7-7-2022

The Effects of Overshadowing in Drosophila melanogaster with Experimentally Evolved Preference

Jill Lee
University of Missouri-St. Louis, jsld3x@umsystem.edu

The effects of overshadowing in *Drosophila melanogaster* with experimentally evolved preference

Jill S. Lee

M.S. Biology, University of Missouri-St. Louis, 2022

A Thesis Submitted to The Graduate School at the University of Missouri-St. Louis
in partial fulfillment of the requirements for the degree
Master of Science in Biology
with an emphasis in Ecology and Evolution

August
2022

<u>Advisory Committee</u>

Dr. Aimee Dunlap, Ph.D.

Dr. Lon Chubiz, Ph.D.

Dr. Zuleyma Tang-Martinez, Ph.D.

Chapter 1

Introduction

Preferences can influence many aspects of the behavior of animals, from mate choice to making the decision where to live and what to eat. At its most simple, a preference is expressed when an animal favors one option over another or others. A preference, for one option or another over potential alternatives, can come in different forms, and many of these have to do with the role of experience and learning. For instance, stating that an option is simply preferred because you always have preferred it, is a little different from saying an option is preferred because you have experience with potential options and have a learning history and familiarity with them. This being said, how does an innate preference develop and how is it modified by learning? Are there explicit selective forces behind most preferences within a given species? The evolution of preferences has been studied for several years. Literature on the topic of preferences has become quite broad over time to incorporate choice preferences, as well as biases in types of decision making. For instance, scrutiny within the psychology of preferences exposed variations in perceptions of decision making. Kahneman et al. found in their classic 1982 study that if a threat of losing something is present, it has an impact on the decision-making process that is greater than likelihood of a potential gain that is equal. Although economically equal, loss is valued differently than gain in this work. These types of basic bias then influence preferences as measured in choice scenarios. As seen in decades of psychology research, preferences play a major role in what drives the decision-making process in both humans and animals. Animals, in particular, alter their behavior and learn from their environment, often in ways that enable them to maximize their fitness under environmental change. Thus preference, molded by evolution and shaped by individual experience, can allow animals to better match future environmental states, even as those states change across the lifetime of an individual.

More recently, researchers have advocated for using the technique of experimental evolution as a way to address some of the foundational questions on the topic of preferences. As Burnham et, al. (2015) argue, advances in the experimental evolution can be the leading force in a course of action for finding the origins of economic preferences. Experimental evolution allows us to test previously intractable problems like the evolution of salience. While preference is a technical term that is used to choose between alternatives, the word salience is usually mentioned as an important component of what could influence preferences. If there are options to manipulate a predicted parameter for the evolution of preference in the laboratory, experimental evolution can support interesting ways to study preferences (Marcus et al., 2018). At times preferences can be considered as an action reflecting pre-existing salience. While salience has been used to study perception and cognition continuously over the years, its meaning can be vague. For instance, it can refer to anything that is prominent enough to stand out from its surroundings. Salience can also be recognized as a notion of a stimulus or of experiences previously in connection with a stimulus, that can then cause organisms to turn their attention toward it (Rumbaugh et al., 2007). Although we do not always have all the information in regard to how salience is used by animals of a given species across different aspects, salience is considered to be absolutely important to evolution and learning. How large a role salience plays within the evolution of learning is still a subject of ongoing research efforts. It is known that selection can help salience evolve to be expressed without experience, as an innate bias, and that salience can be modified, to some extent, by learning.

