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### Cover Page Footnote

We would like to thank Tyler Brandon, Deysi de la Rosa, and Malcom S. Mikkelsen for help with data collection. We would also like to thank Benjamin Reed and Joshua Smith for comments on the manuscript. Funding for this project was provided by the Washburn University Biology Department.

## Influence of Age on Decision Making by Ovipositing *Pieris rapae* (Lepidoptera: Pieridae)

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### Abstract

Due to its effect on the time available for host selection and learning, butterfly age is expected to alter the degree of host specificity and potentially niche breadth. Here, we use the small cabbage white, *Pieris rapae* (L.) (Lepidoptera: Pieridae), to test the effect of age on ovipositional specificity and decision-making time. Specifically, we examined the ovipositional behavior of *P. rapae* 4, 8, and 12 days post-emergence. Females were recorded in thirty-minute trials using leaves of two hosts, mustard leaves, *Brassica juncea* (L.) Czern., and collard greens, *Brassica oleracea* L. Acephala group, and the non-host common bean, *Phaseolus vulgaris* L.. Subsequently, we measured the duration of drumming events (a proxy for decision-making time) and whether the leaves were accepted or rejected as ovipositional substrates. As would be expected if prior experience influenced ovipositional behavior, we saw a reduction in the duration of drumming events as females aged. In particular, we saw a reduction in duration of drumming events when rejecting the non-host between days 4 and days 8 and 12. We also detected a decrease in drumming time between days 4 and 8 when accepting hosts, but an increase in drumming time between days 8 and 12 when accepting hosts. These results suggest both an increased ability to recognize hosts and an increase in selectivity with age.

**Keywords:** host selection, egg limitation, host specificity, oviposition behavior

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Adult ovipositional behavior can determine the larval environment and consequently the realized host range of many plant-feeding insect species. Therefore, understanding the factors that affect ovipositional behavior can help us understand how host breadth may evolve. Over half a century ago Levins and MacArthur (1969) proposed that the costs imposed by decision-making on foraging herbivorous insects were a major component determining host breadth in plant feeding insects. Bernays and Weislo (1994) formalized this concept into a “neural hypothesis”, which proposes that specialization (e.g., narrower host range) may arise from limitations in the ability to gather and process information. In particular, foraging herbivorous insects with fewer number of choices (competing stimuli) require a lower attentional load, leading to decreased error rate and decision-making time (e.g., Bernays 2001, Egan and Funk 2006, Tosh et al. 2009).

For many butterfly species, ovipositional behavior of adult females determines larval resource use and therefore represents a foraging decision expected to experience high selective pressure. Host selection by ovipositing females under the “hierarchical threshold model” (Courtney et al. 1989) is

believed to be determined by genetically fixed preference hierarchies (Miller and Strickler 1984, Courtney et al. 1989, Mercader and Scriber 2007). Therefore, within the “hierarchical threshold model” each host plant is expected to have an intrinsic acceptability threshold determined by positive and negative stimuli and plants low in the acceptability threshold will only be accepted when the insect’s state has changed. Consequently, while the relative preference ranking between hosts is fixed, how tightly host preferences are adhered to (i.e. specificity) is believed to be plastic and may be modified by intrinsic factors such as physiological state, adult experience, age, and fecundity (Miller and Strickler 1984, Bossart and Scriber 1999, Mercader and Scriber 2005, Gamberale-Stille et al. 2019). These non-genetic intrinsic factors act as a form of phenotypic flexibility that can influence the host breadth of species (Miller and Strickler 1984, Mercader and Scriber 2005, Fordyce 2006) and lead to within species variability.

Age has long been considered an important factor affecting host selection (Gossard and Jones 1977) due to its effects on insect physiological state (Miller and Strickler

1984), time available for host selection (West and Cunningham 2002; Rosenheim 1999 a,b), and experience (e.g., Papaj and Prokopy 1989, Snell-Rood and Papaj 2009, Jones and Agrawal 2019). Models suggest that as ovipositing insects age, a decrease in specificity is expected due to a heightened risk of not finding any suitable hosts, even towards the end of a lifetime for organisms initially limited by available eggs (Rosenheim, 1999 a,b). However, organisms that consume limited resources as adults may experience egg limitations later in life, ultimately leading to increased specificity (Rosenheim 1999 a,b; Rosenheim et al. 2008; Rosenheim 2011). In addition, as adult females age, they also have the potential to become more efficient foragers due to prior experience (e.g. Papaj and Prokopy 1989, Snell-Rood and Papaj 2009, Jones and Agrawal 2019), thereby also increasing specificity. Therefore, as ovipositing adults age, specificity may decrease due to time limitation or increase due to experience and/or egg limitations.

