for dispersal capability. Pearson's r was used to test for correlations between (a) choosiness and thorax protein, (b) fecundity and thorax protein, and (c) egg size and thorax protein, to determine if females that invest less in reproduction may be able to

invest more in dispersal capability. There were no significant correlations or evidence of trade-offs between thorax protein and any of the reproductive traits we measured.

DISCUSSION

After investigating the effects of nutrition on a range of life history traits in female cabbage white butterflies, we found that poor nutrition had variable effects on life history traits. Poor adult nutrition decreased both fecundity and choosy oviposition behavior in these butterflies (Figures 10 and 11). Nutrition did not affect egg size, regardless of nutritional quality experienced during larval or adult life stages (Figure 12). Females that experienced poor adult nutrition were perhaps able to acquire enough resources to maintain some but not all traits, such as choosiness and fecundity, at the level of adults from high nutrition treatments. By contrast, poor larval nutrition led to an increase in thorax protein used in flight muscle (Figure 13). These results support the hypothesis that nutrition simultaneously affects choosiness and other forms of reproductive investment. Specifically, they most closely match the prediction for the hypothesis that energetic stress leads to potentially adaptive shifts in life history strategy and energy allocation. Females in our study that were nutritionally stressed decreased investment in reproductive traits, including choosiness during oviposition and fecundity, but increased investment in flight muscle. This result suggests that butterflies may have shifted allocation away from reproduction to dispersal in low-nutrition environments (Karlsson and Johansson, 2008; Stjernholm et al., 2005). However, there were no negative correlations between any traits, including dispersal and reproductive traits, so it is unclear if these traits were linked via the competing energetic demands that shape an animal's life history. There was, however, a positive correlation between choosiness and egg size, indicating that butterflies that invest more in individual offspring do so in multiple ways. It might benefit individuals that produce large eggs to ensure that the eggs will survive by laying them in prime locations, or allocating energy to other forms of individual offspring investment (Snell-Rood, Burger, Hutton, & Moczek, 2016).

Fitness consequences of reduced fecundity and choosiness

Low fecundity resulting from poor adult nutrition may have a direct, deleterious effect on fitness, while less choosy behavior could have indirect but similarly negative fitness consequences. Low lifetime fecundity limits the number of surviving offspring that females produce, but it is possible that females with low early life fecundity could simply live, achieving an equally high fecundity by the end of their lifetime. Fecundity in our experiment was only measured across the first six days of adult life. Thus, females from the low-adult nutrition treatments in our experiment might have adopted a life history strategy of lower early-life fecundity but increased lifespan. Indeed, increased lifespan with nutrient restriction is a well-established phenomenon in insects and other animals (Bishop and Guarente, 2007; Grandison et al., 2009). We measured fecundity across a time period that approximates the lifespan of a wild butterfly because we were unable to measure full adult lifespan in our experiment for logistical reasons. Therefore, further experiments are needed to determine if nutrition mediates a relationship between lifespan and life history traits such as fecundity, or choosiness.

In our experiment, butterflies that were not choosy laid many of their eggs near conspecific females, a behavior which may also have fitness costs in the wild. In North America, wild *P. rapae* are abundant and can occur in very high densities, for example in agricultural fields of host plants. Competition among developing caterpillars can have severe fitness costs, including lower body size and pupal mass, longer development time, and decreased survival (Gibbs et al., 2004; Kivela and Valimaki, 2008). Female cabbage whites tend to lay eggs singly (Root and Kareiva, 1984) and avoid ovipositing on plants with conspecific cues (Jaumann and Snell-Rood, 2017; Rothschild and Schoonhoven, 1977; Schoonhoven et al., 1990). Thus, poor adult nutrition in our experiment led to a change in behavior that would likely be maladaptive for this species in the wild. In addition to exposing their offspring to competition, less choosy female butterflies may lay eggs on host plants that are low in nutrients essential for caterpillars (Chen et al., 2008; Chen et al., 2004; Hwang et al., 2008). At the worst, indiscriminate butterflies may make oviposition “mistakes,” laying eggs on non-host plants that do not support larval growth at all (Nylin et al., 2000). More generally, animals that are less choosy in their decisions risk mating with poor quality mates that provide minimal genetic or direct benefits (Jennions and Petrie, 1997), laying eggs or rearing young in locations with poor nutritional resources or high rates of predation (Battin, 2004; Chen et al., 2008), or even rearing the offspring of other females (Lotem et al., 1995).

