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Reproductive tradeoffs of learning in a butterfly

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The evolution of learning has long been hypothesized to be limited by fitness trade-offs such as delays in reproduction. We explored the relationship between host learning and reproduction in the cabbage white butterfly, *Pieris rapae*. The cabbage white female is innately biased to search for common green hosts but can learn to search for rare red hosts. Host learning was shown previously to vary among full-sibling families and to incur costs in terms of host search efficiency and brain size. In the present study, we show that butterflies from full-sib families with relatively better learning performance on red hosts tend to emerge as adults with relatively fewer and less-developed eggs. We also used methoprene, a juvenile hormone mimic, to advance reproduction in female cabbage whites. We found that methoprene-treated butterflies improved host-finding ability less with experience, relative to controls, providing independent evidence of a link between learning and timing of reproduction. Finally, we show that the learning experience itself is associated with additional decreases in lifetime fecundity. These results support a range of theoretical and comparative studies highlighting the importance of fitness tradeoffs in the evolution of learning and cognition. *Key words:* juvenile hormone, learning, life history, *Pieris rapae*, ovary maturation, trade-off. *[Behav Ecol]*

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Learning is a common form of phenotypic plasticity that allows an organism to adjust its behavior rapidly and usually reversibly in response to spatial and temporal variation in its environment (Stephens 1992; Frank 1996; West-Eberhard 2003). Learning has been shown to vary among species in ways that are consistent with differences among species in the benefits of learning. For example, corvid bird species that rely more on recovery of seed caches to survive winter food shortages have a greater capacity to remember multiple cache locations than species that rely less on such caches (Olson et al. 1995; Bednekoff et al. 1997). Such species differences provide evidence that the benefits of learning drive its evolution. However, these differences also imply that learning has costs or else all species enjoying any benefit to learning at all would be expected to learn maximally well. In fact, there is growing evidence that learning is costly (Mery and Kawecki 2003, 2004, 2005; Snell-Rood and Papaj 2009; Snell-Rood et al. 2009). Comparative studies further suggest that the degree of learning ability in a species is positively correlated with the magnitude of costs related to early exploration (Greenberg 1983), high frequency of mistakes early in life (Laverty and Plowright 1988), and investment in metabolically expensive neural tissue (Sherry et al. 1992; DeVoogd 2004; Lefebvre and Sol 2008). For instance, high performance on spatial learning tasks by seed-caching birds is correlated with increased volume of hippocampal tissue (Krebs et al. 1989; Sherry et al. 1992), an energetic cost to the extent that neural tissue is metabollically expensive (Laughlin et al. 1998).

Such costs of learning are hypothesized to have life-history consequences such as direct trade-offs with life span (Burger et al. 2008) or with competitive ability early in life (Mery and Kawecki 2003). Possibly the most frequently proposed life-history trade-off associated with learning is a delay in reproduction (Mayr 1974; Johnston 1982; Dukas 1998; Ricklefs 2004); reproductive delays have been suggested to be especially important in the evolution of human life history and cognition (Kaplan et al. 2000; Kaplan and Robson 2002). Resources that might be invested in development of reproductive tissue, such as production of oocytes, may instead be diverted to development and maintenance of costly neural tissue required for learning and memory (Laughlin et al. 1998). Aside from such tissue-based costs, the learning process itself is also costly, requiring energy for active exploration and sampling of the environment. Extended early development in vertebrates involves complex experiential processes, including acquisition of foraging skills (Marchetti and Price 1989; Wunderle 1991; Gurven et al. 2006; MacDonald 2007), which are energetically costly and may delay reproduction.

Despite interest in the life-history consequences of learning spanning almost 3 decades, our understanding of the fitness trade-offs associated with learning remains incomplete. A large literature on vertebrates has reported correlations between brain size of a species and timing of reproduction as measured by gestation length, incubation period, weaning time, postnatal dependence on parents, and/or age at first reproduction (Sacher and Staffeld 1974; Mace and Eisenberg 1982; Pagel and Harvey 1988; Iwaniuk and Nelson 2003; Lefebvre et al. 2006; Barrickman et al. 2008). However, inference of cause and effect from such interspecific correlations is complicated by the often large array of confounding variables that differ among species and the fact that brain size is not necessarily a direct measure of learning ability (Healy and Rowe 2007). Furthermore, it is often difficult to determine whether a fitness trade-off reflects a constitutive global cost or an induced environment-specific cost. Global costs of learning—those that are independent of the environment in which learning occurs—are more likely to play a role in limiting the evolution of learning (Snell-Rood and Papaj 2009). Induced costs of learning, those costs specific to the learning

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process itself (Mery and Kawecki 2004), are hypothesized to be less important in explaining variation in learning ability because these costs are only experienced in the environments in which the benefits of learning are also present (see discussion of the costs of plasticity in DeWitt et al. 1998). Thus, it is important to determine whether a correlation between fitness and learning is due to a constitutive or induced cost of learning.

In this study, we explored possible links between learning ability and the timing and extent of reproduction, using a full-sib analysis of female cabbage white butterflies. This system offers 3 main advantages: 1) we can study variation in learning ability within a species, 2) we can experimentally manipulate reproductive delay to further test the link between learning and reproductive timing, and 3) using analyses at the family and individual level, we can distinguish between constitutive and induced costs. The cabbage white, *Pieris rapae*, oviposits exclusively on plants in the family Brassicaceae (Courtney 1986; Renwick and Chew 1994). Host plants generally bear green foliage but are occasionally reddish (Chalker-Scott 1999; Gould 2004; Snell-Rood and Papaj 2009). Females in the genus *Pieris* are innately disposed to search for common green-colored host plants but as in many butterflies, can learn a range of colors, including red, associated with hosts or host surrogates (Kolb and Scherer 1982; Traynier 1984; Papaj and Prokopy 1989; Hern et al. 1996; Smallegange et al. 2006; Snell-Rood and Papaj 2009). Learning allows butterflies in a red host environment to progress from an initial random host search to performance comparable with that of butterflies in the green host environment (Snell-Rood and Papaj 2009; Snell-Rood et al. 2009). Thus, learning allows these butterflies to cope with temporal and spatial variation in host visual characteristics. However, learning comes with increased neural investment, a presumptive cost of learning (Laughlin et al. 1998). In particular, individuals from families that were better able to learn to locate red host plants tended to emerge with greater relative brain volume of brain regions involved in learning (Snell-Rood et al. 2009). The cost of neural investment should be outweighed by the benefits of learning in rare (red) host environments but not in the common (green) host environment where learning plays a minor role in locating hosts (Snell-Rood and Papaj 2009). Previous studies also found additional costs of the learning process itself such as increased neural investment following the learning experience (Snell-Rood et al. 2009).

