

EXPLORING VARIATION IN LEARNING ABILITY IN *PIERIS RAPAE*, THE CABBAGE
WHITE BUTTERFLY

A Master's Thesis
submitted to the Faculty of the
Graduate School of Arts and Sciences
of Georgetown University
in partial fulfillment of the requirements for the
degree of
Master of Science
in Biology

By

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Washington, DC
May 22, 2013

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ABSTRACT

Learning abilities allow animals to modify their behaviors based on experience; such plasticity provides an adaptive mechanism for responding to environmental unpredictability. As with any heritable trait subject to natural selection, learning ability can vary both within and across insect species, suggesting costs to maintaining the ability to learn. In this thesis I explored two different aspects of variation in learning ability in the Cabbage White Butterfly, *Pieris rapae*. First, I evaluated whether *P. rapae* learn two different visual stimuli (color and shape) at the same rate and proficiency in a foraging context. While learning abilities of different stimuli within a given sensory modality have been studied in other insects, visual learning abilities aside from color in butterflies have received little attention. I found that while *Pieris rapae* is capable of learning shape in association with a food reward, color is a more salient cue in a foraging context. Second, I compared life history traits, foraging behavior, and learning abilities of a wild and a laboratory population of *P. rapae* to assess how differences in both genetic makeup and environment can alter phenotypes within a species. I found that the two populations differ in timing of development for several life stages, and that foraging behavior and learning abilities diverged based on population origin. The findings from these two studies provide new evidence for how variation in learning abilities depends on type of stimulus, and can also be affected by environmental pressures and genetic processes.

The research and writing of this thesis
is dedicated to my family, who have been an incredible support during my graduate career.

Many thanks,
Lillian D. Power

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Introduction

The ability to interact with dynamic, unpredictable environments is crucial to an organism's survival and fitness. Learning ability, one of the most common mechanisms for dealing with environmental stochasticity, is found across the animal kingdom, from large, long-lived mammals to small, short-lived insects. Broadly defined as the acquisition of new neuronal circuitry to represent new information stored in the brain, learning occurs when an animal interacts with the surrounding environment and adapts its behavior based on experience (Dukas 2008, Matthews & Matthews 2010). Such alterations in behavior can have large impacts on an organism's fitness, as efficient use of resources and manipulation of ones' environment is important for everything from predator avoidance to mate and food location (Hinton & Nolan 1987, Matthews & Matthews 2010, Barbosa & Castellanos 2005, Kamil & Roitblat 1985).

While insect behavior was long regarded as being primarily driven by instinct (Fabre et al. 1918), research on a multitude of species has disproved this idea. Just as it is important for long-lived vertebrates to learn about their surroundings in order to adapt to unpredictability over both the short and long term, short-lived animals such as insects must also be able to process and interpret environmental information quickly in order to adapt behavior to maximize their fitness. As processing and incorporating external information into short-term memory takes a matter of moments (Burns et al. 2011, Foucaud, & Mery 2011, Dukas & Visscher 1994), animals can use new information from their environment regardless of life span (Carew&Sarley 1986, Dukas 2008, Krasne & Glanzman 1995).

Foraging behavior has been of particular interest in the insect learning literature, as quantity and nutritional quality of food can have great impacts on fecundity and survival (Dukas & Bernays 2000). Insect pollinators are particularly important for maintaining floral diversity, as the success of the plants they pollinate is intimately tied to their own fitness. Up to 87% of angiosperm species worldwide are pollinated by insects, and 75% of global agricultural crop species (translating to 35% of global food production) are estimated to be pollinated by insects and other animals such as birds and bats (Klein et al. 2007, Ollerton et al. 2011). Pollination success is critical for crop productivity, thus making these insects extremely important in terms of global biodiversity as well as human quality of life. Thus, understanding how pollinators make foraging decisions can provide insight into reproductive and evolutionary success of both plants and pollinators, an interesting area of research from theoretical as well as applicable perspectives (Vet et al. 1995, Ferdy et al. 1998, Lewis 1993).

While locating nectar sources may seem like a relatively easy task for an insect, particularly in habitats containing a multitude of floral choices, research has shown that the decision-making process is not so simple. Each plant species has a unique floral morphology, and a pollinator must successfully identify and process a complex array of cues, such as floral color, scent, shape, and height, in order to receive a nectar reward. In order to extract nectar, an insect must learn how to navigate the morphology of a flower, which is different for each flowering plant species. As such, learning to associate floral cues with the presence of a reward could increase foraging efficiency if it allows the pollinator to locate a new flower of the same species and quickly extract the nectar.

Floral constancy, in which an insect that has successfully fed from a particular floral species is more likely to continue visiting that species than other available floral options (Gegeer & Lavery 2001, Kunin & Iwasa 1996, Waser 1985), has been reported for a variety of insects, including bees and butterflies. One can infer that 'constant' pollinators perceive and attend to cues emitted by the flower in order to make foraging decisions. Which cues are used, and their relative importance to different pollinators, has been of considerable interest to entomologists, agricultural scientists, and evolutionary biologists alike (Fenster et al. 2004, Hebets & Papaj 2005, Raguso 2004).

Eusocial insects such as honey bees and bumble bees have been a major focus of research in foraging behavior of insect pollinators. Honey bees have been of particular interest given their rigid caste system and the hive's dependence on the foragers to procure enough food to maintain the entire colony. The ability to learn various stimuli, such as color and odor, has been explored in detail in a foraging context in these insects. Bees can learn a range of colors in association with a food reward, but the rates of learning vary based on a hierarchy of preferred colors for this species; violet is learned most rapidly and blue-green most slowly. Such hierarchies in color preferences have since been shown to exist in both social and solitary insects, though preferred colors vary between species (Menzel 1985, Blackiston et al. 2011, Briscoe & Chittka 2001). Hymenopterans can learn visual patterns, locations, and shapes in association with food rewards (Campan & Lehrer 2002, Cartwright & Collett 1983, Giurfa et al. 1999, Gould 1986, Lehrer et al. 1995). Olfactory learning has also been studied in insects, showing that bees, moths, and a wide range of insect pollinators can learn scents associated with

the location or presence of food (Bitterman 1983 et al., Menzel et al. 1990, Ray & Ferneyhough 1999, Wright & Schiesti 2009).

Solitary insects have traditionally taken a backseat in the literature on pollinator learning, as eusocial insects have conventionally been viewed as better learners. Yet one could make the argument for the alternative (Weiss 2001). While social insects have a highly organized caste system in which each member of the colony has one distinct task at a given time (Winston 1991), solitary insects must forage, locate mates and oviposition sites, and avoid predators more or less simultaneously. Solitary insects such as butterflies have been shown to exhibit the same floral constancy so often seen in honey bees and other hymenopterans, signifying their ability to learn and use floral cues for efficient foraging (Blackiston et al. 2011, Briscoe & Weiss 2011, Goulson & Cory 1993, Goulson, Ollerton & Sluman 1997, Lewis 1989, Weiss 1997). Several butterfly species have been shown to be able to learn color as well as scent in association with food rewards in a manner similar to bees, further supporting the importance of floral recognition and choice for foraging pollinators regardless of sociality (Andersson & Dobson 2003, Omura & Honda 2005, Raguso & Schiesti 2009, Wright & Schiesti 2009). While odor learning has not been as extensively researched within Lepidoptera compared to Hymenoptera, multiple studies on hawkmoths show they are capable of learning odor paired with nectar reward (Balkenius & Kelber 2006, Riffel et al. 2008). Unlike the honeybees, visual cues aside from color have not been explored in detail in the Lepidoptera learning literature in a foraging context, though some studies conducted suggest an ability to learn other visual cues, such as pattern and shape in these insects (Kelber 2002, Allard & Papaj 1996, Weiss unpublished). For example, hummingbird hawkmoths exhibit innate preferences

for particular floral patterns, but can be trained to non-preferred patterning while foraging (Kelber 2002), Monarch butterflies can be trained to recognize some floral patterns in a foraging context, and *Battus* butterflies have been shown to learn leaf shape in an oviposition context (Allard & Papaj 1996, Cepero et al, submitted).

The tremendous diversity in certain floral characteristics, such as color and odor, have received considerable attention in pollinator learning studies. While insect pollinators are able to distinguish and learn floral color and scents, the relative importance of other cues, such as shape, are not as well understood. Floral shape could be important for foraging insects for multiple reasons. Petal or corolla shape can be a distinguishing factor for different floral species and may therefore be useful to pollinators who show high specificity in their floral choices. In addition, shape can change with floral size and may signal differences in floral quality. Torn petals or leaves alter floral shape and may be used by pollinators as a measure of nectar availability. Understanding whether insect pollinators use shape as a distinguishing factor while making foraging decisions could shed light on insect decision-making processes. The presence of shape learning has been found in a foraging context for honey bees (Lehrer et al. 1995) and in an oviposition context for butterflies (Allard & Papaj 1996), but shape learning in butterflies has rarely been explored in a foraging context (Cepero et al. submitted). While evidence would suggest that butterflies should be able to learn shape in association with a food reward, concrete experimental evidence is needed.

Individual Variation In Learning

As with any heritable trait subject to natural selection, individuals vary in learning ability, and several researchers have found genetic variation in learning abilities in insects.

Brandes has documented heritable variation in learning ability in Cape honey bees (Brandes 1988, Brandes 1991). Snell-Rood has found family-level effects of learning ability in oviposition search in the Cabbage White Butterfly, *Pieris rapae* (Snell-Rood et al. 2009). In addition, selection for learning abilities over numerous generations has resulted in the creation of lines that differ dramatically in learning abilities. Mery and Kawecki have selected for learned avoidance of oviposition substrate associated with an aversive odor for *Drosophila melanogaster*, and McGuire and Hirsch conducted a selection experiment in which they conditioned different lines of blow flies to either a saline solution or water (McGuire & Hirsch 1977, Mery & Kawecki 2003). Both studies showed large changes between lines selected for learning compared to lines undergoing no selection for learning, indicating a heritable component to learning.