One of the biggest applications of the idea of the salience of stimuli affecting preference comes from sexual selection, and this example can help outline some of the aspects involved in how salience and preference affect decision making. While sexual selection has multiple components, mate choice is an extremely important component of this process. Mate choice is frequently a decision process where, for instance, a female chooses between two or more males, with this choice being dependent to some extent on the attractiveness of the male’s phenotypic traits. The phenotypic traits being assessed by this female can include things such as colors, shapes, sizes, sounds, and olfactory cues. For example, during a sensitive period during the early stages of a songbird's life, their exposure to specific variants of song influences their later choice of mates. In some species, females match the songs heard during their early sensitive period; while in other species, females may prefer novelty in comparison with familiar songs. However, across sexual selection, preference for many traits appear to be strongly inherited. A classic example is mate choice in guppies. Females typically prefer males with more orange coloration than males who do not have this coloration (Macario et. al, 2019). Similarly, females in swordtail fish species prefer males with sword tails; and, even in closely related species without this male feature, females display a preference for males with sword tails (Ryan and Wagner, 1987). In this brief example in the context of mate choice, both unlearned preference and learning play important roles.

Preferences can be shaped by learning, and can also modulate learning

Over the years, learning has been defined in many different ways. Most researchers in the field today define learning as a relatively permanent behavioral change, resulting from prior experiences. In psychology, for instance, many of the topics that are related to learning, spotlight how people learn and connect with their environments. Learning is considered a basic behavior that is crucial for animals to behave adaptively in various situations throughout their lives. And there are some experiences, either with good or bad outcomes, that could affect behavior more than others. With these experiences, animals can develop preferences that are interrelated with their learning abilities. In terms of experience, preferences can most importantly be affected by the environment an animal is in. Their surroundings, geographical location, prior experiences, and learning abilities, are all able to shape the preferences of an animal (Dunlap and Stephens, 2016). These preferences can, in turn, also affect what is later learned and how well new experiences are learned. Because of this, preferences are known to change over time, and are not considered necessarily stable through an individual's lifespan. How specifically do preference and salience affect learning? According to Rumbaugh et al. (2007), when organisms are put into a specific environment or natural situations repeatedly, this can allow for associations to occur which will become more salient over time. Because of this, behaviors that develop due to common relationships become more likely to form, forming the basis of learning. This, in turn, can create preferences that are "intuitive," or more salient, based on comparing current stimuli to others that may have previously been relevant. An organism that is "aware" of the possible choices when deciding, has a magnified ability to facilitate learning. While this section focuses on preference and associative learning, it is important to note that salience should also affect non-associative learning processes of habituation and sensitization. These, in turn, can also affect preference. Habituation is a form of non-associative learning in which an innate response to a stimulus decreases after repeated or prolonged presentations of that stimulus. A stimulus that is evolutionarily important should be less prone to habituation and should show higher levels of sensitization. Sensitization is a non-associative learning process in which repeated administration of a stimulus results in the progressive amplification of a response. And a stimulus that is more salient should, perhaps also, result in enhanced sensitization.

Taste Aversion Learning

Conditioning in animals is often split into two categories: aversive conditioning and appetitive conditioning. Aversive conditioning is based upon unconditioned stimuli that are avoided because they are unpleasant or painful, such as shock, nausea, or potential predation. Appetitive conditioning is in play when the unconditioned stimulus is considered positive and is the motivation behind behavior; examples are food, water, and even the opportunity to mate. In both situations, actions can then reinforce the well-being of the subject. Strong learned aversions are prevalent across animals, and often come in one well-studied type of learning: taste aversion learning. Taste aversion learning is distaste or aversion for a particular taste or smell that has been previously associated with something considered to be negative. One of the best-known examples of this, and covered in nearly every textbook of animal learning, is the Garcia effect. The Garcia effect is a classic example of how evolutionary history might enable some pairings to be associated more easily than others.