The present study had 3 aims. First, we were interested in determining whether full-sib variation in host learning reported in these earlier studies was accompanied by variation in the timing and extent of reproduction. In light of the cost of brain size, we predicted that reproductive development would be delayed in full-sib families with superior learning abilities. Specifically, we predicted that a full-sib family's ability to learn to search for host plants would be correlated negatively with the degree of ovarian maturity at emergence. Given that learning and associated costs are more pronounced when butterflies are learning to locate red hosts (relative to green hosts), we expected that correlations between learning and reproductive delays would be more pronounced for the ability to learn to locate red, relative to green, hosts. Second, we experimentally manipulated the timing of reproduction to provide an additional test for associations between learning and reproductive delays. In butterflies, juvenile hormone (JH) plays a role in reproductive development in both sexes (Karlinsky 1963; Benz 1970, 1972; Herman 1973, 1975; Herman and Bennett 1975; Herman et al. 1981). Thus, after finding evidence of reproductive delays in the full-sib analysis, we used methoprene, a JH analog, to advance reproductive development and assess its effect on learning performance. Third, we were interested in whether the learning process itself had additional fitness trade-offs. We compared lifetime fitness of butterflies with experience host finding in environments that differed in the amount of learning required for a given level of performance.

MATERIALS AND METHODS

Experiment 1: correlations between ovary maturity at emergence and learning

In Experiment 1, we tested for correlations between reproductive delay and learning ability at the family level. We collected females from 5 field populations (Nevada, Northern and Southern California, Michigan, and Massachusetts) and reared their offspring in a common garden design on artificial diet (Troetschler et al. 1985; Webb and Shelton 1988). Because this species exhibits nearly complete last male sperm precedence (Wedell and Cook 1998), we assumed offspring from field-collected females to be full siblings. Siblings were split into independent treatment groups, with a subset sacrificed at emergence for measurements of ovary maturity, a proxy of reproductive timing. We measured the ability of butterflies to learn to locate either green or red hosts (see Snell-Rood et al. 2009). Correlations between learning ability and ovarian maturity at the family-level avoid confounding effects of the host experience itself on ovary development (see Experiment 3).

Ovary maturity at emergence

Experiment 1 (conducted June–September 2005) tested a total of 12 families from 5 populations (Nevada, Northern and Southern California, Michigan, and Massachusetts; controlling for "population" in analyses did not change the results). At least 4 butterflies in each family were sacrificed at emergence to estimate the number and size of oocytes in ovaries at emergence. All butterflies were stored in glassine envelopes encased in plastic containers at −4 °C, until dissection. Abdomens of butterflies were dissected in lepidopteran saline (Riddiford et al. 1979). All ovarioles were dissected and mature oocytes (fully yolked with a developed chorion) counted. Oocyte size was measured in 5–10 oocytes (mature or immature) within the distal 5 follicles. In total, 418 oocytes from 54 individuals of 12 families were measured. Because the size of immature oocytes is strongly correlated with position in the ovariole, we also recorded the follicle position for each oocyte. Models estimating oocyte size of a family (full-sibling group; $F_{11,350} = 33.2$, $P < 0.0001$) controlled for effects of follicle position ($F_{1,350} = 211.5$, $P < 0.0001$) and individual (nested within family; $F_{53,350} = 13.2$, $P < 0.0001$). Because a substantial number of butterflies sacrificed at emergence had no mature oocytes, the estimate of oocyte number was transformed for these butterflies by adding a constant of 2 units and taking the natural log, which resulted in a normally distributed measure of oocyte number. The (transformed) number of mature oocytes at emergence also varied by family ($F_{12,41} = 2.99$, $P < 0.001$).

Behavioral measurements

The siblings of butterflies used for ovary measurements were tested for host learning ability 2 days after emergence. Behavioral analyses for Experiment 1 are described in complete detail in Snell-Rood et al. (2009), but we summarize our measurements here. Host-searching behavior was assayed using standard observation techniques for butterflies (Snell-Rood and Papaj 2009). Mated gravid females were allowed to search for either a green host (kale, *Brassica oleracea* var. *viridis*) or a red host that also differed in size and shape (*Barbarea*

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vulgaris, grown in the sun). Hosts were distributed in either a simple nonhost environment (containing 8 green nonhosts of one species, *Mimulus guttatus*, and 8 individuals of one of the host species) or a complex nonhost environment (containing 16 green nonhosts of 4 species, *M. guttatus*, *Phlox subulata*, *Oxalis rubra*, and *Aristolochia fimbriata* and 4 individuals of one of the host species). Environments were chosen that were presumed to span a range of difficulty for host search, being easiest when hosts of the innately preferred color green (Hern et al. 1996) were presented in a simple nonhost environment and most difficult when red hosts were present in a complex nonhost environment. Choice of plants during host search was recorded for 1-h periods over 1–2 days. In between (and for 2 days prior to) these learning periods, butterflies were held in 1-m^3 cages in a greenhouse with access to mates and nectar sources.

We measured a butterfly's effectiveness in distinguishing hosts from nonhosts prior to landing. "Host-finding efficiency" was estimated as the proportion of all landings that were made on host plants, of all host and nonhost landings. An individual butterfly that made 4 host landings and 6 nonhost landings would receive a host-finding efficiency score of 0.4 (= 4/10). Host-finding efficiency was measured over all landings and, to control for time during a learning sequence, for consecutive bins of 10 landings made during host search by individual females. To maximize power (i.e., number of siblings observed per family), we focused on the first 20 landings on day 1 of host search and the first 10 landings on the second day of host search. We also considered behavior during the second 10 landings of the second day of host search when describing general patterns of behavior over time and between environments.

We measured learning as changes in host search behavior over time, with respect to an individual's initial naive behavior (i.e., first 10 landings on day 1). In particular, we focused on changes in host search efficiency, or performance, within the first day of host search and between the 2 days of host search. "Within-day change in host finding" was measured as the difference in host search efficiency between the second 10 landings of host search on day 1 and the first 10 landings of host search on day 1. "Between-day change in host finding" was measured as the difference in host search efficiency between the first 10 landings of host search on day 2 of host search and the first 10 landings of host search on day 1. Our measures represent short-term and long-term changes in behavior not distinct memory processes. For instance, within-day changes likely represent a composite of short-term, medium-term, and amnesia-resistant memory, whereas between-day changes likely represent a composite of amnesia-resistant and long-term memory (Margulies et al. 2005).

Experiment 2: manipulation of reproductive delay

In Experiment 2, we attempted to manipulate reproductive timing to provide an independent test of the association between timing and learning. At emergence, individuals were either treated with a JH mimic or, as controls, the hormone solvent alone; the learning ability of these individuals was then evaluated in greenhouse assays similar to Experiment 1, although several changes were made in the greenhouse assays (detailed below) that should considered when directly comparing the 2 experiments. In this experiment, we were controlled for genetic background and larval developmental conditions.

Manipulation of reproductive development

Experiment 2 (conducted March–May 2006) sampled 8 full-sibling families from 3 populations (Arizona, Northern California, and full-sibling families originating from a Carolina Biological laboratory population; controlling for population source in analyses did not change the results). The JH analog methoprene (VWR Inc., West Chester, PA) was used to manipulate ovarian development. For each full-sibling family, females were randomly assigned to 1 of 8 treatment groups, in a fully factorial design: individuals were either control or treatment individuals (with respect to hormone application) and were exposed to a red or green host in a simple or complex environment. One microliter of 1 μg/μl methoprene: acetone solution was topically applied to each treatment female's abdomen within 24 h of emergence. The same volume of acetone, without methoprene, was applied to each control female's abdomen. Females were then held in 1-m^3 greenhouse cages, with access to mates and nectar sources, for 2 days prior to observation of host-finding behavior.