Importance of larval vs. adult nutrition for life history traits

The effects of larval and adult nutritional stress differed greatly in our experiment, with a much stronger effect of adult nutritional stress on reproduction. Adults with ad lib access to honey showed higher reproductive investment than adults with restricted access, regardless of larval nutrition. This pattern suggests that butterflies may be able to alter allocation of the resources they consume as adults. In butterflies, carbohydrates consumed by adult females contribute to egg production (Boggs, 1997; Hill, 1989; O'Brien et al., 2004). When adult resources are limited, butterflies allocate fewer carbohydrates to reproduction (e.g. Bauerfeind and Fischer, 2005a; Boggs and Ross, 1993; Hill and Pierce, 1989), which could explain the lower fecundity and levels of choosiness in our treatments with low adult nutrition. Conversely, high nutrition during the adult stage may compensate, to some extent, for poor larval nutrition. Since adult-derived nutrients contribute to reproduction, females may be able to allocate fewer larval resources to reproduction under larval nutritional stress, making up for the deficit later by allocating more to reproduction as adults (Boggs and Freeman, 2005; Mevi-Schutz and Erhardt, 2005). Interestingly, honey solution has a low but non-negligible protein content, similar to nectar (White Jr, 1975). Adult-derived protein from nectar enhances fecundity in female butterflies reared in protein-poor larval environments (Cahenzli and Erhardt, 2012). Since honey also contains some protein, females with ad lib access to honey in our experiment could have consumed more protein as adults, compensating for protein deficits from poor larval nutrition. This could at least partially explain why adult nutrition seemed to have stronger effects than larval nutrition on fecundity and choosiness in our study, and why adult females that had ad lib access to honey were choosier and more fecund than females that did not. To investigate the importance of protein across life stages in more detail, future experiments could perform larval manipulations similar to this experiment in addition to a range of dilutions of either honey or sugar water as an adult nutrition manipulation.

Although adult nutrition was the primary driver of variation in reproductive traits in our study, larval nutrition did play a role in our measure of dispersal capability, with larval nutritional stress leading to higher thorax protein. If adult butterflies can compensate for poor larval conditions, as our study suggests, then it might benefit a larva reared in poor conditions to invest in the flight muscle necessary to disperse to a potentially better environment as an adult (e.g. Boggs and Niitepold, 2016; Brown et al., 2017). Despite this possible role of larval nutrition for dispersal, it is somewhat surprising that the effects of nutrition on reproduction were limited to the adult stage in our experiment, especially given previous work illustrating the importance of larval nutrition (e.g. Boggs and Niitepold, 2016; Fischer and Fiedler, 2001a; Tigreros et al., 2013). However, other work in butterflies has also revealed larger effects of adult nutrition than larval nutrition in the context of reproduction. Variation in larval nutrition may impact adult survival, rather than reproductive traits, in butterflies that allocate adult-derived resources to reproduction (Boggs and Freeman, 2005). Adult nutrition, on the other hand, may play a larger role in reproductive investment, especially in insects low on the ovigeny index, such as cabbage whites, which emerge with few to zero mature eggs (Watanabe and Ando, 1993). Nutritional congruence across life stages, in addition to poor nutrition during one or the other stage, might influence life history traits (Bauerfeind and Fischer, 2005b). In our study, the largest difference in choosiness was between the low larval/high adult nutrition and high larval/low adult nutrition treatments, indicating that changes in nutritional state across life stages might add to variation in life history traits such as choosiness.

Although we may have obtained significant effects of larval nutrition on choosiness or other reproductive traits with a larger sample size, an alternative is that effects of larval nutrition were obscured by other forms of adult compensation for poor larval nutrition. For example, all butterflies had ad lib access to honey water during the three days that they were in mating cages, so butterflies from low-nutrition larval treatments may have been able to compensate for poor larval nutrition to some extent during that time. One other way that females from low-nutrition larval treatments may have been able to partially compensate for nutritional deficits as adults is through spermatophores acquired from males during mating. Nutrients from male spermatophores are known to contribute to egg development and maturation (Boggs, 1990; Wedell and Karlsson, 2003), in conjunction with nutrients from nectar. Thus, nutrients from spermatophores could have provided additional nutrients to adult females in our study, which in turn could have impacted reproductive traits such as fecundity and egg size (Gwynne, 1984; Oberhauser, 1989; Rutowski et al., 1987; Watanabe, 1988). However, our experimental design should have eliminated any systematic bias stemming from spermatophore nutrients. Females in each mating cage in our experiment had access to males from both larval nutrition treatments and were free to mate with males for three days. Since the females didn’t have access to host plants when they still had access to males, they likely also didn’t re-mate. Unmated females or females that mate with a very low quality male usually produce few mature eggs and lay few, if any, so females that laid less than 10 eggs a day in the behavioral assay were not included in our dataset. This stringent criterion should have minimized variation in male spermatophore quality stemming from larval nutrition or other factors, because all females included in our dataset likely mated with males of reasonable quality.