The Garcia effect, or otherwise known as conditioned taste aversion, is a reaction that is acquired due to a smell or taste that an animal happens to be exposed to before becoming ill. This effect was discovered by John Garcia after his experiment with studying the effects of radiation on mice (Garcia and Koelling, 1967). Throughout his experiment, he noticed that mice would avoid drinking water in a plastic bottle if given directly before radiation exposure. If the same rats were given water in a glass bottle, they would drink. This experiment showed a great example of radiation sickness. It was speculated that the plastic gave a novel taste to the water that allowed for the rats to conclude that this was the reason behind their sickness. Garcia also noted that the rats did not seem to avoid the area in which the radiation was given; but if they did, it took the rats longer to learn this association than just aversion of taste. In the 1967 study by John Garcia and Robert Koelling, they used experimental groups consisting of 10 rats placed in a light and sound shielded box with a drinking spout connected to an electronic drink meter that was used to count each touch of a rat's tongue to the spout. Garcia and Koelling were seeking to determine the differential effectiveness of cues, due either to the nature of the radiation or toxic effects and peculiar relations, which a gustatory stimulus has to the drinking response. Garcia and Koelling essentially came up with the idea to make an auditory and visual stimulus dependent upon the animal having to actually lick the waterspout. This was done in four experiments: "bright-noisy" water, "tasty" water paired with radiation, a toxin, immediate shock, and delayed shock, as punishers. The capacity of these response-controlled stimuli, to inhibit drinking in the absence of reinforcement, was tested later. The experiment first consisted of a one-week habituation to drinking in the apparatus without stimulation. There were pre-tests to measure the intake of bright-noisy water and tasty water prior to training. In addition, there was an acquisition training with reinforced trials, where the stimuli were paired with reinforcement during drinking and non-reinforced trials, where rats drank water without stimuli or reinforcement. The training then terminated when there was a reliable difference between water intake scores on reinforced and non-reinforced trials. There were post-tests to measure intake of bright-noisy water and tasty water after training. The results of their experiment indicated that all reinforcers were effective in producing discrimination learning during the acquisition phase, but obvious differences occurred in the post-tests. The avoidance reactions produced between the post-test scores were statistically significant in both experiments. Gustatory stimuli produce nausea and gastric upset when they are paired with agents that acquire secondary reinforcing properties, which might be described as "conditioned nausea." Auditory and visual stimulation did not acquire similar properties, even when they are contingent upon the licking response.

Both Garcia and Koelling did exceptional work with taste aversion, even though their research questioned what was previously understood about classical conditioning. Today, their work has opened the door for new experiments with the concept of taste aversion and gastric upset. Over the years, conditioned taste aversion has been proposed to be linked directly to an animal's fitness. If the animal can learn to avoid something that brings forth illness or nausea, the animal eventually increases its chance to survive.

What Are Blocking and Overshadowing?

Overshadowing is defined as a term from classical conditioning, where learned associations can be decreased for one conditioned stimulus due to the presence of a second conditioned stimulus (Mackintosh, 1976). Overshadowing is typically considered a result of the differences between characteristics of a pair of stimuli like intensity or the strength of any behavior, such as an impulse or emotion. Overshadowing is usually seen when there happens to be two or more stimuli present and one of those stimuli produces a stronger response than the other. This can happen when one stimulus appears to be more prominent in some way to the animal than the other stimulus. Overshadowing can also occur when an organism fails to learn or ignores a stimulus. When overshadowing happens, subjects trained to a compound stimulus (stimuli A and B, presented together) that are paired with an unconditioned stimulus, like quinine, will learn less about one of those components, for instance, B, than if they had been trained with B alone. Specifically, if A overshadows B, the test of A alone will result in stronger learning of B than that of B alone, even though subjects had identical experience with each stimulus.

A great real-life example of overshadowing is using a treat, as a way to teach your dog to sit. Normally, when training your dog, most pet owners will start by saying a verbal command for their dog to "sit." But an owner could also use a second cue simultaneously, like pointing at the floor. The next step, as the owner, would be to add a treat once the dog sits, after saying the verbal command to “sit,” while also pointing at the floor. Now the dogs may have learned this compound cue, and sits upon hearing the word and seeing the gesture. But it is also possible for one cue to overshadow the other. In this case, the dog has learned the association between “sit” and sitting, but not the association between the hand gesture and sitting, or vice versa. By testing each potential association separately, we can reveal how the dog is using two potential cues for learning to sit and receive a treat.