Differences in learning between individuals can be measured in various ways (Blackiston et al. 2011, Dukas 2008). Learning abilities can be defined as the level of proficiency (maximum percent correct response) an individual and/or population reaches given a certain amount of time. Learning proficiency can be calculated by measuring the number of landings on a trained stimulus, or time spent probing the trained stimulus, in the absence of the associated reward or punishment (Blackiston et al. 2011, Briscoe & Chittka 2001). Rate of learning can be assessed by measuring the time it takes for an individual to reach a certain criterion of proficiency on the trained stimulus. Confusion is the extent to which an individual visits a non-trained stimulus (Blackiston et al. 2011). These measures have been used repeatedly in the literature to compare learning between individuals or populations (Blackiston et al. 2011, Goulson & Cory 1993, Kandori et al. 2009, Snell-Rood et al. 2009, Weiss 1997, Weiss & Papaj 2003).

Costs to Learning

Many biologists have proposed a cost to learning as an explanation for the variation in learning abilities we see across the animal kingdom (Johnston 1982). Costs to learning can be considered constitutive or induced (Burns et al. 2011). Constitutive costs are always incurred whether or not learning takes place in an individual's lifetime. Large brain size and the complex neuronal circuitry required for learning and memory require energy expenditure that may otherwise be invested in other important traits. Induced costs, in contrast, occur during the process of learning itself, as learning requires at least the temporary building of connections between neurons, as well as growth of new neurons in the brain (Burger et al. 2008). Therefore, we might expect life history trade-offs between learning and other important fitness traits.

Though the concept of such trade-offs is commonly mentioned in the learning literature, proving the existence of these trade-offs has been difficult. Correlational studies have shown a constitutive trade-off between learning ability and survivorship in *Drosophila melanogaster* (Burger et al. 2008). The strongest evidence for trade-offs between learning and other traits comes from Mery and Kawecki, who found that *Drosophila* that had been selected for higher adult learning abilities exhibited reduced larval competitive ability when food supply was low, signifying a constitutive cost to maintaining high learning abilities in this species (Mery & Kawecki 2003). Induced costs were shown with the same selected lines when flies from the high learning lines that had been forced to learn cues associated with new oviposition media each day showed an operating cost in the form of reduced egg laying (fecundity) compared to individuals not required to use learning abilities daily (Mery & Kawecki 2004). The process of creating

long-term memory, which requires protein synthesis, has also been thought to involve trade-offs (Dukas 1999). *Drosophila* that had been selected for improved memory exhibited lower desiccation resistance when forced to build long term memory compared to individuals of the same line who were subjected to the same aversive conditioning, but under conditions that did not create long-term memory (Mery & Kawecki 2005).

Snell-Rood and Papaj have examined constitutive versus induced costs to learning in *Pieris rapae* (Snell-Rood et al. 2009, Snell-Rood et al. 2011). One study found both constitutive and induced costs to oviposition color learning (Snell-Rood et al. 2009). The size of adult mushroom bodies, areas of the insect brain known to be involved in learning and memory, varied at the family level, signifying constitutive costs to maintaining larger mushroom bodies (Snell-Rood et al. 2009). Butterflies that been trained to a host plant color also had larger mushroom bodies relative to control butterflies that were not forced to learn during their lifetime, signifying an induced cost to learning in this context (Snell-Rood et al. 2009).

In one of the few empirical papers to examine costs to learning, Snell-Rood explored costs to learning in *Pieris rapae* (Snell-Rood et al. 2011). The study reports that better learners have later ovary maturity than poorer learners. When the authors treated caterpillars with Juvenile Hormone, which decreases time to reproductive maturity, adults were less likely to find host plant when compared to non-JH-treated individuals. The authors argue this indicates a direct trade-off between learning ability and ovary maturity beyond correlative evidence previously collected. The findings based on costs to learning are flawed in this publication, as the correlation between ovary maturity and learning abilities occurred only in one of the two host environments, and JH is known to affect a

wide array of insect physiological and behavioral components that could have impacted the butterfly's host plant search flight. As such, very few studies have truly shown costs to learning, not surprisingly given the difficulty of the subject matter.

While maintaining extensive learning abilities may theoretically come at a cost, there are also costs to reduced learning. All animals are capable of learning to some extent, but species with larger brain size and more complex neuronal circuitry are capable of more complex learning and memory storage (Aboitiz 2008). Floral constancy, described above, has repeatedly been used as an example of insect learning, but can also be interpreted as representing limitations to insects' memory capacity (Gegear & Lavery 2001, Kunin & Iwasa 1996, Lewis 1986, Waser 1983, Waser, 1986). Lewis explored limits to memory capacity in *P. rapae* and found that these butterflies exhibited floral constancy and increased efficiency of extracting nectar from one species over time, but the introduction of another floral species reduced their foraging efficiency on both the newly introduced flower as well as the previously learned flower (Lewis 1986). Learning to extract nectar from the second species interfered with memory of nectar extraction from the first, indicating a constraint to memory in this species.

Research Question One:

Can Pieris rapae butterflies learn shape in association with a food reward?

Butterflies, as well as other insect pollinators, are capable of learning a variety of floral cues, which contribute to efficient foraging decisions. The majority of learning studies in insects, however, has focused on hymenopterans, and in particular, the honey bee. While butterflies have been shown to be capable of learning color in a foraging context, I was interested in whether other visual cues, such as shape, are important in a

butterfly's search for food. Bees can learn shape and complex patterns while foraging, and butterflies can learn shape in an oviposition context, so it seems plausible that butterflies could learn a shape in association with a food reward. I investigated whether *P. rapae* could learn shape in a foraging context, and if so, whether there were differences in the strength of response to shape stimuli versus color stimuli (i.e., do these butterflies learn shape as well as color?). I hypothesized that *P. rapae* would, like the honey bee, be able to learn shape in a foraging context, but that shape would not be as salient a cue as color, as has been shown in bees (Lehrer et al. 1990, Menzel 1985, Wackers & Lewis 1999). The differences in saliency of shape and color as foraging cues, if similar in *P. rapae* and the honeybee, are likely due to variation in the strength and importance of the two visual signals. Relative to floral shape, color can be seen more easily from a distance, and may be the primary visual cue used to distinguish between flowering plant species (Briscoe & Chittka 2001, Menzel 1979). Shape, if used at all, may be of secondary importance for identifying specific flowers (for example, floral species of similar coloration with different petal or corolla shape).

Laboratory Evolution in Insects and a Comparison to Wild Populations

Insects are a popular animal class used to study a wide variety of biological research topics. Evolutionary biologists and ecologists take great interest in the integral roles insects play in a variety of biological communities and ecosystem functioning (Funk et al. 2002). Additionally, many insects are intimately associated with applied agricultural and medical research. Insects play a major role in both the maintenance and degradation of annual crop output, as insect pollinators are vital for crop pollination, and herbivores

and seed predators reduce crop quality and output (Kevan et al. 1990, Agrawal et al. 1999). Other insects, such as mosquitos and triatomine bugs, are vectors for life-threatening diseases such as malaria, chagas disease, and dengue fever (Smart et al. 1973). Geneticists and molecular biologists study insects because many of their pathways are conserved across animal kingdom and are useful models for vertebrates (Keil & Steinbrecht 2010).

The characteristics that make insects ideal for laboratory study also leave them vulnerable to both intentional and accidental alterations to their physiology, genetics, and behavior. Additionally, insect traits such as rapid generation times and small body size allow for relatively easy and cost-effective maintenance of insect colonies in the lab. While insects are well suited to laboratory studies, there are inevitable changes in lab populations as a result of selective changes and random processes. The selection imposed by an artificial environment with limited space, coupled with both deliberate and inadvertent selection as a result of laboratory husbandry practices and genetic forces such as drift and founder effects, can lead to a multitude of physiological, developmental, and behavioral changes in animals maintained in a laboratory environment.

Random Genetic Processes

Changes in the laboratory phenotype can result from neutral or random genetic processes such as genetic drift and inbreeding. Many lab colonies are maintained with little to no gene flow between wild and laboratory populations, thus creating a closed laboratory population susceptible to a variety of genetic processes capable of altering allele frequencies from generation to generation. Genetic drift can be a strong force affecting allele frequencies and reducing genetic variation in populations with small

effective population sizes as is typical of laboratory colonies. Small initial population size at the establishment of the laboratory colony can lead to founder effects, a special case of genetic drift that occurs when the few individuals founding a new population do not genetically represent the allele frequencies of the larger population from which they came (Templeton 2008). Reduced genetic variation relative to wild populations has consistently been a concern for insect breeders and has been shown to be a common pattern in laboratory colonies (Kim et al. 2007, Mackauer 1972, Mason et al. 1987). For example, a study assessing changes in allele frequencies at the ADH locus in a lab population of *Dacus oleae*, the olive fruit fly, found a 40-fold increase in the frequency of a rare allele with the majority of genetic changes occurring in the first two generations, a likely consequence of Founder effects in this population (Zouros et al. 1982).

Long-term laboratory colonies are highly susceptible to inbreeding depression as a result of small population size. Inbreeding affects allele frequencies by reducing heterozygosity (Armbruster et al. 2000, Roush et al. 1986), which has been shown to negatively impact a multitude of phenotypes, including reduced fecundity and survivorship (Ehiobu et al. 1989, Latter & Mulley 1995, Oosterhout et al. 2000). These genetic changes resulting from non-selective processes can affect an organism's phenotype in unpredictable ways.

Inadvertent Selection

Inadvertent selection is an inevitable consequence of the drastic differences between laboratory environments and the natural habitat of a given species and can impact an organism's physiology as well as behavior. The typical laboratory environment is designed to maximize the organism's survival and fecundity while minimizing cost and

effort of maintaining the colony as well as variation in the organisms. As a result, laboratory colonies are typically held under constant light:dark photoperiods, temperature, and humidity in order to maintain year-round activity and reproduction of the species of interest. Animals are often kept in cages, vials, or tubes with limited or no contact with other species, maximizing environmental homogeneity. Such constant conditions sharply contrast the unpredictable nature of an organism's native habitat. Natural abiotic stochasticity imposes environmental stress such as low food availability and fluctuations in temperature and humidity. Reduced selection for stress resistance has been shown to occur under the predictable conditions of the laboratory environment. Long-established laboratory stocks of *Drosophila melanogaster* show decreased resistance to starvation and desiccation when compared to wild populations (Hoffman et al. 2001).