Concluding remarks

This work demonstrates the potentially large impacts of nutrition on performance in butterflies, including a novel connection between nutrition and behavior. Organisms may be able to cope with poor nutrition by re-allocating resources and utilizing alternative life history strategies (Boggs, 2009). We found some evidence for this in that butterflies from low larval nutrition treatments increased investment in thorax muscle. However, the findings of this experiment and others (Bauerfeind and Fischer, 2005b; Hunt et al., 2005; Scott and Fore, 1995; Wong and Kolliker, 2014) also suggest that poor nutrition may have simultaneous adverse effects on a range of reproductive traits, including behavioral traits such as choosiness. While it is possible that the butterflies in our experiment were investing more in additional traits that we did not measure, we prioritized traits relevant for fitness in nutritionally poor environments. Nutritional stress decreases availability of energetic resources, potentially making it difficult for butterflies or other animals to successfully cope with nutritional deficits, especially if there are not better environments within a feasible dispersal distance. The life stage during which an animal experiences nutritional stress may amplify negative effects of poor nutrition. For example, low nutrition during the adult rather than larval stage seemed to have larger effects in cabbage white butterflies, especially for reproduction. Adult nutrition could be particularly important for these butterflies if they rely on adult nutrition to compensate for poor nutrition during the larval stage. These results highlight the need to further investigate the effects of adult nutritional stress in butterflies and other pollinators, and the extent to which adequate adult nutrition is key for maintaining fitness in these animals. Such studies could inform our knowledge of which plants provide the best nectar for pollinators, which is especially relevant in regions where pollinators or their nectar resources are declining.

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CONCLUSION

My dissertation addressed the hypothesis that variation in choosy behavior can arise from different life history strategies. Chapter 1 of my dissertation showed that there is variation in choosiness during oviposition in cabbage white butterflies, specifically in the extent to which they preferred highly fertilized host plants and plants without conspecific cues. There was a trade-off between fecundity and choosiness, and the trade-off was present for both types of choices the butterflies made about where to lay eggs, suggesting that variation in choosiness may be part of a life history strategy. Next, I wanted to induce a shift in life history to see if there would be a concurrent shift in choosiness as well as other life history traits. Since food acquisition is known to affect energy allocation and life history (Van Noordwijk and de Jong, 1986), Chapters 2 and 3 investigated two environmental factors- conspecific density and nutrition, respectively- that affect food availability. It seems unlikely that conspecific density affects choosiness or life history in cabbage whites, given that variation in density during the larval and adult stages failed to produce variation in choosiness or other life history traits. Instead, they appear to use a fixed avoidance strategy with respect to variation in density. However, variation in adult nutrition led to variation in choosiness and fecundity, reinforcing the idea that variation in choosy decision-making could be part of an animal's life history strategy.

On the other hand, I did not find any evidence of life-history trade-offs in Chapter 3, which does not provide support for the idea that such trade-offs could be driving variation in choosiness or other traits. This was somewhat surprising, given that I did find evidence of trade-offs in Chapter 1, and life history trade-offs have been established in other behavioral traits (e.g. Hebets et al., 2008; Hunt et al., 2005; Pruitt et al., 2011; Snell-Rood et al., 2011). There reasons for this inconsistency are unclear because there were few differences in experimental setup between experiments. The host plants in and timeframe of behavioral assays were similar across both years, and both experiments were performed during the same time of year. Photoperiod, temperature, humidity, and other environmental factors were controlled and similar between experiments. However, one factor I did not control was genotype. The genetic composition of the population from which I collected mothers likely varied from year to year and was not something that I could control. In addition, although I kept track of families in Chapter 1, I intentionally did not do so in Chapter 3 due to the time commitment it requires. However, if genotype is responsible for some of the variation in life history traits, and if that variation could affect the detection of trade-offs, then genetic differences that I did not account for in Chapter 3 could explain why I did not detect trade-offs. Indeed, other studies have shown evidence of genetic variation in other behavioral life history traits such as learning (Snell-Rood and Papaj, 2009). Regardless, the fact that I did not find trade-offs among any pairs of traits in Chapter 3 only indicates that there were not trade-offs in general, not that behavioral traits such as choosiness specifically do not exhibit trade-offs. Thus, while it is a puzzling result, it does not necessarily provide support against my overarching hypothesis.