A related phenomenon, which can happen when multiple stimuli are present, is known as blocking, which is a result of prior experience with one part of a compound stimulus. Blocking broadly refers to failing to learn new information, due to remembered past experience. The cross species learning effect of blocking has been studied using classical conditioning and can have similarities to overshadowing effects (Cassaday, 2014). This principle of conditioning was first introduced by Kamin (1969). Kamin (1969) explained that having a conditioned stimulus that predicts an unconditioned stimulus is satisfactory for learning. If an animal is able to learn that a conditioned stimulus gives a solid indication of an unconditioned stimulus, then the animal does not need to become conditioned with another conditioned stimulus. The term blocking comes in because, in this scenario, the animal will not learn any other conditioned stimulus that will predict that unconditioned stimulus; the original learned association blocked the formation of the new association. Blocking is tested identically to overshadowing, except a pre-phase is present where only one stimulus (A only) is paired with an unconditioned stimulus; once this association is learned, the animal is presented with the compound stimuli (A and B together). And like with overshadowing, animals are tested for learning to A alone and to B alone. If performance with B is lower than performance with A, then the blocking of B by A has occurred. Blocking is typically considered more likely to occur when the first stimulus presented is more salient than the second.

A great real-life example of blocking is when your pet is originally exposed to the sound of a bell that tells them their food being poured into a bowl to eat. After the bell and food have been associated together reliably, the dog approaches the food bowl whenever the bell is rung. Now a second stimulus will be added to the pairing, say a flashing light over the bowl. So, the dog is approaching the food bowl when the bell sounds and the light flashes. When tested with only a flashing light, however, the dog doesn't approach the bowl. Assuming the dog still approaches the bowl when only the bell sounds, we would say that the bell-food association has blocked the learning of the bell-light association. It isn't a given that this blocking would happen, but it is a potential outcome of this training scenario.

What unifies both blocking and overshadowing, other than the presence of multiple stimuli, is this emphasis on the importance of salience of the stimulus. The more salient of the two stimuli is what is more likely to overshadow, as well as block the other stimulus. But this explanation can be problematic because salience is typically defined post hoc: something is important because the animal acts as if it's important. And this salience is then often assumed to have evolved. For this reason, I am approaching these classic aspects of learning in a system where salience has been experimentally evolved. Thus, the full recent evolutionary history of the animals being tested is known. Our lab has a model system where fruit flies have been evolved to have innate biases towards certain stimuli, and I now discuss the biology of these choices for flies.

How Do Flies Decide Where to Lay Eggs?

For animals that lay eggs without any additional maternal or paternal care, it is very important to be selective when deciding where to lay those eggs. Flies, in particular, are very selective with the environments that they choose to lay their eggs and this choice affects the survival and development of their offspring. Substrate texture, microbial composition, fermentation volatiles, color, density, and temperature are all selected for or against in the oviposition decisions made by flies (Dweck et al, 2013). With this being said, can we reliably predict certain cues or conditions that result in a fly laying her eggs in one place over another? What role does the ancestral history of *Drosophila melanogaster* play? This is an active area of research. From previous studies, it has been found that olfactory and gustatory cues play a huge role as an oviposition stimulant and in guiding choice. In the 2013 study of Dweck et al, the experimenters set out to find if certain fruits have an influence over oviposition preference and if those fruits acted as oviposition stimulants. Flies were exposed and have unrestricted access to different fruits, six at a time, using a multiple-choice oviposition assay. All of the fruits used in their experiment were ripe and undamaged, as a way to reduce the amount of yeast the flies could be exposed to that could then influence the decisions made by the flies. Across their experiment, the flies consistently chose oranges as a site for oviposition over 15 other fruits that were tested. None of the flies has had any previous experience with any kind of fruit. With this, the experimenters determined that the observed preference for orange by the flies must be innate. With further work, Dweck and colleagues showed that this orange preference was due to a single class of olfactory sensory neurons, Or19a, which detect characteristics of terpenes in the citrus fruits. Terpenes are aromatic compounds found in many plants that create their scents and are usually found in the colored rinds of citrus fruit. Dweck et al. chose the terpene limonene to single out and test in their experiment. They tested a transgenic line of sweet oranges with a reduced content of limonene against a control and multiple different fruits. Compared to the other fruits, the line with the reduced content of limonene had no difference in the number of eggs flies laid on the substrates. This meant that having a normal abundance of limonene is very important for an increased oviposition seen in fruits containing citrus. Limonene may be an oviposition attractant instead of being an oviposition stimulant (Dweck et al, 2013).