The artificial diet and oviposition substrate presented to laboratory insects can have immediate behavioral and physiological effects on the colony, thus creating differences between lab and wild populations. For example, alterations in visual and chemical sensory perception as a result of artificial larval diet have been recorded in *Manduca sexta* (Schoonhoven 1967, Yamamoto 1974, Blaney et al. 1986). Individuals raised on an artificial diet developed polyphagous behavior, accepting multiple host plant types that would otherwise be rejected by individuals raised on natural host plants (Yamamoto 1974). Experimenters hypothesized that the change in sensitivity to host plant types is a result of alterations in the sensitivity of contact chemoreceptors, particularly during the first instar (Schoonhoven 1967, Yamamoto 1974). Additionally, differences in adult feeding behavior of *Manduca sexta* between larvae fed artificial and natural diets has been shown to be due to a difference in visual sensitivity, as artificial diet tends to be

limited in beta-carotene, a micronutrient known to affect eye development (Goyret et al. 2009, Raguso et al. 2007). These results suggest that certain physiological responses, particularly chemosensory variation, can be unintentionally induced within an individual's lifetime as a consequence of dietary differences.

Changes in environment can also inadvertently affect an organism's behavior and have been documented in numerous species as a consequence of a laboratory environment. Mating behavior has repeatedly been shown to change in a laboratory setting. Aggression related to competition for mates increased two-fold in laboratory-reared male bollweevils compared to wild males, and wild females found laboratory males to be significantly more attractive (Villavaso & Mcgovern 1986). Similarly, male corn earworms maintained in the lab for over 120 generations were less discriminating than wild males when exposed to female sex pheromone, and the requirement for low light intensity for pheromone response shown by wild males was highly reduced in lab-maintained males (Raina 1989). The heightened sexual response of laboratory-reared male insects may be due to a variety of factors, including reduced sensory sensitivity and increased selection for mating activity, since constant laboratory conditions allow for mating year-round. Female reproductive behavior and development have also been shown to vary between laboratory and wild populations. Multiple studies have shown that laboratory-reared females prefer to mate with members of the same strain, often failing to recognize wild males as potential mates (Fye & Lebreque 1966, Dame et al. 1964). Such differences in mate recognition and sexual aggression are likely unplanned consequences of environmental differences between the laboratory and wild habitat.

Intentional Selection

Differences between laboratory and wild populations can occur as a result of intentional selection instituted by the domesticator. Individuals in a colony may be selected for specific traits that maximize colony productivity, such as shortened development time and increased survivorship and fecundity. *Manduca sexta* larvae from a long-established laboratory colony developed more quickly and with fewer instars than wild hornworms, a consequence of differences in diet as well as selection (Kingsolver 2007). Changes in reproductive timing have also been documented, as females from laboratory colonies of the tobacco budworm *Heliothis virescens* (Raulston 1975) and the olive fruit fly *Dacus olea* (Economopoulos et al. 1967, Voyadjoglou & Giannakakis 1976), oviposited earlier than wild females. Earlier maturation and faster development times are likely a result of artificial diet as well as well as selection (Economopoulos et al. 1967, Voyadjoglou & Giannakakis 1976, Raulston 1975).

Fitness factors such as increased survival and fecundity are often selected upon to maximize colony output. While survival in the lab can initially decline dramatically as a result of rapid environmental change between wild and laboratory environments, average survivorship often increases through time as adaptation to the lab selects for genotypes that will be most successful in the given environment (Hoy 1976, Zouros et al. 1982). Laboratory colonies can show an increase in survivorship over time (Armbruster et al. 2000, Kingsolver 2007). Fertility can increase under laboratory conditions as a result of environmental factors, such as climate homogeneity, or it can decrease, as inbreeding depression is known to decrease fertility over time (Armbruster et al. 2000, Henter 2003).

Increases in fertility are likely due to changes in both larval and adult diet and reduced competition. Decreases in fertility may be an outcome of inbreeding.

Behavioral changes can occur as a necessary adaptation to a new lab environment. The artificial diets and oviposition substrates used in the laboratory may not be universally accepted by all individuals at the founding of the colony, thus creating a selection regime for individuals that readily accept artificial media critical for survival in this environment. Wild and lab-reared Caribbean fruit flies show different oviposition preferences in response to physical stimuli provided for oviposition. Wild individuals show specific preferences for egg laying on domes rather than flat discs, whereas lab-reared individuals show no sensitivity or preference between stimuli (Greany & Szentesi 1979). Such results are likely due to rearing practices selecting genotypes that reduce discrimination between oviposition site types and stimuli.

Conclusions Regarding Wild and Laboratory Insect Populations

Differences in selection pressures and the strength of random genetic forces between wild and laboratory insect populations can lead to a divergence between these two population types that can be manifested in a multitude of ways. The differences in behavior and physiology between laboratory colonies and wild populations may not be of any consequence to researchers studying highly conserved genetic and developmental processes, but could be problematic for evolutionary biologists and ecologists using insect colonies to make conclusions about their wild counterparts. Understanding the limitations laboratory colonies pose is critical for making valid statements based on lab research.

Research Question Two:

*Do learning abilities differ between wild and laboratory-reared *Pieris rapae*?*

Variation in environmental predictability and stress can alter the importance of learning to an individual's fitness. I have explored whether learning abilities vary between two populations, one derived from a laboratory colony and the other derived from wild-caught individuals, which experience extremely different environmental conditions, in order to see how such differences may cause variation in behavior. I examined learning in butterflies from a laboratory colony of *P. rapae* that had been maintained under constant conditions (light, temp, day-length, season), in a homogeneous and predator-free environment with an unlimited food supply in both larval and adult stages of life for over ten years. The environmental consistency experienced by the lab population contrasts with unpredictable daily and seasonal variation experienced by wild populations of the same species, which must navigate complex habitats in order to forage efficiently. In addition, the laboratory population has been maintained without the introduction of new genetic material for the past ten years, yielding a small, closed population susceptible to a variety of random genetic processes such as founder effects, genetic drift, and inbreeding. These genetic factors could also lead to differences in a variety of phenotypes, including changes in learning abilities, when compared to the larger wild populations of *P. rapae*.

I hypothesize that laboratory *P. rapae* will be weaker learners in a foraging context compared to a wild population, as selection to maintain learning abilities is more relaxed in the predictable and consistent laboratory environment. The laboratory population may have undergone changes due to the stronger impact of drift on small,

closed populations. Thus learning abilities may differ through both alterations in selection and random processes. To my knowledge, only one other study has compared learning abilities between wild and laboratory insect populations, in a study using the Mediterranean Fruit Fly, *Ceratitidis capitata* (Papaj et al. 1987). Studies comparing learning abilities provide insight into how environmental pressures and random genetic processes may interact to shape behavior differently among populations of a single species.

Using the Cabbage White Butterfly, *Pieris rapae*, as a Study Organism

Pieris rapae is a small butterfly native to Europe, North Africa and Asia that has spread globally in the past 150 years. *P. rapae* spread to North America via Montreal in the 1860s (Scott 1992), and has since successfully spread across the continent. *Pieris rapae* larvae feed on members of the crucifer family, making them an agricultural pest on a variety of Brassicaceae crops such as brussel sprouts, kale, turnip and broccoli greens, and cabbage, as well as a variety of non-agricultural native and invasive plants (Chew & Renwick 1995). Adults are generalist foragers and pollinators of a variety of nectar plants.

The Cabbage White Butterfly is a suitable study species for my questions on learning behavior for many reasons. Multiple studies have shown that *P. rapae* is capable of learning to feed from a specific flower, exhibits floral constancy, and can learn color in association with a food reward (Kandori & Ohsaki 1996, Kandori et al. 2009, Lewis 1989). Because we know that this species can learn visual cues in a foraging context, we can explore whether shape may be used for floral discrimination and choice. In addition, at least one laboratory colony of *Pieris rapae* has been maintained for commercial purposes for the last ten years, allowing a comparison of learning abilities between wild

and laboratory populations. *Pieris rapae* is also relatively easy to catch from the wild and maintain in a laboratory environment. For these reasons, I use *Pieris rapae* to explore shape learning in a wild population, and compare shape and color learning in wild and laboratory *Pieris rapae*, in order to understand how variation in learning can depend on the type of stimulus as well as differences in environmental and genetic processes.

Methods

Population Origins

One wild and one laboratory population of *Pieris rapae* were used for experimental comparisons and are described below. The comparison between wild and laboratory populations acts as a case study in population variation in learning behavior, and the experiment must be replicated with multiple populations of wild and laboratory insects. I plan on replicating the laboratory and wild learning comparison study either with additional populations of *Pieris rapae* or other butterfly species with readily available laboratory and wild populations, such as *Vanessa cardui*.

Lab population

Pieris rapae eggs were purchased from Carolina Biological Supply Company of Burlington, North Carolina in June of 2012 and will hereafter be referred to as the laboratory population. Additional Carolina Biological butterflies were ordered the summer of 2013 to collect additional egg hatch data, but were not used for learning experiments. The Carolina Biological *P. rapae* colony was started with butterflies from a research colony initially maintained by the University of Wisconsin beginning in 1998 and has been managed year-round for over 9 years. Carolina Biological Supply rears *P. rapae* at a constant temperature of 75 degrees and a 16-hour light cycle using fluorescent

light, eliminating seasonal variation from colony experience. Six hundred to 1500 butterflies are kept in the colony at one time, with 100 to 150 butterflies in a single 35.5 x 29.5 x 18” cage. Larvae are fed artificial diet primary composed of wheat germ, casein and vitamin mix. Adults nectar on a sponge soaked in orange Gatorade.

Lab population at Georgetown University

The adults of the initial laboratory population were used for learning assays (described below) and were bred for two additional generations through summer and late fall 2012 in order to maximize sample sizes. Breeding occurred after individuals were used for learning assays. Sexes were kept separate during learning assays, then placed in mixed sex cages containing brussel sprouts for oviposition and a sponge soaked with 15% honey solution for feeding, after completion of the experiment. Mixed dormitories contained one female and multiple males in order to keep track of maternal family origin while maximizing potential for matings. If there were more females than available cages, multiple females were placed in a single mixed dorm. Eggs were collected and reared as described below.