Verification of hormone manipulation

Although previous studies suggest that topical methoprene application will significantly advance the timing of reproduction in a range of butterfly species (Karlinsky 1963; Benz 1970, 1972; Herman 1973, 1975; Herman and Bennett 1975; Herman et al. 1981), we wished to precisely quantify the effect of our hormone manipulation on *P. rapae* egg development. Thus, following Experiment 2 (January–March 2010), a subset of butterflies were reared from the Carolina Biological laboratory stock and treated with either 1 μl of 1 μg/μl methoprene:acetone solution (treatment individuals) or 1 μl of acetone within 24 h of emergence. Females were then held in a 1-m^3 flight cage with ad libitum access to food (15% honey water solution) and mates (all females included in the final analysis mated with at least one male). During this period, females had no exposure to host plants. Forty-eight hours after treatment, butterflies were sacrificed and stored at −20 °C. Individuals were dissected in 1× phosphate-buffered saline, and the number of mature eggs counted. Body size (hind wing area) was included in all analyses.

An additional verification of the hormone treatment was conducted by testing for differences between control and treatment butterflies in egg production during testing (see Experiment 2: Behavioral measurements) and following testing (see Experiment 3).

Behavioral measurements

In Experiment 2, control and treatment butterflies were allowed to search for 1 of 2 hosts in 1 of 2 nonhost environments. This experiment used 2 hosts that differed in color and brightness but not in leaf shape or nutritional quality to offspring (Slansky and Feeny 1977): green and red cabbage (*B. oleracea* var. *capitata*: Brassicaceae). Cabbages were relatively young (1.5–2 months old), and thus color differences were not as pronounced as in some previously described experiments (Snell-Rood and Papaj 2009). This change in the hosts used (relative to Experiment 1) allowed us to focus on color learning by minimizing olfactory and shape differences between hosts. Both number and density of nonhosts increased between the simple and complex treatments of Experiment 2. Both simple and complex treatments included red and green varieties of nonhosts so as to estimate color choice during host search. The simple treatment included red and green varieties of lettuce (Lactuca sativa: Asteraceae); the complex treatment included both varieties of lettuce in addition to red and green varieties of swiss chard (*Beta vulgaris* var. *cicla*: Amaranthaceae), basil (*Ocimum basilicum*: Lamiaceae), wood sorrel (*O. stricta*, *O. rubra*: Oxalidaceae), and gaura (*Gaura lindheimeri*: Onagraceae). The simple environment contained 16 nonhosts and 8 hosts; the complex environment

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included 40 nonhosts and 8 hosts. The changes in nonhosts in the greenhouse assay (relative to Experiment 1) allowed us to estimate color learning (through changes in the choice of green and red nonhosts) and also kept the absolute number of hosts the same between simple and complex treatments. The various greenhouse assay changes in Experiment 2 allowed us to focus on color learning but did limit direct comparisons between the 2 experiments.

Learning performance of control and treatment females was assayed using previously described behavioral observation techniques (see above for Experiment 1 and Snell-Rood and Papaj 2009; Snell-Rood et al. 2009). We primarily focused on changes in host-finding efficiency over time. Additionally, we assessed a butterfly's ability to vary the colors used when searching for hosts of different colors. "Color choice" was estimated as the proportion of all nonhost landings made by an individual that were on green nonhosts versus red nonhosts. Values greater than 0.50 indicate a preference for green over red (see Snell-Rood and Papaj 2009).

Because hormone treatments may affect a wide range of physiological and behavioral traits, we measured several components of fitness during the test. Treatment and control butterflies differed in overall landing frequency (treatment butterflies had fewer landings, see RESULTS); thus, all measures of fitness were a function of overall landings. First, we quantified "independent oviposition frequency," as the proportion of total landings consisting of independent host landings—a landing on a given host plant was counted as "independent" when it was separated by a landing on a different plant (either host or nonhost). This measure is analogous to the criteria for counting "host landings" in calculating host-finding efficiency (Snell-Rood and Papaj 2009), and thus, the measure of "total hosts" calculated in Experiment 1. Second, we quantified "repeat oviposition frequency" as the proportion of independent ovipositions that were followed by the female leaving the host plant but then immediately returning and ovipositing again on the same host plant. Third, we defined "batch oviposition frequency" as the proportion of ovipositions that were followed by the female immediately ovipositing on the same host plant without leaving the plant (a measure of laying eggs in clusters). Finally, we determined overall fitness during testing as the sum of all ovipositions (independent, repeat, and batch) divided by total host and nonhost landings during testing.

Experiment 3: induced costs of learning

In Experiment 3, we tested for induced, or environment-specific, costs of learning. A subset of butterflies that went through learning trials in Experiment 2 was held for egg collection for 4 days following behavioral testing. This allowed us to measure 1) effects of the learning process itself on overall fecundity (using control butterflies) and 2) take measurements of hormone treatment on lifetime egg production (using both control and methoprene-treated individuals) providing an additional verification that methoprene advanced reproductive timing.

Each butterfly was individually housed in a 2-l plastic container modified to be a cage. Each cage contained a 3-cm diameter section each of red cabbage, green cabbage, red-leaved lettuce (nonhost), and green-leaved lettuce (nonhost). Each plant was secured over a water-filled plastic cup using a rubber band; plants were removed and replaced daily. Every day, for 4 days after behavioral testing, all eggs were counted and removed from the cage. Each female had ad libitum access to 15% honey water, presented in a plastic cup filled with a red or yellow scrub sponge. Cages were kept humid with a wet-paper towel. Food was refreshed daily, and individual females were placed on their food each day to ensure they remained well fed.

We tested whether the experience of a butterfly was related to lifetime egg production. We considered not only the test environment (host color, nonhost complexity, see Experiment 2) but also changes in host-finding efficiency of each individual.

Statistical analyses

Family-level correlations between behavior and life-history traits were used to measure reproductive delay as a cost of learning (Experiment 1). For these analyses, estimates of a family's learning ability and host search behavior were taken from previous analyses (Snell-Rood et al. 2009). Briefly, mixed-model analyses of variance (ANOVAs) were performed in JMP 7.0, where "family" and "family by host color" were treated as random effects, whereas "host color" and "nonhost complexity" were treated as fixed effects. The least-square means from the family by host color effect were used to estimate a family's behavior in each host environment. Family-level measures of behavior were treated as dependent factors in tests of ovary characteristics at emergence. Body size (hind wing area, see Snell-Rood et al. 2009) of a family was included in each analysis: families exhibited significant genetic variation in body size (e.g., Experiment 1: $N = 123$, $F_{11,111} = 5.66$, $P < 0.0001$). ANOVA was used to test for effects of hormone treatment in Experiment 2 and testing environment in Experiment 3. All proportional measures of behavior were arc-sin square-root transformed for statistical analyses, although uncorrected values are presented in figures.