Here we take advantage of the small cabbage white, *Pieris rapae* (L.) (Lepidoptera: Pieridae), a specialist on members of the Brassicaceae family that has long been the subject of studies on ovipositional behavior (e.g., Richards 1940, Gossard and Jones 1977, Renwick and Radke 1988, Lund et al. 2019), to investigate the effect of aging on decision-making by ovipositing females. Specifically, we test a) the effect of age on ovipositional specificity and b) if ovipositing females exhibit reduced decision-making time with age/experience. Upon alighting on a plant, females of many butterfly species searching for oviposition sites will engage in a behavior known as “drumming”, which consists of rapidly moving their forelegs across the surface of the leaf (Schoonhoven et al. 2005). This behavior allows females to evaluate the ovipositional substrate, by exposing contact chemoreceptors in the tarsi to stimulant and deterrent compounds on the leaf surface. This behavior is well described (Schoonhoven et al. 2005) for *P. rapae* and provides an identifiable behavior linked to host plant evaluation. In addition, *P. rapae* are known to exhibit adult learning (Snell-Rood and Papaj 2009) and experience reduced egg laying with age (Gossard and Jones 1977), making them ideal candidates for this study.

### Materials and Methods

**Study organism:** *Pieris rapae* individuals were purchased as eggs from Carolina Biological Supply Co. and reared on green cabbage leaves *Brassica oleracea* L. var. capitata. Larvae were maintained at room temperature under an 18:6 L:D

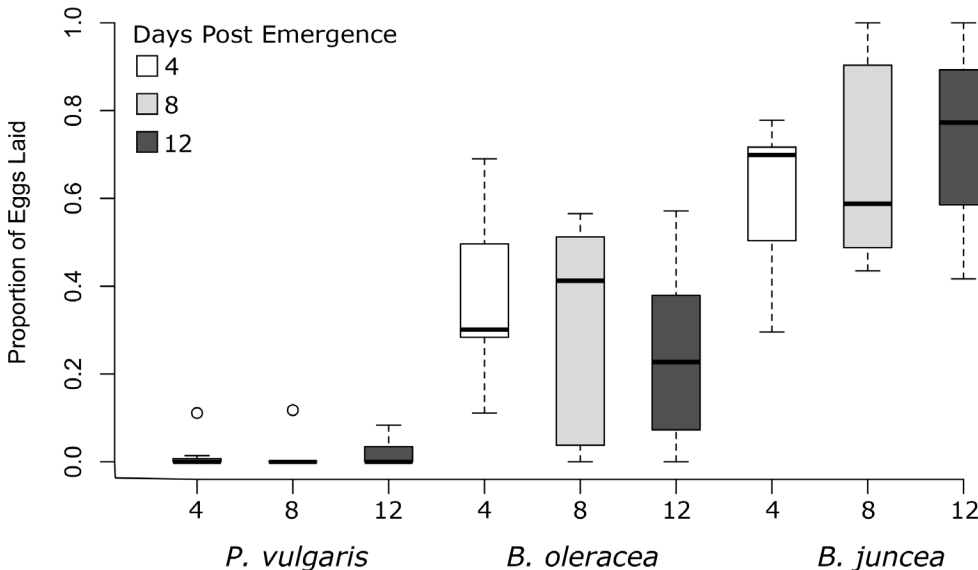
photoperiod, allowed to pupate, transferred to individual emergence cages, and females were numbered on the front wings prior to introduction to a common cage along with males. Butterflies were provided an artificial nectar solution of 15 % honey-water solution and a lepidopteran Ringer’s solution (following Lederhouse et al. 1990) to ensure male fertility. Cabbage leaves were introduced daily for a one-hour period between 10 AM and 2 PM to allow females to oviposit. Females that were a part of the ovipositional assays were removed from the common cage on days in which they were assayed as they were presented ovipositional substrates during the assays.

#### Three-choice oviposition assays:

Three choice assay using leaves of two hosts, mustard leaves, *Brassica juncea* (L.) Czern., and collard greens, *Brassica oleracea* Acephala group, and the non-host common bean, *Phaseolus vulgaris* L. were performed and filmed on 17 butterflies at 4, 8, and 12 days post emergence. *B. juncea* and *B. oleracea* were chosen as two hosts based on experience from prior assays using *B. juncea* and *B. oleracea* with *P. rapae* females from the same source. In particular, previous assays had indicated a preference for *B. juncea* under similar conditions, but not an overwhelming preference for *B. juncea* (*B. juncea* preferred in most, but not all assays). For each assay, a single fresh leaf of each of the three plants was placed individually over small glass cylinder (6 cm diameter and 7 cm height) and secured with Parafilm® exposing only the upper circular surface area for each assay; ensuring identical leaf surface area was exposed for each leaf and each assay. Cylinders with leaves were placed on the far side of a 60 × 30 × 30 cm (L × W × H) glass aquarium in a row with the sides and back walls covered in black cardboard. The order of each leaf in the row (i.e., left, middle, or right) was randomized between assays. A video recorder was placed on the front wall of the aquarium, and a light placed above the leaves. Prior to each assay butterflies were cooled for 2 minutes at 6-8 °C, placed in the enclosure, and allowed to heat up and then filmed for 30 minutes.