Wild population

Wild *Pieris rapae* adults were caught on the Georgetown University campus in Washington, D.C. throughout the summer and fall of 2012 and will hereafter be referred to as the wild population. Females were caged and allowed to lay eggs on brussel sprout leaves and fed on honey-soaked sponges. Only the adult F1 offspring were used in learning assays. *Pieris rapae* are active in the region from early spring to late fall. Larvae feed on a wide variety on plants belonging to the mustard family and adults nectar on a

diversity of local flowers. Outdoor temperature, humidity, and photoperiod vary by season.

Within-population genetic relatedness

The Carolina Biological population is composed of the offspring of at least eight different females. The exact number of families could not be determined, as laboratory butterflies were bred either in individual cages with one female per cage, or in communal cages with multiple males and females, suggesting the number of families is in fact higher than eight. Wild population butterflies came from the F1 generation of wild-caught females caught continuously over the course of the summer. While the specific number of families could not be determined due to the use of communal cages for egg collection, the wild population is represented by a minimum of five families. The use of individuals from at least eight families from the laboratory population and five families from the wild population suggests the individuals used in this study vary in genetic relatedness both within and across populations and are representative of the populations of origin.

Larval rearing

Eggs from lab and wild populations were hatched and larvae reared in a common garden laboratory setting with the same oviposition substrate, larval host plant, light, humidity and temperature. Leaves bearing eggs from mated laboratory females and gravid wild caught females were placed into separate 16 oz. plastic deli containers labeled with the identity of the mother (if possible) and lay date. Every morning, containers were checked for hatchings and first instar larvae were transferred onto fresh brussel sprout leaves (using a fine soft-bristled paintbrush) and placed in individual

containers. One larva was placed in each individual container with multiple brussel sprout leaves in order to record its individual growth, death, and pupal weights. Larvae were fed brussel sprouts *ad libitum* on average every other day, and cages cleaned twice a week. Each larva was numbered in order to track population origin and record individual descriptive data as described below. Once pupation occurred, frass and old leaf material were removed and the container was checked daily for adult emergence.

Egg and larval development

Larval containers were checked daily for deaths, pupation, and low food supply. Each container was labeled with individual number and population origin. Individual egg lay date, hatch date, and pupation date were recorded in order to compare development times of different life stages between populations. In addition, pupal weights were recorded 24 hours after pupation in order to allow time for the pupal case to harden.

Construction of paper models

Models for innate, color, and shape learning trials were created using Black, Orange-Hue, and Blue-Ex Coloraid paper (www.coloraid.com). Black triangles were made for innate training periods, orange and blue circles were made for color training and testing periods, and blue squares were paired with blue circles for shape learning training and testing periods. All models were 9 cm² in area. The colors orange and blue were chosen for the color learning assays based on innate preference testing conducted by Kandori and colleagues for *P. rapae* showing that out of twelve offered colors, yellow was the most highly preferred, followed by blue and then orange (Kandori et al. 2009). I did not include yellow in color learning assays in order to avoid strong innate preferences, which might have overridden or masked the need any evidence of learning. I chose to

use blue and orange because, as preferred colors I anticipated that they would motivate butterflies to interact with the models without dominant innate preferences confounding learning abilities. Blue was used for the shape models because the high contrast blue makes with the white background has been shown to enhance insect abilities to distinguish between edges (Lehrer et al. 1990).

To create the model, a 10 μ l eppendorf pipette tip was pushed through the center of the paper so the base of the pipette tip lay flush with the paper. A hot glue gun was used to secure the base of the tip to the paper as well as close the tip. The flower models were then inserted into the training or testing board, 8 to 9 cm apart.

Innate Preferences

We conducted innate preference assays to determine whether the lab and wild populations had a strong innate preference for one of the two colors (orange and blue) used specifically in the color learning assays, as the use of additional colors during innate preference testing could skew or complicate the data. The laboratory innate preference experiment was conducted in the summer of 2012, while the wild innate preference experiment was conducted in the summer of 2013.

A subset of adult butterflies was randomly assigned to innate preference testing. The innate preference experiment was comprised of two phases: a two hour priming phase in which butterflies were allowed to feed on black paper models in order to enhance their association between artificial flower models and the presence of a honey reward, and a testing phase, in which an individual's preference for orange or blue was assessed. The testing period occurred every morning following a priming period, for a total of four consecutive priming and testing periods. All priming, training, and testing

phases were completed under fluorescent lighting set to a 16 hour light cycle in a room with natural light coming from windows as well as a central skylight.

Forty-eight hours after emergence, adults were separated by sex, and groups of four to six butterflies were placed into 60 x 60 x 60 cm mesh cages with a 33.5x20 cm innate preference training board on the cage floor. The board was covered in white printer paper and held six 9 cm² black, triangular flower that each contained a 15% honey solution in a centered pipette tip. Butterflies were allowed to forage on the black models for two hours per day for a total of four consecutive days. Fresh honey solution was placed in the models each day, and refilled during priming if any empty models were observed. At 30 minute intervals we noted which butterflies were feeding on the models; at the end of the priming period, butterflies not observed feeding were hand-fed by placing a butterfly on a black model and carefully unrolling the proboscis into the honey solution. Hand-fed butterflies were allowed to feed for 30 seconds, then placed back into the cage. In this manner, butterflies were allowed to associate the models on the board with a food reward without influencing innate preferences for blue or orange.

Every morning following training, butterflies were tested for innate color preference, yielding a total of four testing periods. Each of the 4 to 6 butterflies from the priming period was placed individually in a cage with a testing board identical to the training board described, but containing six rewardless circular color models (three orange and three blue) instead of black triangles. *Pieris rapae* increases activity levels in cages with other butterflies, three additional naïve butterflies ('buddies') were added to the testing cage in order to maximize activity from the focal butterfly without sacrificing statistical independence. Data were collected only from the focal butterfly; 'buddy'

butterflies were not used for any data collection or assessment. The focal butterfly was then observed for a period of five minutes, and the number of landings on each color model as well as the time spent probing each model was recorded. A complete landing is defined as a landing on a model from flight in which a probe occurred. A probe, or a feeding attempt, is defined as the unraveling of the proboscis and contact with the paper model.

Shape and color learning/ training and participation

Butterflies used for learning assays were not used for innate preference data. Learning assays were comprised of two phases: a training phase and a testing phase. Methods are identical to those explained in the innate preference experiment, except instead of a priming phase, there is a training phase explained below. Adult butterflies were randomly assigned to learn either a color stimulus (orange or blue) or a shape stimulus (circle or square).

Two days after emergence, adults were separated by sex and placed into cages in groups of four to six according to treatment. Training boards were placed at the bottom of each cage and consisted of six paper flower models. Three models were the assigned stimulus and contained a 15% honey solution; the other three were the non-assigned stimulus of the same treatment type and contained a 10% salt solution, known to be unpalatable to *P. rapae* and most butterflies. Butterflies were hand-fed on their assigned stimulus for 5 seconds immediately prior to their first training period, then allowed to forage on the board for two hours a day for four consecutive days during mid-morning to early afternoon. The position of each butterfly was recorded every 30 minutes during training in order to document which butterflies were self-feeding. Butterflies that were

not documented as having fed from a rewarding model were fed for 30 seconds on their assigned stimulus after the 2 hr training session ended. Training participation information was later used to compare participation rates between wild and laboratory populations.

Shape/ color testing

Every morning following a training period, butterfly shape or color choices were evaluated. Similar to innate preference tests, focal butterflies were individually placed in a testing cage with three naive ‘buddy’ butterflies and a testing board. The testing board was identical to the training board except floral models did not contain honey or salt solution. Butterflies were hand-fed on their trained stimulus for 5 seconds immediately prior to testing, then released into the testing cage and allowed to explore the testing board for five minutes. The number of landings on each color or shape type as well as the time spent probing each model were recorded. Landing and probing events are defined in the innate preference methods section. Butterflies that failed to make a complete decision during the testing period were recorded as having made no decisions and were excluded from analysis for that testing day.

Statistical analysis

Egg and larval development times and adult survival

Population differences in egg and larval development time as well as pupal weights between wild and laboratory butterflies were compared using Student’s T tests. In order to determine whether laboratory-reared butterflies were more likely to feed on artificial models than wild individuals, a chi-squared test was conducted comparing the number of butterflies from each population feeding themselves on day 1 as well as day 4 of training. A chi-squared test was also conducted comparing the number of butterflies

within each population feeding themselves on day 1 and day 4 of training in order to determine whether butterflies increasingly recognized the artificial model as a food source over time.

Innate color comparisons

To determine whether the laboratory and wild populations had an innate preference for orange or blue, the first complete landing was recorded during testing periods for the innate preference experiment. In addition, the average percentage of landings on each color across all four testing days were recorded for each individual.

Learning comparisons

All butterflies that made at least one complete decision during testing (described above) were used for analysis. Butterflies that made no decisions were excluded from the analysis for that testing day. In this way, butterflies that only made decisions on one or a few days, or died before the final day of testing, could still be used for analysis using daily testing data.

Learning Measures

The following measures were used to compare learning abilities between shape and color-trained butterflies:

Presence of Learning: Prior to comparing shape and color treatments, we evaluated whether butterflies successfully learned their stimulus type. Learning is said to have occurred if there was a significant increase in median percent correct landings between days one and four of testing.

Maximum Proficiency: Proficiency measures how successful the treatment group was in learning the trained stimulus, and can be measured by calculating the number of errors (landings on the non-trained stimulus) that occur after training. Maximum proficiency was defined as the maximum median percentage of landings on the trained stimulus on the final day of testing.

Learning Rate: Learning rate is defined as the time, or number of testing days, necessary to decrease error rate and correctly associate the trained stimulus with a food reward.

Learning rate was evaluated by comparing median percent correct landings on day one versus day two for butterflies trained to color and shape. Testing days one and two were used because they show the biggest difference in learning rates when comparing shape and color treatments.