RESULTS

Experiment 1: correlations between ovary maturity at emergence and learning

Butterflies showed improvements in host-finding efficiency both within and between days, although this improvement was more pronounced in the red host relative to the green host environment (Figure 1; statistics presented in Table 2 of Snell-Rood et al. 2009). Host-finding efficiency was initially higher in the green host environment relative to the red host environment, but by the second day of host searching was comparable between host environments. Host-finding efficiency was consistently higher in the simple nonhost environment relative to the complex nonhost environment, but performance improved over time in both environments (Figure 1). The effect of nonhost complexity—a function of both nonhost density and diversity—on host-finding efficiency (Figure 1) was greater than in another experiment where these environments varied in only nonhost diversity (Snell-Rood and Papaj 2009).

Butterfly families that showed a greater between-day increase in host-finding efficiency in the red host environment were significantly more likely to emerge with smaller eggs and marginally significantly more likely to emerge with fewer mature eggs (Table 1 and Figure 2). The relationship between measures of ovary maturity and proxies of learning were specific to between-day changes in host-finding efficiency in the red host environment. There were no family-level correlations between egg number or size at emergence and total hosts located, changes in host-finding efficiency within days or changes in host-finding efficiency in the green host environment (Table 1 and Figure 2).

Experiment 2: manipulation of reproductive delay

In Experiment 2, changes in host-finding efficiency of untreated (control) butterflies improved over time in all host

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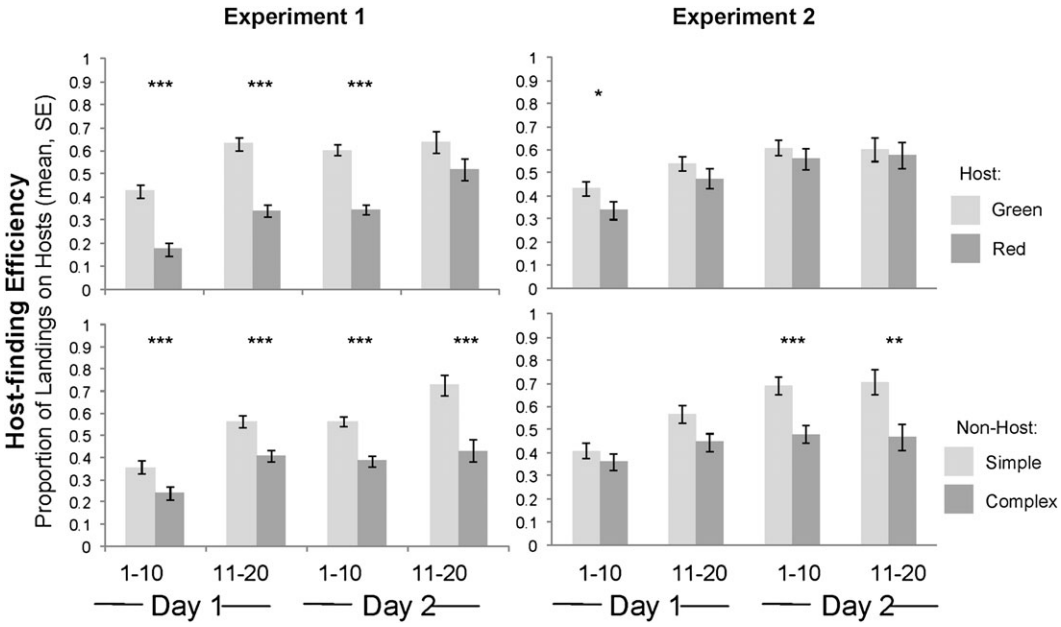

Figure 1
Patterns of host-finding efficiency over time. Shown are results summarized from Experiments 1 and 2 measuring butterfly host search behavior over time (data analyses from Experiment 1 are presented in Table 2 of Snell-Rood et al. 2009; data analyses from Experiment 2 are presented in Supplementary Table 1). Graphs present least-square means from ANOVAs that consider the effects of host color, nonhost complexity, and their interaction on host-finding efficiency (the proportion of landings on hosts) in environments that vary in host color or nonhost complexity. Significant and marginally significant differences between host environments are indicated by 3 ($P < 0.01$), 2 ($P < 0.05$), or 1 ($P < 0.10$) asterisks. Only butterflies with at least 20 landings on the first day and 10 landings on the second day of host searching were included in the analyses. For Experiment 2, only control butterflies were used in these analyses (not methoprene-treated individuals).

and nonhost environments (Supplementary Tables 1 and 2; Figure 1), similar to changes in performance observed in Experiment 1. Initially, host-finding efficiency was marginally higher in the green host environment but rapidly improved in the red host environment to a comparable level. Furthermore, host-finding efficiency was higher in the simple relative to the complex nonhost environment, although this difference was most pronounced on the second day of host searching (Supplementary Table 1; Figure 1). The effect of nonhost complexity—a function of both nonhost density and diversity—on host-finding efficiency (Supplementary Table 1; Figure 1) was greater than in another experiment where simple and complex environments varied in only nonhost diversity (Snell-Rood and Papaj 2009). The use of both green and red nonhosts also allowed us to quantify color choice in this experiment (proportion of nonhost landings on green nonhosts). Butterflies chose a higher proportion of green nonhosts than red nonhosts in the green host but not the red host environment (Supplementary Table 1 and Figure 1).

Table 1
Relationship between ovaries at emergence and a family's host finding

	Egg number		Egg size	
	Egg effect ($F_{1,9}$)	Body size ($F_{1,9}$)	Egg effect ($F_{1,9}$)	Body size ($F_{1,9}$)
Green, Δw/in:	1.78	0.10	2.38	0.06
Red, Δw/in:	0.43	0.53	0.20	0.45
Green, Δbtwn	1.38	0.50	0.65	0.35
Red, Δbtwn	3.58* (−)	13.6*** (−)	5.17** (−)	14.5*** (−)
Green, total	1.69	0.38	1.13	0.26
Red, total	0.37	1.16	0.28	1.26

Shown are F values from ANOVAs that test for effects of either egg number or egg size, while controlling for body size, on measures of learning and total hosts located. Siblings from 12 families (Experiment 1) were either sacrificed at emergence for ovary measurements or allowed to find either a red or green host plant in a simple or complex nonhost environment. Host finding was measured for the red and green hosts as either the change in host finding ability across 2 days or as the total hosts located, controlling for total landings. The slope of a significant effect is indicated with a (−) or a (+).
*$P < 0.10$; **$P < 0.05$; ***$P < 0.01$.

At day 2 of adulthood (when butterflies underwent behavioral testing), butterflies treated with methoprene at emergence had significantly more eggs in their ovaries relative to control females that were treated only with the solvent, acetone ($N = 13$, $F = 17.6$, $P = 0.002$; ANOVA controlling for body size; Supplementary Figure 2). We also compared egg production after behavioral testing, reasoning that if methoprene advanced reproduction, egg production should decrease at an earlier age in methoprene-treated butterflies relative to controls. In an ANOVA controlling for body size (hind wing area), family, host color, and nonhost complexity during learning, control and treatment butterflies did not differ in egg production just after host learning (day 5: $N = 86$, $P = 0.53$, Supplementary Figure 3). However, shortly thereafter, egg production dropped significantly in methoprene-treated butterflies (day 6: $N = 86$, $P = 0.009$; day 7: $N = 77$, $P = 0.002$), before dropping to comparable levels in control butterflies (day 8: $N = 69$, $P = 0.47$). Taken together, these results suggest that our hormone treatment significantly advanced the timing of reproduction.