The collective results from this dissertation provide some support for the idea that variation in choosiness can be costly and thus part of an animal’s life history, since investing in choosy behavior during oviposition can lead to reduced investment in other traits. A life history perspective allows for an organizational framework for thinking about variation in choosiness. Such a framework is needed to gain insight into why there might be increases in choosiness in some cases and decreases in other cases, novel insights. Previous work investigating behavior in other contexts also suggests that variation in choosiness might stem from life history. Degree of choosiness during mate choice, for example, may be part of a life history strategy (Hebets et al., 2008; Hunt et al., 2005), and choosiness during habitat choice can be energetically costly and associated with trade-offs (Pruitt et al., 2011). Resetarits (1996) even theorized that oviposition site choice may be part of life history evolution, similar to traits such as fecundity or egg size, but the idea had not been investigated in depth. Thus, this dissertation contributes a previously-lacking detailed evaluation of the concept that variation in choosiness during oviposition can be explained by life history. In addition, it further substantiates the argument that choosiness in general can be considered part of a life history strategy.

This work focused on choosiness as part of life history as an explanation for variation in choosy behavior, but it did not address the question of which mechanisms underlie that variation. The findings from Chapter 1 that choosiness exhibits trade-offs with other life history traits such as fecundity and egg size indicates that choosiness is likely a costly trait, and there are constraints preventing butterflies from investing in all traits simultaneously. However, the exact nature of these constraints is currently unknown. Limited availability of time or energy could drive the observed trade-offs. The result from Chapter 3 that nutrition affects choosiness and fecundity indicates that energy availability may play a role in the expression of those traits. However, I found limited support for the idea that variation in nutrition drives trade-offs. It may be that more traits need to be measured in order to detect trade-offs driven by nutrition, or the spatial scale over which butterflies were searching for host plants may not have been energetically costly enough to induce detectable trade-offs. Alternatively, trade-offs could be driven by genetic or hormonal pleiotropy (Flatt et al., 2005; Gutteling et al., 2007; Leroi et al., 1994; MitchellOlds, 1996), explanations which my dissertation was not designed to address. Additional studies are needed to isolate the mechanisms underlying trade-offs between choosy behavior and other life history traits.

Another limitation of this work is that all three chapters focused on one species with a particular behavioral repertoire and life history strategy, even though there is a wide range of behavioral complexity and life history strategies across eukaryotes. It may be difficult to directly translate the results of these experiments to other species, since life history strategies are influenced by evolutionary past. For example, costs associated with brains and cognition may be different in social and solitary species (Dunbar, 1998), which could affect the expression of trade-offs. Species that experienced a wide range of environments in their evolutionary past may be more plastic and thus less sensitive to current variation in environmental factors like conspecific density or food availability (Moran, 1992; Snell-Rood, 2013). When these factors also influence life history trade-offs, then trade-offs in these species may be less pronounced. The *P. rapae* butterflies I studied are invasive and widespread in North America (Scott, 1992). They are also more resistant to variation in nutrition compared to the closely related but more specialized species *P. napi* (Jaumann, unpublished data). In Chapters 2 and 3 of my dissertation, it is possible that conspecific density and nutrition would have produced stronger effects on choosiness and more pronounced life history trade-offs in other, more specialized species like *P. napi*. Future studies using a comparative approach to study reaction norms and trade-offs would reveal the extent to which the results of my dissertation generalize to other species, both butterflies and other taxa. I am currently in the process of analyzing data from a side project comparing the nutritional plasticity of these two species. Regardless of potential interspecific differences, the results of my dissertation indicate that choosy behavior can factor into an animal's life history and is subject to the same shifts as other traits with environmental variation.