Statistical Analysis for Learning Experiments

Color Learning

A Mann-Whitney U test comparing the median percentage of correct landings on testing days one and four was conducted to determine presence of learning for orange and blue-trained butterflies for wild and laboratory populations. Colors were tested individually in case learning was impacted by the color assigned.

Shape Learning

Just as for the color treatment group, a Mann-Whitney U test comparing days one and four median percentage of correct landings was conducted to determine presence of learning for wild and laboratory butterflies. Circle and square data were combined for shape analysis due to low sample sizes.

Color and Shape Learning Comparison in the Wild Population

In order to determine whether learning rates between color and shape-trained butterflies differed, we compared the learning measures described above for both treatment groups. First, we determined whether each group learned their stimulus by running Mann Whitney U tests comparing median percent correct landings between days one and four for both color and shape treatments. Second, we compared maximum proficiencies for color and shape treatments by using a Mann Whitney U test to detect statistical differences between median percent correct landings on day four for both treatment groups. Third, we compared learning rates between color and shape treatments by running Mann-Whitney U tests comparing days one and two median percent correct landings for each treatment. Statistical differences between days one and two were then compared for each treatment.

Laboratory and wild learning comparison

In order to determine whether learning abilities differed between laboratory and wild butterflies, the learning measures described were used to compare wild shape-trained butterflies to laboratory shape-trained butterflies, and wild color-trained butterflies to laboratory color-trained butterflies. Colors were compared individually, as in the color and shape learning comparison. First, we measured presence of learning for wild and laboratory butterflies trained to color or shape by running Mann Whitney U tests comparing median percent correct landings between days one and four for each treatment group (Wild shape, wild orange, wild blue, lab shape, lab orange, lab blue). Second, we compared maximum proficiencies for laboratory and wild butterflies by using a Mann Whitney U test to detect statistical differences between median percent correct landings

on day four of testing within shape and color treatments. Third, we compared learning rates between laboratory and wild color and shape treatments by running Mann-Whitney U tests comparing days one and two median percent correct landings for each treatment. Statistical differences between days one and two were then compared for each wild and laboratory butterflies trained to shape, and wild and laboratory butterflies trained to color.

Learning Comparison Between the Sexes

In order to determine whether learning abilities differed between the sexes, the mean percentage of correct landings were calculated for all butterflies that lived through all four days of testing, and individual mean percent correct landings were compared between males and females. While comparing day four data would have been a more suitable measure to compare maximum proficiencies among the sexes, the sample sizes were too low for the wild shape treatment for this comparison to be conducted.

Comparing individual means allowed for standardized sex comparisons across all four treatment groups (wild color, wild shape, laboratory color, laboratory shape).

Results

Egg and larval development times

The mean days to hatch was significantly longer for the wild *P. rapae* population when compared to the laboratory eggs (Laboratory, n=371, mean days to hatch=3.29 days; wild, n=1581, mean days to hatch =4.17, t=-6.71, p<0.0001) (Fig. 1). Days to hatch ranged from under 24 hours to 7 days from lay date for both populations, though a higher proportion of laboratory eggs hatched within 48 hours of oviposition compared to wild eggs (Fig. 1). Laboratory egg hatch rates were spread more evenly across potential days to hatch when compared to the wild populations. The highest proportion of laboratory

eggs to hatch on a single day was 26% on day four of post oviposition, compared to 59% of wild eggs taking four days to hatch (Fig. 1).

Wild larvae developed faster than laboratory larvae (time to pupation: Laboratory, $n=225$, mean=14.50 days ; Wild, $n=144$, mean= 13.89 days, $t=3.13$, $p=0.0018$)(Fig. 2), but took longer to emerge from the pupal stage when compared to the laboratory population (Laboratory, $n=135$, mean=7.79 days; wild, $n=112$, mean=8.07 days, $t= -3.31$, $p=0.001$)(Fig. 2). Days to pupation did not differ between the sexes within each population (Laboratory, female $n=66$, female mean=14.17 days; male $n=64$, male mean=14.14 days, $t=0.08$, $p=0.936$; Wild, female $n=58$, female mean=13.43 days, male $n=59$, male mean=13.92 days, $t=-1.77$, $p=0.076$)(Fig. 2). Pupal weights did not differ between populations (Laboratory, $n=201$, mean=0.1599 g; wild, $n=133$, mean=0.1593, $t=0.27$, $p=0.789$), but differed significantly among the sexes within each population, with males being heavier than females (Laboratory, female $n=64$, male $n=58$, female mean=0.1534 g, male mean=0.1665, $t=-4.16$, $p<0.0001$; Wild, female $n=58$, male $n=56$, female mean=0.1541 grams, male mean=0.1652 grams, $t=-2.82$, $p=0.005$).

Adult survivorship / participation in training

More laboratory butterflies lived through all four days of training than wild butterflies when survivorship to day four of training was compared for the two populations. Thirty percent of wild butterflies died by training day four compared to 12% in the laboratory population (Laboratory, $n=204$; Wild $n=114$, $X^2=6.93$, $p=0.006$).

Laboratory butterflies fed themselves more often than wild butterflies on the first day of training, with 37% of laboratory butterflies and 14% of wild butterflies coming down to feed from the model flowers in the array on day one of training (Number of

individuals feeding on day 1: Laboratory, n=325; wild, n=167, $X^2=29.32$, $p<0.0001$)(Fig. 3). Laboratory butterflies participated more often than wild individuals on day four of training as well, with 50% of laboratory butterflies and 29% of wild butterflies feeding themselves (Laboratory, n=179; Wild, n=79, $X^2=9.47$, $p=0.002$)(Fig. 3).

A significantly higher percentage of laboratory butterflies fed themselves during training on day four (50%) than on day one (37%) (Laboratory day 1, n=325; laboratory day 4, n=179; $X^2=7.41$, $p=0.006$). While wild butterflies participated less during training than did laboratory butterflies, their visitation to models also increased over the course of the 4 days; 29% of wild butterflies fed themselves on day four (n=79) and only 14% fed themselves on day one (n=167) ($X^2=8.3$, $p=0.004$). These data suggest that both lab and wild butterflies were able to learn to associate artificial models with food reward over time.

Innate color preference data

Of the 21 laboratory butterflies that participated in the innate preference experiment, 19 chose orange for their first feeding attempt, signifying an innate preference for that color. The laboratory butterflies made a mean 76.99% landings on orange when data from each individual was combined across all four testing dates, also indicating a preference for orange.

No innate preference was found to exist for the wild population. Out of the 26 butterflies that participated in the innate color preference experiment, ten butterflies landed first on blue, and 16 landed first on orange. On a population level, 54% of decisions were made for orange when data for all individuals across all four days of testing were combined. The lack of innate preferences at a population level is likely a

consequence of variation in individual preferences, with 10 butterflies making all their landings on orange, and seven making all their landings on blue (Fig. 4). The remaining nine butterflies ranged from 20% to 71% of their landings on orange.

Learning results

Color learning in the wild population

Blue-trained butterflies showed a significant increase in median percent correct landings from 60% on day one to 100% on day four, indicating that the butterflies learned to associate color with reward (Day 1, $n=11$; Day 4, $n=10$, Mann Whitney U test: $z=1.61$, $p=0.05$)(Fig. 5). Learning could not be evaluated for butterflies trained to orange, as the median percent correct landings were 100% across all four days (Day 1, $n=7$; Day 4, $n=5$, Mann Whitney U test: $z=0.6761$, $p=0.25$), reflecting the population's strong innate preference for orange. Color learning did not differ between the sexes when the percentage of correct landings was averaged for each individual across all four days (Female $n=12$, male $n=14$, Mann Whitney U Test, $z=0.36$, $p=0.719$).

Color learning in the laboratory population

Laboratory butterflies trained to orange could not be evaluated for learning abilities as they maintained a median of 100% percent correct landings across all four days of testing. Blue-trained butterflies, on the other hand, showed evidence of learning, as the maximum proficiency of 73.68% correct landings on day four was significantly higher than 37.5% correct landings on day one (Day 1, $n=21$; day 2, $n=19$, Mann Whitney U test: $z=3.453$, $p<0.001$)(Fig. 5). Color learning did not differ between the sexes when the percentage of correct landings was averaged for each individual across all four days (Female $n=30$, male $n=31$, Mann Whitney U Test $z=-0.76$, $p=0.447$).

Shape learning in the wild population

Circle and square data were combined for shape analysis due to low sample sizes. Butterflies trained to shape showed a significant increase between days one (50%) and four (65%) in median percentage of correct landings on the trained shape (Day 1, $n=11$; day 4, $n=6$, Mann-Whitney U test: $z=2.18$, $p=0.01$), indicating learning (Fig. 6). Shape learning did not differ between the sexes for percent correct landings averaged for each individual across all four testing days (Female $n=7$, male $n=8$, Mann Whitney U Test, $z=0.06$, $p=0.952$).

Shape learning in the laboratory population

Laboratory butterflies showed no evidence of shape learning, with no differences in median percent correct landings between days one and four (Lab day 1, 50%, $n=18$; Lab day 4, 50%, $n=27$, Mann Whitney U test: $z=0.3958$, $p=0.6922$) (Fig. 6). Shape learning did not differ between the sexes when individual percent correct landings were averaged across all four testing days (Female $n=23$, male $n=22$, Mann Whitney U Test, $z=-0.27$, $p=0.787$).

Color vs. Shape Comparison in the wild population

As we saw evidence of learning only in blue-trained butterflies, we compared data from the blue-trained butterflies to data from the shape-trained butterflies in order to assess differences in butterflies' ability to learn color versus shape. Learning rate differed between the two stimulus types, as the median percentage of correct landings for blue-trained butterflies jumped from 60% to 100% between days one and two and stayed at 100% through day four, yielding a significant increase of 40% in median correct landings by the second day (Fig. 7) (Day 1, $n=11$; day 2, $n=6$, Mann Whitney U test: $z=2.315$,

$p=0.0342$). Butterflies trained to shape started with a median of 50% correct landings on day one of testing, and increased 5% to a median of 55% correct landings on day two, indicating a slower learning rate when compared to blue-trained butterflies over the same period (Fig. 7). Butterflies trained to shape reached a median maximum proficiency of 65% by day four, considerably lower than the median of 100% correct landings blue-trained butterflies achieved by day four, though the difference was not statistically significant (Shape, $n=6$; blue, $n=10$, Mann Whitney U test: $z=-1.8683$, $p=0.0617$) (Fig. 3).