Not only did the experimenters find that flies preferred citrus, but additional work from other labs found that these aspects of oviposition preference are an ancestral trait from their native southern African habitats (Mansourian et al, 2018). In the present day, those habitats are known as Zimbabwe and Zambia. The Miombo and Mopane Forest are found in this area and are home to many plants that bear fruit. Of all of the fruits that are found in this area, the marula stands out because of its thick rind that is similar to that of citrus. The Marula fruit contains a juicy pulp and a pH similar to orange, which are both physical and chemical properties that are the known preference of *Drosophila melanogaster*. Above all other fruits tested, flies showed a preference for ovipositing into marula fruit, and this preference is extremely evolutionarily conserved, being found in populations of flies that left Africa hundreds of thousands of years ago. Mansourian and colleagues showed that marula fruit is locally abundant in these areas of Africa and are preferred by local flies, as well. They hypothesize that these patterns of resource abundance and availability have shaped the evolution of oviposition preference for marula.

Resources availability is not the only selective pressure for oviposition preference; avoidance of risk can be equally important. Dweck and colleagues furthered their work on citrus, asking adaptive questions about why the preference for citrus is so strong. Preference for citrus may thus have more meaning than long gone ancestral history and nutritional aspects of the substrate. They hypothesized that the choice of citrus may be a response to parasitism, which is another factor that can influence the decision-making process of where flies are choosing to lay their eggs. Populations of >80% paratization rate that are caused by endoparasitoid wasps are reported as the greatest cause of mortality in *Drosophila melanogaster* (Dweck et al, 2013). Citrus fruits have a rind that is thicker than most fruits and this may be a characteristic that flies seek out as a source of protection for their eggs against parasitoid wasps, as parasitoid wasp ovipositors cannot penetrate fruits with this thick epicarp. Dweck and colleagues found that at least one parasitoid wasp indeed shows a preference against citrus odorants when tested. In addition, Mansourian and Stensmyr (2015) found in their work that across the oviposition neurobiology work, there are some aspects of preference are determined by responses of specific olfactory receptor neurons, which can include things such as with the citrus receptor, but that this choice can also modulated in the brain. There is also evidence that some of these preferences have evolved in response to resource availability and risk.

<u>Experimental Evolution of Oviposition Preference in Flies</u>

Displaying animals in worlds that were created specifically by experimenters, then allowing those populations to evolve based off of their responses to the environmental conditions created is in fact experimental evolution (Dunlap et al, 2019). In addition to the evidence covered above, preference has proved a tractable behavioral trait for selection laboratory evolutionary experiments. Although in sum, these experiments show that evolving an innate preference for an oviposition substrate is not completely straightforward, as originally though. I reviewed some of these experiments here.