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APPENDIX

CHAPTER 1 SUPPLEMENTARY MATERIAL

Methods

Larval rearing methods

Focal butterfly larvae were reared in the lab in an incubator set at a 14-hour photoperiod, 23°C, and 70% relative humidity until the eggs hatched. Second-instar larvae (7-10 days after egg-laying) were transferred in groups of three from the organic cabbage leaves to 4-oz plastic cups containing artificial agar-based diet modified from established methods (Troetschler et al. 1985). Briefly, wheat germ (5% by weight), casein (3%), sucrose (2.4%), Wesson salt mix (0.9%), torula yeast (1.2%), cholesterol (0.36%), linseed oil (0.47%), vitamin mix (1%), sorbic acid (0.15%), and ascorbic acid (0.3%) met the nutritional needs of the caterpillars, and cellulose (1%) and cabbage powder (1.5%) were added to mimic the chemical composition of a cabbage host plant. Minute quantities of methyl paraben and streptomycin were added to control fungi and bacteria in the diet. All ingredients were purchased from Frontier Agricultural Sciences- Entomology Division, except for the streptomycin, which was purchased from Sigma-Aldrich.

Statistical Analysis

Table 3. List of all models included in Chapter 1 AICc analysis. All models tested included nutrition and conspecific density as fixed, additive effects and individual as a

random effect. Although only the top five models are listed in the main text, all eleven models listed below were included in the AICc analysis.

Model	Random effects	Interactions	dAICc
1	day, mother, individual	density x nutrition	0.0
2	day, mother, individual	density x nutrition day x density	2.1
3	day, mother, individual	density x nutrition day x nutrition	2.1
4	day, mother, individual	density x nutrition day x nutrition day x density	4.1
5	day, individual	density x nutrition	13.9
6	day, mother, individual	none	22.2
7	day, individual	none	36.2
8	mother, individual	density x nutrition	253.3
9	individual	density x nutrition	266.2
10	mother, individual	none	275.5
11	individual	none	288.5

CHAPTER 3 SUPPLEMENTARY MATERIAL

Methods

Pilot protein manipulation

Briefly, cabbage white offspring from wild-caught mothers were reared on one of four artificial diets that differed in protein content. Recipes for two of the diets are in Table 4. The remaining two diets contained larger amounts of the protein-containing ingredients listed in Table 4 (casein and torula yeast) and less filler (cellulose), so they had higher total levels of protein. Caterpillars were reared on the diets in cups in climate chambers under controlled conditions in until emergence. Adults were weighed on a balance on the day they emerged by placing them in glassine envelopes, then subtracting off the weight of the empty envelope. In addition, we measured forewing length by sacrificing the butterflies, removing their forewings, taking a picture of the wings, and measuring the length from the point of muscle attachment to the apex of the wing using ImageJ software (see Methods in main text of Chapter 3 for more details).

We tested for differences in body mass and forewing length among protein treatment groups using ANOVAs. Both response variables were log-transformed for normality. The total sample size was approximately 45 females spread across diets. Protein treatment had a moderately significant effect on body mass ($F_{3,33} = 2.367$, $p = 0.089$), and a Tukey HSD post-hoc test showed that the only two treatments that were moderately significantly different from each other were the two we included in the main experiment ($p = 0.064$). Although the overall ANOVA for forewing length was moderately significant ($F_{3,41} = 2.517$, $p = 0.071$), a Tukey HSD post-hoc test did not identify any two diets that were significantly different from each other. However, the two diets used in the main experiment had the lowest p-value ($p = 0.124$) and the largest difference between means.

Table 4. Diet recipes for larval protein manipulation (low protein = low nutrition larval diet and high protein = high nutrition larval diet). Ingredients that were manipulated are shaded in grey. Casein and torula yeast were manipulated because they are sources of protein, whereas cellulose is a filler and was manipulated to keep the total dry weight of the diet constant.

per 800 ml water	**High Protein**	**Low Protein**
	(in grams)	(in grams)
Wheat Germ	50	50
Cellulose	24	31
Cabbage Flour	15	15
Casein	20	15
Sucrose	24	24
Wesson Salt Mix	9	9
Torula Yeast	8	6
Cholesterol	3.6	3.6
Vitamin Mix	10.5	10.5
Methyl Paraben	0.75	0.75
Sorbic Acid	1.5	1.5
Ascorbic Acid	3	3
Streptomycin	0.175	0.175
Flaxseed Oil	5 ml	5 ml
Agar	15	15

Table 5. List of all models included in Chapter 3 AICc analysis. All models tested included larval nutrition and adult nutrition as fixed effects.

Model	Random effects	Interaction	dAICc
1	batch, individual	none	0.0
2	individual	none	1.1
3	batch, individual	larval x adult	6.2
4	individual	larval x adult	6.7
5	batch	none	193.3
6	none	none	193.8
7	none	larval x adult	197.7
8	batch	larval x adult	200.9