Color versus shape comparison in the laboratory population

While we could not evaluate learning in orange-trained butterflies, butterflies trained to blue showed evidence of learning: median percent correct landings increased significantly from days one to four. Butterflies trained to shape showed no evidence of learning, indicating a difference in learning abilities between the two treatment types.

Wild vs. lab comparison

Shape

As described above, wild butterflies showed evidence of shape learning through an increase in median percent correct landings from day one to day four, while no learning was detected in the laboratory population (Fig. 6). Wild butterflies had a significantly higher maximum proficiency than laboratory butterflies when the median percent correct landings on day four of testing were compared (Wild, $n=6$; lab, $n=27$, Mann Whitney U test: $z=2.1055$, 2-sided $p=0.0352$).

Color

As orange-trained butterflies could not be evaluated for learning abilities in either wild or laboratory butterflies, color learning was compared between the two populations

using blue-trained butterflies. Blue-trained butterflies differed between populations both in learning rate as well as maximum proficiency. As described above, wild butterflies increased in the median percentage of correct landings by 40% between days one and two. Laboratory butterflies did not show a significant increase in median percent correct landings between days one and two, starting at 37.5% on day one and increasing to 50% on day two, changing a total of 12.5% in one day (Day 1, n=21; day 2, n=19, Mann Whitney U test: $z=0.4649$, $p=0.6420$). Blue-trained wild butterflies reached their maximum median percent correct landings of 100% on day two and maintained their maximum through day four, while laboratory butterflies reached their maximum median percent correct landings of 73.68% on their trained color on day four of testing (Fig 5). While wild and lab populations do not differ in the median percent correct landings on blue on day 1 (Wild, n=11; lab, n=21, Mann Whitney U test: $z=1.6192$, $p=0.1054$), they do differ significantly on day 4 (Fig. 5) (Wild, n=10; lab, n=19, Mann Whitney U test: $z=2.2966$, $p=0.0216$), indicating a significant difference in maximum proficiency for these two populations.

In order to determine whether rapidly-hatched individuals (under 24-48 hours in the egg stage after being laid) exhibited any differences in their learning abilities when compared to the rest of the population, the day four testing scores for the percentage of correct landings were compared to population medians for rapid-hatch butterflies. All butterflies that hatched within 24 to 48 hours were laboratory individuals trained to color upon adult emergence. The rapid hatching did not influence the learning abilities of the butterflies, as all the orange-trained laboratory butterflies with hatch times under 48 hours reached 100% correct landings by day four, which is comparable to the population

median of 100% by day four (n=7). Butterflies trained to blue with hatch times within 48 hours ranged from a maximum proficiency of 50% to 80% correct landings on day four of testing, also comparable to the population median of 74% correct landings on day 4 (n=3).

Discussion

Egg and larval development times

Laboratory butterflies took a mean of 3.29 days to hatch, which is almost a day earlier than the mean of 4.17 days for wild eggs. Selection for rapid development times in the lab may be a possible explanation for laboratory eggs hatching more quickly than wild eggs, as many laboratory colonies select for rapid generation times. Some *Manduca sexta* laboratory populations have reduced development times, as wild individuals pupate after six instars, and laboratory larvae typically take five instars to complete the larval stage (Kingsolver 2007). The reduction in the number of instars in the *M. sexta* laboratory population is likely due to inadvertent selection for rapid development, which may occur in the laboratory population, though it does not fully explain the more even spread of days to hatch as a whole.

P. rapae eggs hatched anywhere from within 24 hours to seven days after being laid. While wild and laboratory eggs reflected the same range of days to hatch, the majority (88%) of wild eggs hatched within a relatively narrow range of three to five days, compared to 61% of laboratory eggs hatching within the same range. While only one wild egg was documented as having hatched within 24 hours, 10% of laboratory eggs hatched in under a day. The comparatively even distribution of days to hatch for the laboratory population may be a consequence of the laboratory environment during this

stage of the *P. rapae* life cycle, as the homogeneity of the laboratory may release the laboratory population from environmental pressures such as predation and seasonal variation that are known to impact development time (Abrams & Rower 1996, Nylin & Gotthard 1998). Perhaps the majority of wild eggs hatch within a narrower range of days due to stabilizing selection creating a window for maximized fitness in the wild. Stabilizing selection may result from a multitude of predatory, competitive, and ecological pressures that create an optimal window for egg development and hatching time (Nylin & Gotthard 1998). Finally, genetic drift may have altered allelic variation determining time to hatch, potentially increasing the number of individuals capable of both rapid and slow development and emergence in the laboratory population.

Adult participation in training

Butterflies from the laboratory population consistently fed themselves more often than did wild butterflies during training periods. According to the *P. rapae* lab manager at Carolina Biological labs, the laboratory stock population is fed orange Gatorade on an orange plastic scrubbie sponge during the adult stages, a practice that has occurred throughout the past nine years. Butterflies that will be successful in the lab population ought to be able to associate artificial models with a food reward. This component of the laboratory environment could select for individuals that recognize artificial food sources quickly and efficiently, as adults who do not locate a food source will suffer both in survivorship and fecundity. As larvae derived from both populations were fed the same diet and trained in the same arena, the variation in adult acceptance of artificial food sources must reflect genetic differences as a result of genetic drift, selection, or a combination of the two. While acceptance of food sources has been shown to vary

between wild and laboratory populations in previous studies, such differences have typically been found to be based in chemoreceptor sensitivity resulting from changes in larval diet, or from genetic drift (Goyret et al 2009, Greany & Szentesi 1979, Raguso et al. 2007, Zouros 1982).

Wild butterflies have experience with a multitude of natural flowers, and as such I hypothesized they would be less able than laboratory-reared butterflies to identify a food reward from an artificial paper model that lacks so many of the complex components of a flower, including odor, three-dimensional structure, and presence of other plant components such as stems and leaves. Although lab-reared butterflies readily visited the models, the wild populations did not initially recognize models as 'flowers'. Additionally, the percentage of individuals feeding themselves in both populations increased over time, indicating that the number of both laboratory and wild butterflies associating floral models with food rewards increased with experience. As my results show, with experience wild butterflies increasingly visit artificial models, but their initial interactions with the models did not initiate a strong feeding response.

Innate color preferences

Laboratory butterflies showed an innate preference for orange over blue. The innate preference for orange may be an ancestral trait for this population, as founder butterflies used to begin the lab population may have had an orange preference that has been maintained through the generations. Though I have not tested this hypothesis, previous work on *Pieris rapae* color preferences has shown that field-caught butterflies from Japan prefer blue over orange, which is in direct contrast with my results (Kandori et al. 2009). The differences in innate preferences may be a consequence of using papers

with different intensities or reflectances, as many insects have been shown to prefer colors with higher intensities. Because I did not use papers identical to those used in the Kandori study, butterflies in my experiment were exposed to slightly different color options. As innate preferences have been shown to be highly context-specific and adaptable based on the colors available, even minor differences between the orange and blues used in the two studies could impact the outcome of innate preferences of the two study populations.

The differences in innate preferences between populations could be an effect of ecological, environmental, and genetic variation between populations of the same species rather than a side effect of different experimental methodologies. Innate preferences have typically been studied at the species level and population comparisons are rare (Ings et al. 2009). Strong selection for innate color preference may occur if floral variability is limited in a given habitat and finding available nectar sources quickly and efficiently is impacted by recognition and acceptance of the dominant species (Gumbert 2000, Ings et al. 2009, Lunau & Maier 1995). It is possible that there is a higher number of blue nectaring flowers available in Japan than Washington DC, which could select for different innate preferences based on location (Ings et al. 2009, Raine & Chittka 2007), but this explanation seems unlikely, given that *P. rapae* is highly generalist and likely nectars on a wide variety of floral species in both locations. Genetic drift may result in different color preferences across populations as well, if these innate preferences are not under heavy selection to be maintained and any color that contrasts with green might lead a pollinator to a flower.

The innate preference for orange may also be due to the feeding practices conducted in the laboratory. In the Carolina Biological population, adult butterflies are fed orange Gatorade from an orange plastic scrubbing pad placed in a plastic cup, so that selection for individuals that are strongly attracted to the color orange may be present in this environment, and orange may be a strong signal to initiate feeding, thus explaining their strong response to orange in both innate and learning experiments.

While I hypothesized that the differences in learning blue and orange for wild butterflies would reflect the same preference for orange the laboratory butterflies exhibited, this was not the case. The differences in proficiency observed between blue and orange-trained butterflies were hypothesized to be a consequence of a population-level innate preference for orange, which would explain why butterflies responded so strongly to orange across all four days of testing. Individual innate preference varied between the two available colors, with some strongly preferring orange or blue, and others showing no preference for either color.

Color is a more salient cue than shape for *P. rapae*

As *Pieris rapae* innately prefer orange, the orange stimulus would have reinforced their propensity for that color and no learning would be apparent. Butterflies readily learned blue with a maximum median of 100% correct landings by day two. The strong response to color observed in this study comes as no surprise, as color has repeatedly been shown to be an important floral cue for several butterfly species, including *Pieris rapae*, *Danaus plexxipus*, and *Battus philenor* (Blackiston et al. 2011, Goulson & Cory 1993, Goulson et al. 1997, Lewis 1989, Weiss 1997).

In contrast to color learning, butterflies trained to either circle or square floral models showed a significant improvement in their association of a shape stimulus with a food reward over the four day testing period, but reach a maximum proficiency of just 65% on day four, which is clearly not as high as for butterflies trained to color. These results support my hypothesis that *P. rapae* would be able to learn shape in a foraging context, as the ability to learn to associate shape with a rewarding or aversive stimulus could be useful in allowing a butterfly to distinguish between the wide variety of floral structures seen in nature. Although the presence of shape learning in a foraging context has not previously been documented in butterflies, pattern and shape learning have been seen in hawkmoths, as well as in honeybees (Campan & Lehrer 2002, Cartwright and Collett 1983, Giurfa et al. 1999, Gould 1986, Kelber 2002, Lehrer et al. 1995, Raguso 2004).