Hormone treatment had significant effects on measures of host learning. Methoprene-treated butterflies were less likely to improve host-finding efficiency across the 2 days of host search relative to controls (Figure 3, Table 2). However,

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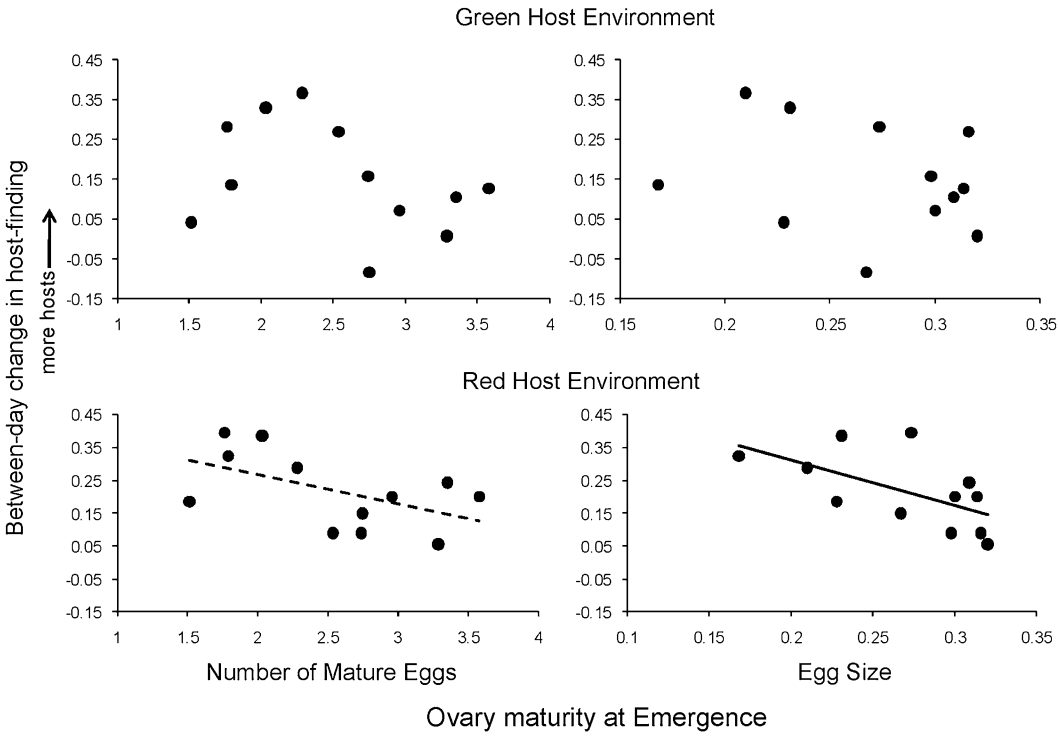

Figure 2
A family's reproductive investment at emergence is related to change in host-finding efficiency in red host environment. Shown are leverage plots from ANOVAs including body size and ovary maturity. Each point represents a group of full-sibling butterflies (Experiment 1). Reproductive investment at emergence was measured as the total number of mature oocytes and the average size of eggs in the most distal ovariole positions. Behavior was measured as the change (across 2 days of learning) in host-finding efficiency (the proportion of landings on hosts). Solid lines represent $P < 0.05$; dashed lines represent $P < 0.10$.

hormone treatment did not affect measures of learning within the first day of host search or changes in color choice (between or within days; Figure 3 and Table 2).

We also tested whether hormone treatment had effects on overall measures of fitness (oviposition frequency) during testing. Because methoprene-treated butterflies had more total landings during testing relative to control butterflies (Table 3; mean [standard error]: methoprene = 22.0 [1.8], control = 37.7 [1.3]), all measures of fitness during testing were corrected for overall landings. Hormone-treated butterflies had a significantly lower "independent oviposition frequency," a function of ovipositions on host plants separated by landings on other plants (Table 3 and Figure 4), analogous to the fitness measure used in Experiment 1 (see Table 1). However, hormone-treated butterflies were more likely to repeat ovipositions on the same host plant, without landing on different host or nonhost plants in between (Table 3 and Figure 4). When all ovipositions were tallied (independent, repeat, and batch), total oviposition frequency (ovipositions/total landings) was not significantly different between hormone-treated and control butterflies (Table 3).

Experiment 3: induced costs of learning

In Experiment 3, we tested for effects of the learning experience itself on lifetime fecundity. Butterflies (control individuals from Experiment 2) were held for 4 days following testing and all eggs laid on both red and green hosts were counted. Butterflies that had searched in the red host environment, relative to those in the green host environment, had significantly lower lifetime fecundity (Table 4 and Figure 5). Butterflies that had searched for hosts in the complex nonhost environment, relative to those that had searched in the simple non-host environment, also had significantly lower lifetime fecundity (Table 4 and Figure 5).

There was also a relationship between the amount an individual butterfly learned and lifetime fitness. Controlling for host color, nonhost complexity, body size, and family, there was a negative relationship between an individual's within-day change in host-finding efficiency and their lifetime fitness (Figure 6; $N = 25$, $F = 4.50$, $P = 0.05$). However, there was no relationship between an individual's between-day change in host-finding efficiency and their lifetime fitness ($N = 16$, $F = 0.05$, $P = 0.82$).

DISCUSSION

Family-level correlations between ovary maturity at emergence and learning

Learning and cognition have long been hypothesized to be a driving force in the evolution of life-history traits, including the timing of reproduction (Mayr 1974; Johnston 1982; Dukas 1998; Kaplan et al. 2000; Kaplan and Robson 2002; Ricklefs 2004). Our family-level study supports this hypothesis by providing an explicit link between learning performance per se and the timing of reproduction. Butterfly families that emerged with relatively less well-developed ovaries—as measured by both total number of mature eggs and size of oocytes at emergence—showed relatively more improvement in host-finding ability across successive days of host experience (Figure 2 and Table 1). This link was specific to the red host—and not the green host—environment, where color learning has been shown to be used in locating hosts (Supplementary Figure 1; Snell-Rood and Papaj 2009).