**No-choice oviposition assays:** To determine whether age decreased rejection time when non-hosts were encountered, we first conducted no-choice assays consisting of a leaf of the non-host *P. vulgaris*. Assays were performed and filmed prior to every three-choice trial on the same females as the three-choice trial. The setup was identical to the three-choice trial, with the exception that only a *P. vulgaris* leaf was available.

**Behavior Coding:** Films were viewed and the duration of each drumming event



**Figure 1.** Boxplots of the proportion of eggs laid on common bean, *P. vulgaris*, collard greens, *B. oleracea* Acephala group, and mustard greens, *B. juncea* by *P. rapae* females 4, 8, and 12 days post emergence. Boxes represent interquartile ranges, heavy lines medians, whiskers minimum and maximum non-extreme outliers, and circles extreme outliers.

and whether it led to egg laying or not were coded for 11 butterflies that laid eggs during at least two of the three choice trials using Solomon Coder (Péter 2011). Eleven butterflies laid eggs during days 4 and 8, and 9 butterflies laid eggs during all three trial days. In total 1494 drumming events were recorded and 872 eggs were laid by these 11 butterflies during the trials.

**Statistical analysis:** All analyses conducted using R (R Core Team, 2019).

**Host specificity:** In our assays, very few eggs were laid on *P. vulgaris*, and we were solely interested in shifts in the proportion of eggs laid between the two hosts. Therefore, as the proportion of eggs laid per day for each butterfly are correlated all analyses were run on the proportion of eggs laid per trial on the preferred host, *B. juncea*. We used General Linear Mixed Effect Models (GLMM) with proportion of eggs laid on *B. juncea* as the response variable, individual butterfly as a random effect, and age as a fixed effect using the lme4 package (Bates et al. 2015). Proportion of eggs laid on *B. juncea* were arcsine transformed to meet distribution assumptions. Statistical significance was determined using likelihood ratio tests (LRT).

**Decision making time:** The effect of age/experience on decision-making time (duration of drumming) was tested in two main formats. We first tested effect of

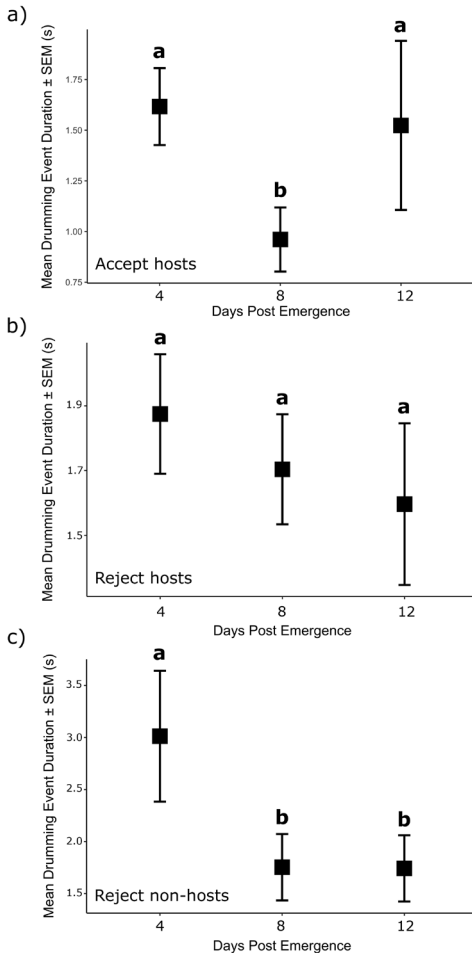
host and age on the duration of drumming events as a GLMM with host, age, and their interaction as fixed effects and individual butterfly as a random effect. Subsequently, we analyzed the effect of age on duration of drumming events on the preferred host and non-host separately as GLMM's with age as a fixed effect and butterfly ID as a random effect. Finally, we contrasted the duration of drumming events when the two host plants (*B. juncea* and *B. oleracea*) were rejected or accepted as GLMM's with choice (rejected or accepted) and age as a fixed effect and butterfly ID as a random effect. Duration of drumming events were square root transformed to meet distribution assumptions and significant effects interactions were tested using likelihood ratio tests (LRT) and pairwise comparisons using Tukey's HSD as implemented in the multcomp package (Hothorn et al. 2008).