Although we saw evidence that *P. rapae* can learn both color and shape, color appears to be a more salient cue than shape during foraging for this species. Why might we see differences in learning rates and abilities between two different visual stimuli within a single foraging context? While a flower has many cues for a pollinator to attend to, not all may be of equal importance when an insect is making foraging decisions. In this instance, shape may be important as one component of a multicomponent visual signal, and may help a pollinator to distinguish between flowers of similar color, but may not be as important as color when distinguishing between floral taxa. Shape learning may be more important in other contexts, such as oviposition, as leaf types may be more readily discriminated based on shape than on a relatively common green color (Allard & Papaj 1996, Rausher 1978, Papaj & Rausher 1987, Weiss & Papaj 2003). Differences in

the learning rates between these two visual stimuli may reflect a hierarchy of visual cues used during foraging in this species. Color has also been shown to be a more salient cue than shape for honeybees (Goyret & Kelber 2011, Lehrer et al. 1990, Menzel 1985, Wackers & Lewis 1999). Hierarchical use of floral cues has been reported for other pollinator species as well, though floral traits studied are typically multimodal (color and scent, floral height and scent) (Andersson & Dobson 2003, Balkenius & Kelber 2006, Bogdany 1978, Raguso 2004). The color versus shape hierarchy may reflect the same pattern of importance in color versus scent seen in honeybees, where color is an important cue from a distance, and scent is used secondarily once the flower is reached (Bogdany 1978).

The apparently weak shape learning may also be an outcome of the protocol used in the experiment with respect to the perceptual abilities of the butterflies. Perhaps circle and square are perceived as being more similar to one another than orange and blue, and thus are more difficult for butterflies to discriminate. Using different shape types, such as a radially symmetrical shape versus a bilaterally symmetrical shape, or convex versus concave curvature, may yield stronger responses and learning abilities in this species. Additionally, flowers are not two-dimensional, so incorporating some form of three-dimensionality into shape learning assays may also improve shape-learning rates.

Wild butterflies learn more readily than lab populations

While the presence of learning could not be assessed for wild and laboratory butterflies trained to orange, population differences were seen for butterflies trained to blue. Both wild and laboratory butterflies trained to blue showed evidence of learning, though wild butterflies exhibited a faster learning rate as well as higher maximum

proficiency. The differences in blue learning signify variation in color learning abilities between the two populations. Shape learning reflected the same trend as butterflies trained to blue, in that wild butterflies showed weak learning, and laboratory butterflies showed no evidence of shape learning at all. Population comparisons for butterflies trained to blue as well as shape signify variation in learning abilities between the two populations, with wild butterflies being better learners in a foraging context.

In order to truly assess whether differences in learning between the two populations of study are a consequence of the laboratory and wild environments, learning abilities in multiple laboratory and wild populations must be studied. As this experiment currently has a sample size of one for each population type, we cannot determine the reasons behind the learning differences observed. Differences in learning may be due to random variation between the two populations, or may be due to the differences in selection pressures and genetic processes described in the introduction. Other lab studies showing something similar support the interpretation that differences may in fact reflect factors associated with laboratory and wild populations, though that currently cannot be determined with the data collected. My explanations for the learning results in the laboratory and wild comparison experiment are further explained below, assuming the mechanisms driving my hypotheses are correct and the differences observed are a consequence of the laboratory environment. In order to determine whether my results are in fact an outcome of the hypotheses proposed in this thesis, I will conduct the same experiment using wild and laboratory populations of the Painted Lady Butterfly, *Vanessa cardui*.

Three possible explanations exist, if indeed my results reflect alterations in learning ability as a result of the differences between wild and laboratory populations. First, laboratory butterflies may be weaker learners than wild butterflies as a result of differing selection regimes between the two environments. Wild butterflies are under constant pressure to locate food sources across dynamic landscapes that require learning and memory. Laboratory adults, however, do not need to search through complex habitat for a food source, as they are consistently provided one food type in a constant location. If there is a cost to maintaining learning abilities, as has been suggested multiple times in the literature, losing learning abilities in an environment where it provides no fitness advantages is possible (Burns et al. 2011, Dukas 2008).

Secondly, differences in learning abilities may be due to random genetic processes. The Carolina Biological population was established from a relatively small number of individuals from a wild population, and so is likely to have experienced founder effects, which could impact a multitude of phenotypes including the learning abilities of the future generations in this population (Barton & Charlesworth 1985). Additionally, genetic drift can impact learning abilities if the alleles controlling learning are drifting towards fixation or loss. Drift can be particularly strong in small, closed populations (Nei & Tajima 1981), such as Carolina Biological's *P. rapae* colony.

Finally, and most likely, the differences between wild and laboratory population learning abilities result from a combination of the above explanations regarding both selection pressures and random forces, leading to reduced learning rate and ability in the laboratory population. While behavioral variation has been documented between wild and laboratory insect colonies before, this is the first account of variation in learning

abilities between these two population types. I plan on repeating this study with multiple laboratory and wild *P. rapae* populations or other butterfly species, such as *Vanessa cardui*, in order to determine whether differences in learning ability seen in this study is a true consequence of differences between laboratory and wild populations. In addition, an experiment investigating color learning in cross-bred butterflies between the laboratory and wild populations is underway in order to further understand the underlying mechanisms for differences in learning seen in this study.

Overall significance

My results demonstrate that learning abilities can vary based on stimulus within a modality and population origin. Not all visual stimuli are equally salient to a pollinator, which has been shown in other moth and Hymenoptera species (Capan & Lehrer 2002, Goyret & Raguso 2006, Menzel 1985, Raguso 2004), but never before for butterflies in a foraging context. In addition, few studies have investigated learning differences between wild and laboratory populations, though learning differences have been shown in wild and laboratory-reared vertebrates (Brown et al. 2003, Harker & Whishaw 2002, Papaj et al. 1987). Such comparisons may help us understand how environmental pressures and population parameters can affect learning abilities, and thus fitness outcomes within a species. I plan on further examining this particular topic by comparing additional laboratory and wild populations in order to understand whether my findings are due to simple differences between two populations, or whether they in fact represent generalizable differences between laboratory and wild environments as I hypothesize. The findings from these two studies provide new evidence for how variation in learning

abilities depend on cue type within modalities (such as shape versus color) as well as environmental pressures and genetic processes.

Appendix A: Acknowledgements

I would like to thank my committee, the members of which have been crucial in experimental design and analysis as well as the writing of this thesis. I would like to extend particular gratitude to my advisor, Martha Weiss, for her continued support and mentorship throughout my graduate career. Her continued friendship and advisement has been a wonderful motivational support that has helped shape me into the scientist and person I am today. I am grateful for her time and expertise, and look forward to working with her in the future! I would also like to thank fellow lab member Jean Tsai for her continued support both as a friend and coworker, who has helped me tremendously with editing this thesis as well as experimental design. I would also like to thank Dr. Art Shapiro and Dr. Snell-Rood for advice and sending *P. rapae* for use in these experiments, as well as the Georgetown Department of Biology, which has been crucial in shaping my scientific career. I would also like to acknowledge Sigma Xi, Georgetown University Center for the Environment, and Washington Biologist's Field club, without whose financial support this work would not have been possible.

Appendix B: Figures

Figure 1. Number of days to hatch after eggs were laid for wild and laboratory populations. The black four-pointed star denotes a mean of 3.29 days to hatch for the laboratory population, and the grey star denotes a mean of 4.17 days to hatch for the wild population.

Figure 2. Mean number of days spent in the egg, larval, and pupal stages for laboratory and wild *P. rapae*. Numbers in the colored bars represent mean number of days spent in that stage.

Figure 3. Percentage of butterflies participating in day 1 and day 4 of training for wild and laboratory populations. Letters denote significant differences between each population on each day using Chi-Squared tests.

Figure 4. The proportion of landings on orange for each individual butterfly used in the innate preference experiment across all four days of testing.

Figure 5. Median percentage of correct landings for blue-trained wild and laboratory butterflies for the four days of testing. Error bars indicate median absolute deviation.

Figure 6. Median percentage of correct landings for wild and laboratory butterflies trained to shape on all four days of testing. Error bars indicate median absolute deviation.

Figure 7. Median percentage of correct landings for shape-trained and blue-trained wild butterflies across the four days of testing. Error bars indicate median absolute deviation.