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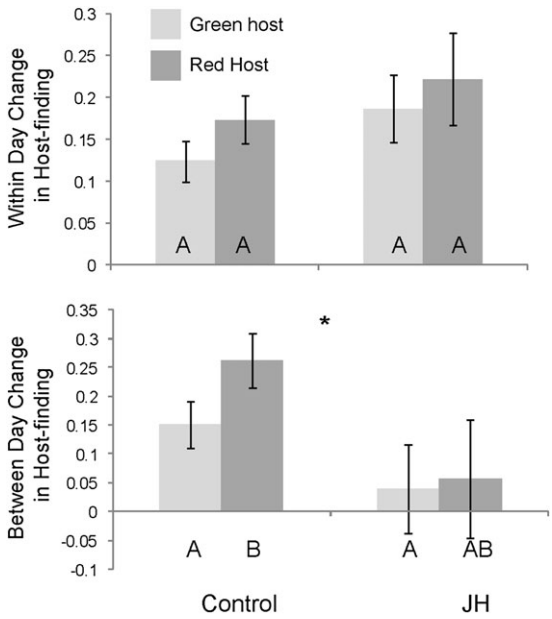

Figure 3
Artificial advancement of reproduction alters learning across days but not within days. In Experiment 2, treatment butterflies were treated with methoprene (JH mimic) upon emergence as adults, advancing their reproduction, whereas control butterflies were treated only with the solvent acetone. Behavior was measured as the change in host-finding efficiency (the proportion of landings on hosts) within the first day of host search or between the 2 days of host search. Shown are least-square means (and standard errors) from ANOVAs controlling for host color and nonhost complexity.

The family-level relationship between learning ability and ovarian maturation is consistent with the notion of a fitness trade-off between these 2 traits. Johnston (1982) proposed such a trade-off over 25 years ago, offering several nonmutually exclusive explanations by which the evolution of learning might result in delays in reproduction. One explanation focused on allocation of resources in development. Johnston reasoned that learning may incur costs which cause resources to be shunted away from reproduction and into processes related to learning. A growing body of research suggests that learning does indeed have costs (Mery and Kawecki 2003, 2005). In *P. rapae*, learning entails costs in terms of brain size:

Table 2
Effects of reproductive manipulation on measures of learning

	N	JH	Host color	Complexity	Color × JH	Family
Δhost w/in	183	2.01	1.21	10.2**	0.03	2.48**
Δcolor w/in	183	3.66	1.12	1.37	0.10	1.78
Δhost btwn	96	5.38*	0.85	5.37*	0.44	1.34
Δcolor btwn	96	0.13	0.10	0.12	0.51	1.69

Shown are *F* values from ANOVAs which include effects of host color (green or red), nonhost complexity (simple or complex), family (full-sibling group), and JH treatment (methoprene application or control) on measures of learning and fitness. Changes in host finding and color choice are measured across the 2 days of learning (the difference in behavior between the first 10 landings of each day) or within the first day of learning (the difference in behavior between the first and second bin of 10 landings).
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 3
Effects of reproductive manipulation on measures of fitness

	N	JH	Host color	Complexity	Color × JH	Family
Total Ldgs	383	50.7***	5.02*	1.41	0.04	5.31*
Ind. Ovi.	383	5.37**	78.5***	65.7***	2.14	3.79***
Rep Ovi.	352	13.4***	10.9**	0.14	4.16*	0.98
Batch Ovi.	352	0.01	1.47	0.07	0.0001	17.9***
Total Ovi.	370	0.08	50.2***	49.2***	1.25	8.35***

Shown are *F* values from ANOVAs which include effects of host color (green or red), nonhost complexity (simple or complex), family (full-sibling group), and JH treatment (methoprene application or control) on measures of learning and fitness. Total landings refer to all landings made during testing on either a host or a nonhost. "Independent Oviposition frequency" (Ind. Ovi.) is measured as the proportion of total landings that were on independent hosts (i.e., landings on a given host were separated by landings on other plants). "Repeat Oviposition frequency" (Rep. Ovi.) was measured as the proportion of independent ovipositions that were followed by the female leaving a given host and then immediately returning to (and ovipositing on) that host without landing on a different plant in between. "Batch Oviposition frequency" (Batch Ovi.) refers to the proportion of independent ovipositions that were followed by the female ovipositing on the same host, without leaving the plant. "Total Ovipositions" refers to the sum of all ovipositions (independent, repeat, and batch) divided by total landings.
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Learning ability of a family is associated with greater neural investment at emergence (Snell-Rood et al. 2009). Although

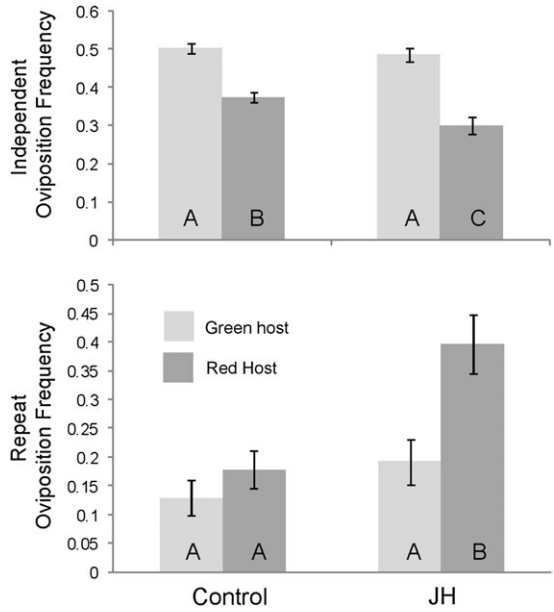

Figure 4
Effects of hormone manipulation on oviposition frequency. In Experiment 2, methoprene-treated and control animals searched for hosts in either a red or a green host environment. "Independent oviposition frequency" refers to the proportion of total landings that were on independent hosts (i.e., landings on a given host were separated by landings on other plants, analogous to "total hosts" of Experiment 1). "Repeat oviposition frequency" refers to the proportion of independent ovipositions that were followed by the female leaving a given host and then immediately returning to that host without landing on a different plant in between. Shown are least-square means from a model that controls for host color, nonhost complexity, and full-sibling family (see Table 3).

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Table 4
Lifetime fecundity varies with learning experience

	Host	Complexity	Family	Body size	*N*
Post-test eggs	5.14**	7.79***	2.91**	0.96	47
Lifetime eggs	5.93**	7.97***	3.09***	0.40	47

Shown are *F* values from ANOVAs which include effects of testing environment (see below), full-sibling family, and body size (hind wing area) on measures of lifetime fecundity. In Experiment 2, female butterflies learned either red or green hosts (Host) in a simple or complex nonhost (Complexity) environment. In Experiment 3, these individuals were then allowed to oviposit freely on red and green hosts for 4 days after learning to estimate lifetime fitness. Post-test fecundity represents the number of eggs laid on red and green cabbage for 4 days after testing while lifetime fecundity also includes the number of eggs laid during the testing itself.
*$P < 0.10$; **$P < 0.05$; ***$P < 0.01$.

direct evidence is lacking, it is conceivable that the additional neural tissue associated with better host learning performance in cabbage whites causes delays in ovarian maturation.

Interestingly, we found no correlation between ovary status at emergence and measures of changes in performance within the first day of host searching. Our measures of learning represent a composite of memory processes (short-term, medium-term, and long-term memory; see Margulies et al. 2005), but the between-day changes in performance are at least somewhat underlain by long-term memory while within-day changes are not. Thus, our results linking changes in performance across days, but not within days, are reminiscent of the idea that long-term memory, which involves protein synthesis and morphological changes at the synapse level (Lamprecht and LeDoux 2004), is costlier than short-term memory (Mery and Kawecki 2005).