## Results

### Three-choice oviposition assays:

**Host preference:** The proportion of eggs laid on the preferred host *B. juncea* remained consistently higher throughout the trials and no significant effect of age was observed (LRT:  $\chi^2 = 2.554$ ;  $df = 2$ ;  $P = 0.279$ ; Fig. 1).

**Decision making time:** We observed a significant reduction in the duration of drumming events with age overall (LRT:



**Figure 2.** Mean duration of drumming events (seconds) by *P. rapae* females 4, 8, and 12 days post emergence a) when accepting hosts (*B. oleracea* Acephala group, and *B. juncea*), b) when rejecting hosts, or c) when rejecting the non-host (*P. vulgaris*). Means followed by the same letter are not significantly different at the  $P < 0.05$  level (Tukey's HSD).

$\chi^2 = 39.488$ ;  $df = 2$ ;  $P < 0.001$ ; Fig. 2). More specifically, we observed an initial reduction in the duration of drumming events leading to hosts being accepted with age (LRT:  $\chi^2 = 39.464$ ;  $df = 2$ ;  $P < 0.001$ ) and an overall reduction in the duration of drumming events leading to non-host being rejected with age (LRT:  $\chi^2 = 17.989$ ;  $df = 2$ ;  $P < 0.001$ ). However, there was no change in the duration of drumming events with age for preferred hosts when rejected (LRT:  $\chi^2 = 1.024$ ;  $df = 2$ ;  $P = 0.599$ ) and insufficient observations of acceptance of the non-host to analyze. Within drumming events leading to the acceptance

of hosts, we saw an initial reduction in drumming duration for accepted hosts between 4-day old and 8-day old butterflies, followed by an increase between 8-day old and 12-day old butterflies (Fig. 2a). Within non-hosts we saw a reduction in drumming time in both 8- and 12-day old butterflies compared to 4-day old butterflies (Fig. 2c).

In addition, we observed an overall greater duration of drumming events when hosts were rejected than accepted (LRT:  $\chi^2 = 94.45$ ;  $df = 1$ ;  $P < 0.001$ ). Suggesting, that within these assays when females were being more selective and rejecting a host plant, decision making times were increased.

**No-choice oviposition assays:** Very few drumming events took place in no-choice assays (< 20 compared ~1500 in three choice assays) and none of the butterflies performed drumming behaviors during all three no-choice assay. In contrast, all butterflies that laid eggs during the three choice assays (described below) also drummed on the non-host *P. vulgaris*. These results suggest that *P. rapae* females foraging for ovipositional substrates are unlikely to meaningfully interact with non-host plants in the absence of neighboring host plants.

## Discussion

Although an overall effect of age on ovipositional preference amongst hosts was not observed, we observed a distinct effect on the duration of drumming events. Tarsal drumming behavior is exceedingly common in butterflies and strongly linked with contact chemoreception (Renwick and Chew 1994), a key component of host plant recognition and acceptance/rejection of hosts. Here we observed both an initial reduction in the duration of drumming events, as expected if prior experience influenced ovipositional behavior, and subsequently an increase in the duration of drumming events when accepting hosts, as would be expected if female selectivity were to increase with age (Fig. 2).

*Pieris rapae* females exhibited an overall reduction in the duration of drumming events with age, indicating faster decision-making time with age/experience. In particular, there was a marked reduction in duration of drumming events when accepting hosts or rejecting non-hosts between days 4 and 8 (Fig. 2), suggesting an increased ability to recognize hosts from non-hosts. However, between days 8 and 12 we saw an increase in the duration of drumming events when hosts were accepted, but not when rejecting hosts or non-hosts (Fig. 2). This suggests that older females were capable of faster decision-making, yet decision-making time was longer when accepting hosts in

the final trial. Tradeoffs between speed and accuracy in foraging herbivores have been well-established (Bernays 2001) and a bias towards accuracy would be expected if an egg limitation were present.

It is worth noting that during no-choice assays using the non-host *P. vulgaris* very few drumming events occurred throughout all assays. This suggests that behaviors leading to oviposition are rare in the absence of suitable hosts. However, host searching in complex environments containing multiple non-hosts is common and increased neural capacity has been previously observed in *P. rapae* foraging in complex compared to simple environments (Snell-Rood et al. 2009). Here we observed within *P. rapae* females primarily exposed to a simple environment (a single host present in non-testing arenas) a decrease in decision-making time when rejecting or accepting hosts in a previously experienced environment. However, we also found that as females age an increase in decision-making time could become present when accepting hosts. Although the design of this study does not allow us to separate the effects of experience and age, the observed patterns suggest that as females age, foraging efficiency may be modulated by both experience and aging.

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