Figure 1

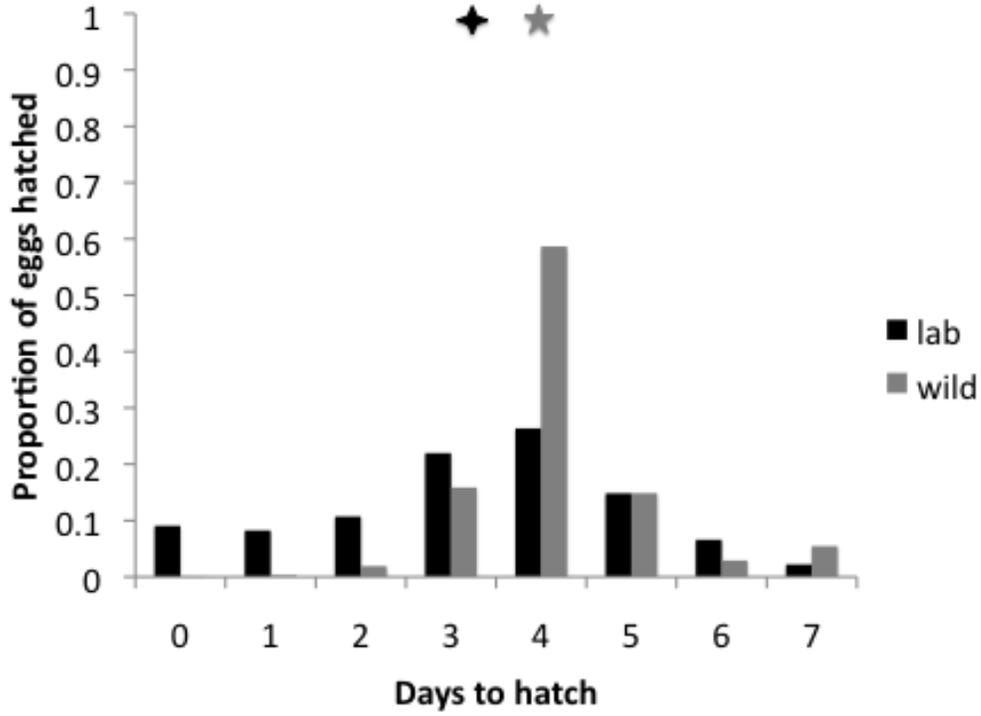


Figure 2

Laboratory



Wild

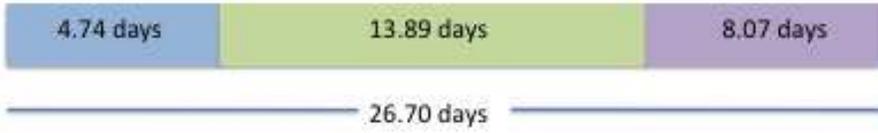


Figure 3

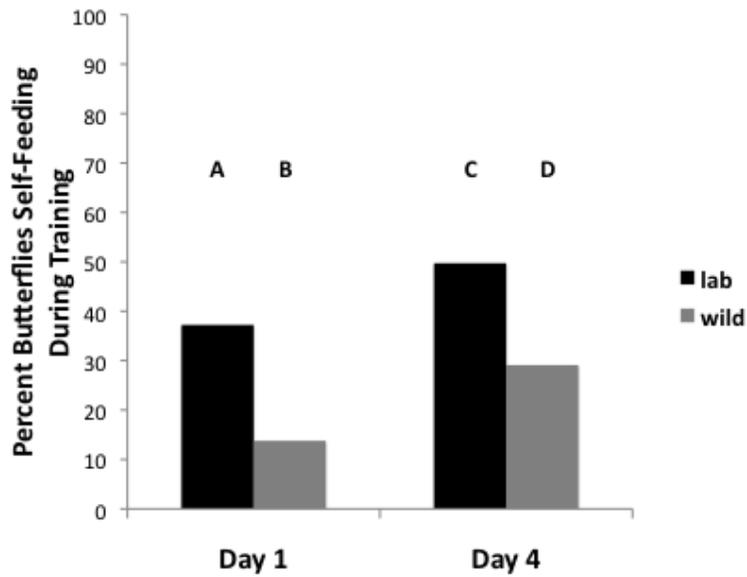


Figure 4

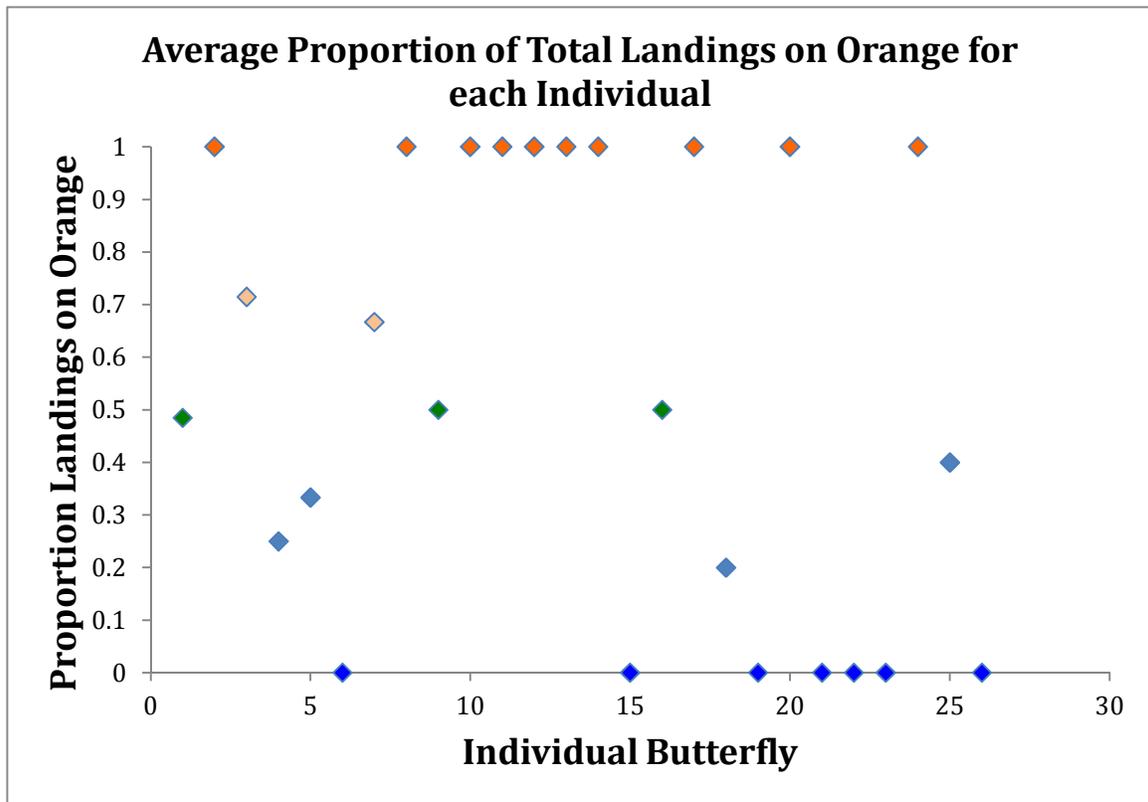


Figure 5

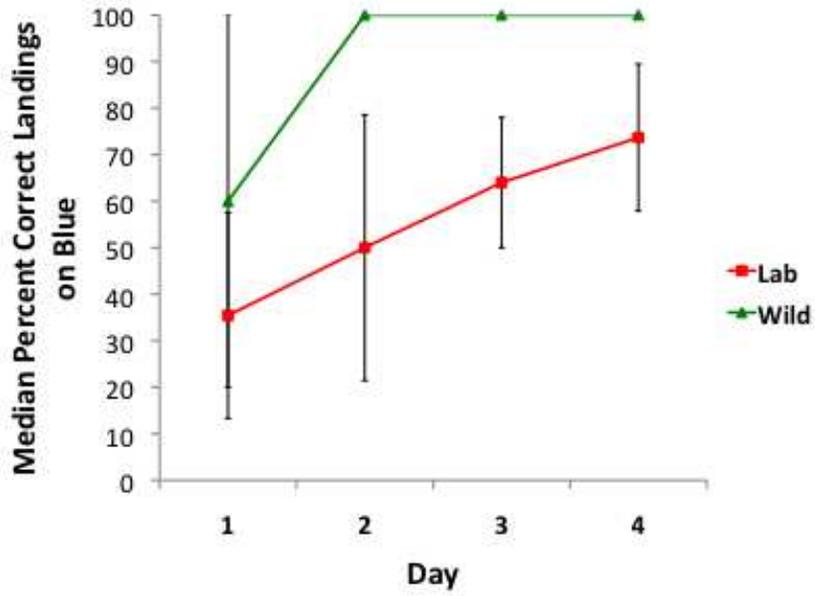


Figure 6

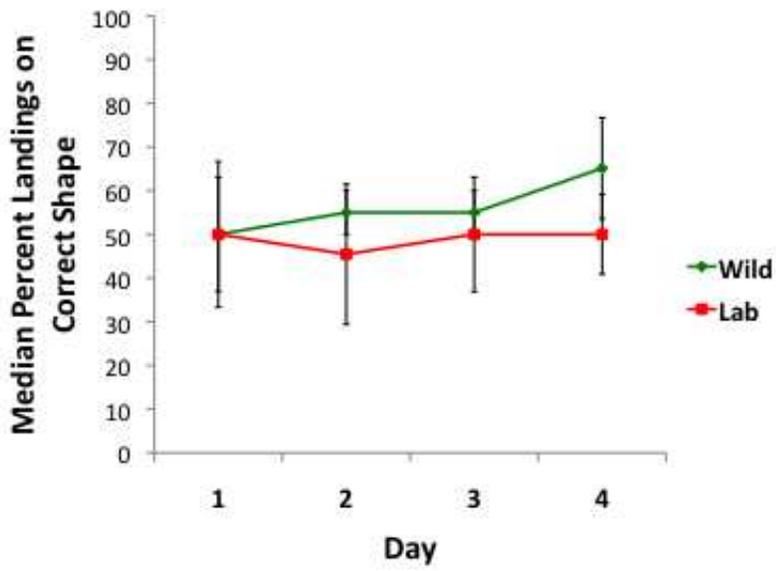
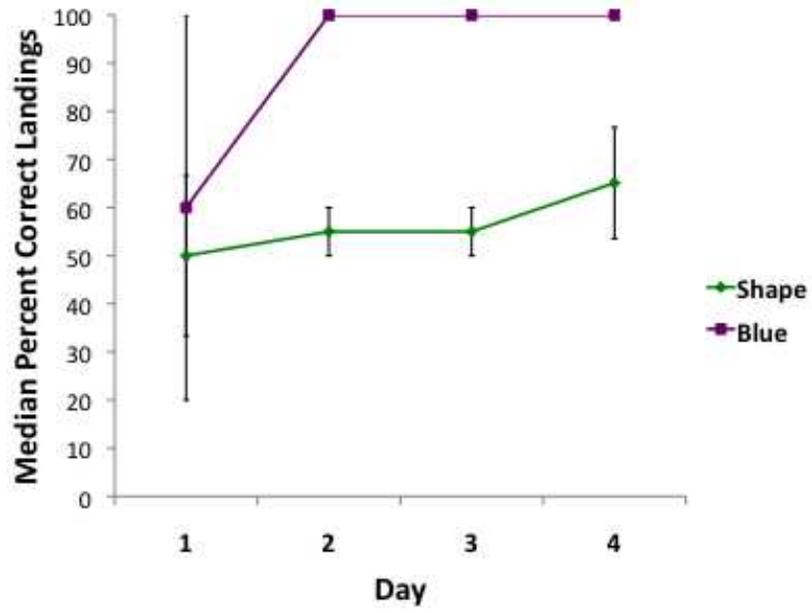


Figure 7



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