In this experiment, the primary fitness trade-off associated with family-level learning ability was a delay in reproduction. Just how costly is a delay in reproduction? Because lepidopteran species mature a large proportion of their eggs during adulthood—many using adult-acquired resources—it is unclear just how long into adulthood our observed reproductive delay is relevant (O'Brien et al. 2002; Jervis et al. 2005). Regardless, in this butterfly system, where adult life span in the field is typically less than 14 days (Suzuki 1978), delaying reproduction by even a day or 2 likely represents a significant cost. Individuals that delay reproduction should have a lower chance of surviving to maximal reproduction. Additionally, delaying reproduction may lead to missed opportunities due to egg limitation upon emergence (Rosenheim et al. 2000; Jervis et al. 2001)—for instance, butterflies may encounter host plants during their first day of adulthood, but ovary immaturity may preclude the use of these plants.

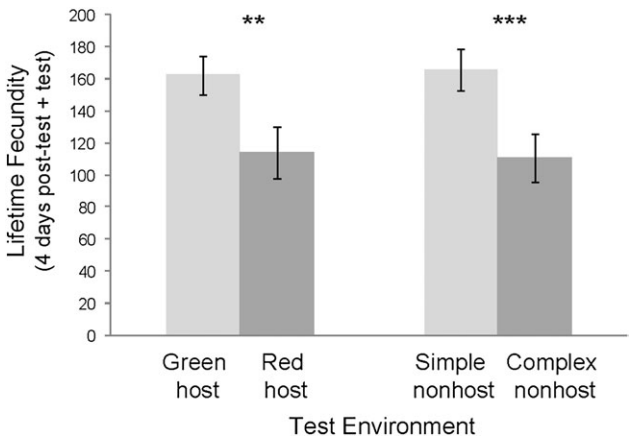

Figure 5
Effects of test experience on lifetime egg production. In Experiment 3, control butterflies from Experiment 2 were held for 4 days after testing with access to both red and green hosts. Total lifetime fecundity was quantified as the total number of eggs laid during these 4 days plus total ovipositions during behavioral testing. Test experience refers to the environment during host searching (red or green host nested within a simple or complex nonhost environment). Shown are least-square means from an ANOVA that also controls for full-sibling family and body size (see Table 4).

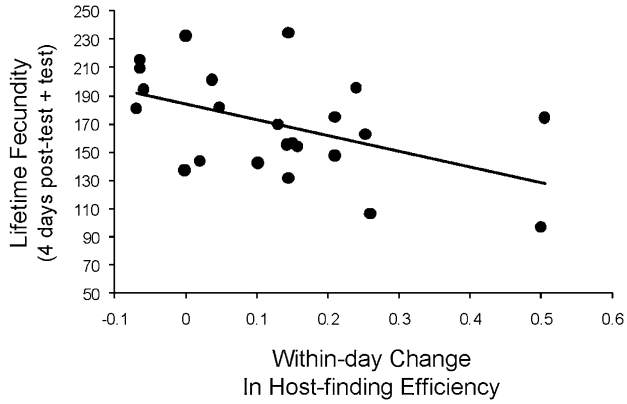

Figure 6
Effects of learning on lifetime fecundity. In Experiment 3, control butterflies from Experiment 2 were held for 4 days after testing with access to both red and green hosts. Total lifetime fecundity was quantified as the total number of eggs laid during these 4 days plus total ovipositions during behavioral testing. The *x* axis shows the learning performance of an individual butterfly during behavioral testing (within-day change in host-finding efficiency). Shown is a leverage plot from a model that also controls for host color, nonhost complexity, full-sibling family, and body size. Learning performance was significantly related to lifetime fitness ($N = 25$, $F = 4.50$, $P = 0.05$).

Although reproductive timing was correlated with changes in performance across days, we found no correlation between ovary maturity at emergence and our measure of overall fitness (total host landings across both days of host searching, corrected for total landings, Table 1). This was a surprising result given that a separate experiment showed that the change in host-finding efficiency between days of host searching was a significant contributor to overall fitness in the red host environment (Snell-Rood and Papaj 2009). There were 3 main differences in the host search environment between these 2 experiments, including 1) the inclusion of both red and green nonhosts versus only green nonhosts, 2) the ratio of hosts to nonhosts, and 3) the degree of color difference between green and red nonhosts. Taken together, these differences suggest that host searching in the present experiment (Experiment 1) occurred under less challenging search conditions than those in Snell-Rood and Papaj (2009) such that the relative benefits of learning (higher fitness in the red host environment) were less pronounced. Although future work will be needed to support this assertion, this discussion underscores the importance of considering the host environment(s) in which selection is occurring. The costs of delayed reproduction may sometimes be offset by the benefits of learning (e.g., in the red host environment), whereas in other conditions (e.g., green host environments), the global cost of

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reproductive delay is experienced, but with few offsetting benefits of learning (Figure 1; Snell-Rood and Papaj 2009). Thus, the competitive outcome between learning and nonlearning genotypes will depend on a range of factors such as host distribution over time and space and butterfly life span.

Artificial advancement of reproduction decreases learning

We used hormonal manipulations to artificially advance reproduction and provide a separate test of the link between reproductive timing and learning ability. Previous studies on butterflies, and in particular those in the genus *Pieris*, suggested that topical treatment with methoprene, a JH analog, could be used to advance reproduction in *P. rapae* (Karlinsky 1963; Benz 1970, 1972; Herman 1973, 1975; Herman and Bennett 1975; Herman et al. 1981). We found that methoprene application did indeed advance egg maturation. Individuals treated with hormone at emergence had, on average, twice the egg load of control females 2 days later (Supplementary Figure 2). Furthermore, when hormone-treated and control butterflies were held for 4 days after behavioral testing, egg production of hormone-treated butterflies dropped off earlier than that of control butterflies (Supplementary Figure 3). These results suggest that our level of hormone application significantly advanced the timing of egg development in *P. rapae* such that egg production peaked earlier in adulthood in treatment relative to control individuals. Because these manipulations occurred prior to, and independent of the host experience, we feel it simulates a manipulation of an environment-independent, constitutive (or global) reproductive trade-off.

Hormone application was accompanied by significant changes in learning performance. Specifically, hormone-treated butterflies, relative to controls, were less likely to improve host-finding efficiency across the 2 days of host searching (Figure 3 and Table 2). However, there was no difference between control and treatment butterflies in changes in host-finding efficiency within the first day of host searching (Table 2). The specificity of this effect to between-day changes in performance paralleled the result of the family-level correlations, where ovary maturity at emergence was related to between-day but not within-day changes in performance (Table 1). The parallel result linking between-day changes in behavior and reproductive delays emerged between experiments despite the differences in host and nonhost species used in greenhouse assays (see MATERIALS AND METHODS), suggesting that this result applies to learning more generally, not certain plant species.

Treatment and control butterflies also differed in their patterns of oviposition across different hosts. Over all landings, control butterflies had significantly more independent host ovipositions relative to treatment butterflies (Table 3 and Figure 4). In contrast, treatment individuals had significantly more repeat ovipositions—an independent oviposition event followed by leaving the host and then immediately returning to (and ovipositing on) the same host without landing on another plant in between (Table 3 and Figure 4). Given that oviposition on separate plants should decrease larval competition and contribute to the "risk-spreading" strategy of *Pieris* (Root and Kareiva 1984; Kivela and Valimaki 2008), these "repeat ovipositions" may be costly. However, overall, total number of ovipositions during testing (corrected for total landings made) did not differ between control and treatment butterflies (Table 3).

Taken together, these behavioral observations suggest that artificial advancement of reproduction affects learning performance directly. The comparable total fitness between methoprene-treated and control butterflies suggests the 2 groups had comparable host-seeking motivation. However, methoprene-treated individuals had a selective reduction in between-day learning ability. This reduction may have reduced independent oviposition frequency, which butterflies compensated for by repeating ovipositions on a given host plant. The fact that these changes in fitness are more pronounced in the red host environment (Figures 3 and 4), where learning is more pronounced (Figure 1; Snell-Rood and Papaj 2009), further implicates the effect of hormone treatment on learning. These results are consistent with the idea that advancement of reproduction trades off against investment in processes or structures necessary for learning, such as neural tissue. Indeed, in a separate analysis, 2 days after emergence (at the time when host searching began in our experiments), hormone-treated animals, relative to controls, tended to have smaller absolute ($N = 7$ individuals; $F_{1,5} = 17.6$, $P = 0.008$) and relative mushroom body volume ($N = 6$ individuals; $F_{1,3} = 7.95$, $P = 0.06$; Snell-Rood EC and Gronenberg W, unpublished data; see methods in Snell-Rood et al. 2009). Future work on more individuals will be required to validate this observation.

Our results suggest that, due to energetic tradeoffs, hormone application has indirect (negative) effects on learning stemming from its direct (positive) effects on reproduction. However, it is important to note that JH regulates many aspects of insect physiology (Wyatt and Davey 1996; Flatt et al. 2005). It is possible that hormonal application also has direct effects on learning and even that our results reflect the JH-mediated coordination of a suite of traits (Flatt et al. 2005). JH has known effects on neural development and learning in other systems such as crickets, where it stimulates neural growth (Cayre et al. 1994). In adult honeybees, increased JH titers are associated with the development of foraging and learning in adult workers (Robinson and Vargo 1997), although in this case, the association was not due to direct effects of JH on neural growth (Fahrbach et al. 1998). Our results echo a recurrent theme across these studies that JH has diverse roles in regulating life history and behavior in insects (Nijhout 1994). Indeed, JH affects short-term memory and not long-term memory in honeybees (Maleszka and Helliwell 2001), which differs from our results. Further studies integrating hormone titer measurements and manipulations (Zera et al. 2007) may have implications for understanding the mechanistic basis of the observed genetic variation in learning and reproduction. In the meantime, our results must be interpreted cautiously, knowing that hormonal manipulations have complex effects on suites of traits.

Additional induced costs of the learning process

Our results suggest that learning ability comes with constitutive fitness trade-offs in the form of reproductive delays. These trade-offs are expressed regardless of the environment in which learning occurs and thus represent global costs of learning, which can explain the maintenance of genetic variation in learning and plasticity. We were also interested in whether there were additional induced costs of the learning process. Given that such costs are specific to the environment in which the benefits of learning are experienced, these costs should, in theory, play less of a role than global costs in limiting the evolution of learning (see discussion in DeWitt et al. 1998). We found that butterflies with host searching experience in the red host environment (relative to the green host environment) and the complex nonhost environment (relative to the simple nonhost environment) had significantly lower lifetime fitness (Table 4 and Figure 5). For instance, butterflies with experience searching for red hosts had a 30% reduction in lifetime fitness relative to individuals with

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experience searching for green hosts (Figure 5). The red host and complex nonhost environments are comparatively more "difficult." A lower host-finding efficiency in these environments can potentially be compensated for by greater investment in learning. For instance, initial host-finding efficiency in the red host environment, relative to the green host environment, can be erased after 10–20 landings in that environment (Figure 1, Snell-Rood and Papaj 2009); indeed, neural investment increased in butterflies with experience in the red host or complex nonhost environment (Snell-Rood et al 2009). Thus, the lower fitness due to experience in the red host and complex nonhost environments may be a direct consequence of increased investment in learning. This interpretation is supported by the correlation between an individuals' change in host-finding efficiency and their lifetime fecundity (Figure 6). The fact that this correlation was specific to changes in performance within days, as opposed to between days, suggests that these induced environment-specific fitness trade-offs associated with learning may be fundamentally different from the global constitutive trade-offs associated with learning observed in Experiments 1 and 2.

It is known that the learning process itself can result in fitness trade-offs (Mery and Kawecki 2004). These results add to this work by suggesting that both induced and constitutive fitness trade-offs of learning can be acting simultaneously and that they may be tied to separate components of learning such as short-term versus long-term memory processes.

Alternate hypotheses: learning as an indirect result of life-history variation

The observed correlation between learning and reproductive delays supports the hypothesis that learning is costly and results in direct life-history tradeoffs. However, it is also possible that learning may co-vary with reproduction as an indirect result of life-history variation. There are several mechanisms by which this can occur. First, a delay in reproduction may be a strategic response to a learning strategy that does not necessarily result from the costs of learning, a scenario that Johnston (1982) put forward. When what is learned is relevant to reproduction in particular, a delay in reproduction may be strategic if successful reproduction is better assured after sufficient experience is gained. If performance improves over time (e.g., Figure 1) and early egg maturation trades off against fitness later in life, learning genotypes that find hosts slowly at first might benefit by maturing ovaries later than nonlearning genotypes. Second, studies in a variety of insects suggest that individuals with more eggs to lay are less discriminating (Heimpel and Rosenheim 1998; Papaj 2000). Butterflies from families with advanced reproduction—for instance, hormone-treated individuals—might assign less weight to experience and consequently sample more. By sampling the environment more widely, they may discover just as many host plants as a more discriminating butterfly from a family of good learners. This hypothesis would predict that hormone-treated butterflies made more landings during oviposition trials; however, this was not the case (Table 3).

These alternative explanations bring up the general point that studies linking learning to life-history variation should carefully interpret correlations. We complemented our correlative approach in Experiment 1 with a manipulation of reproductive delay in Experiment 2 to infer causal links between timing and learning. However, most manipulations of life-history traits invariably manipulate suites of life-history traits, again complicating interpretations. A promising avenue of future research would be contrasting individuals with and without large pools of available energy. Any energetic trade-off between learning and reproduction early in life should be more pronounced in energy-stressed individuals (or families). Regardless, future work will help to clarify alternate hypotheses.

Conclusions

In conclusion, our results support the hypothesis that learning should directly trade off with components of life history such as early reproduction and total fecundity. We used both correlative and manipulative approaches to link learning and reproductive timing within a species, complementing a wide range of existing comparative studies linking life history and proxies for learning (brain size). Our study is the first to demonstrate explicit links between variation in learning per se and delays in reproduction. Our results also highlight an additional induced cost of the learning process itself in terms of a direct reduction in lifetime fecundity. This study emphasizes that learning ability comes with global costs and the learning process itself comes with separate induced costs.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at http://www.beheco.oxfordjournals.org/.

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