The first research, on experimentally evolved oviposition preferences in flies, was published nearly 20 years ago, Mery and Kawecki evolved learning, as well as preference flies in two experimental evolution studies (2002, 2004). In their first study, their populations of *Drosophila melanogaster* were maintained under conditions that they proposed, would favor the evolution of learning in connection with oviposition substrate choice (2002). In their experiment, the flies have the option of two media (orange and pineapple), one of which was paired with quinine, a chemical that is aversive to flies. In a second stage, 30 minutes later, quinine was no longer present and flies choosing to lay eggs on the substrate that had not been paired with quinine were exhibiting behavior consistent with learning. By alternating each generation which substrate was paired with quinine, they evolved populations of flies with enhanced learning abilities. Their second study attempted to evolved oviposition preference in a completely fixed environment, where the same substrate always corresponded with fitness and, also always, corresponded with the choice consistent with learning (2004). Once again, they succeeded in evolving enhanced learning, but not the enhanced preference they were predicting.

Dunlap and Stephens (2009) were able to evolve innate preference for oviposition in flies and did this by placing preference at odds with learning, as predicted by their model. Essentially, while an innate preference might be predicted in an environment that never changes, learning is also a successful strategy because cues for learning are reliable. Both strategies can be favored in a fixed environment. However, if learning is unreliable, but the best place to lay eggs every generation is fixed, then preference can evolve. And this is the scenario in which Dunlap and Stephens evolved preference in flies. Experimental evolution gives us a controlled and known evolutionary history. We can't go back in time to see how a preference for marula fruit evolves, but we can use experimentally evolved populations with evolved bias and then ask questions about how that bias affects decision making and learning. For this thesis, I am using populations of flies which have evolved innate preference towards orange and against pineapple; and, populations of flies, which have evolved innate preference towards pineapple and against orange. How will these evolved biases affect how flies acquire new information and learn? According to predictions from salience, this evolved bias should affect how multiple cues will interact in blocking and overshadowing paradigms.

How evolved flies can be used to test questions about innate bias and learning

Drosophila melanogaster has been used as a model organism to study genetics for over a hundred years. These flies have a very short life span; and because of this, large quantities of flies can be produced in a small amount of time. For an adult fly to develop, it only takes 10 days from fertilization. 1500 eggs can be produced by a female fly in their lifetime. Since fruit flies are very small, they are relatively easy to maintain and have very little requirements. Fruit flies can be raised and tested even in the smallest amount of space.

Using populations of flies with a known evolutionary history (for the previous 200 generations), I am asking questions about how learning and preference interact. I am doing this by specifically comparing two different stimuli in populations with a history of making choices about those stimuli, with a third stimulus, which these populations have not encountered in over 500 generations, if not more. Our basic prediction is that when a preference to a specific substrate is evolved, that stimulus should become more salient, and should thus overshadow other learning. We are able to directly test this major assumption in animal learning because we have experimentally evolved populations of flies to have different substrate preferences for where females will lay their eggs; because this evolutionary history is known, and has been directly manipulated, we have an excellent ability to test this assumption. We use a basic paradigm of taste aversion. Here a fly has the opportunity to learn about two substrates: one which is “good" or rewarding and one which is paired with quinine, a bitter substance which flies avoid. In psychological terms, we have set up discrimination learning with a rewarding (good) option (the S+) and a punishing (aversive) option (the S-). In this paradigm. we then test overshadowing in two contexts: either the S- pairing may have overshadowing possible, or the S+ pairing may have overshadowing. And overshadowing is made possible because in those cases, a compound stimulus is present.

Chapter 2

Introduction

Preferences arise within decision making when one option is favored over one or others. For instance, if an individual favors one stimuli over another, it has a preference. A preference can also refer to specific characteristics that are desired by certain individuals. Mate choice, favorite foods or favorite colors are all examples of contexts where individuals show inherent preferences. Preferences can also be influenced by learning, and here preferences are formed when individuals remember a specific feature of a stimuli during the learning process. Because of the role of experience, preferences are highly affected by choices that an individual makes. Preferences can also be considered as an expression of salience. As such, salience is considered to be a crucial part of both evolution and learning.

Evolutionary history can also carry information about salience and preference, and these preferences can be robust to the experiences inherent in environmental change for an organism. This interaction between evolution, preference and learning are all tractable through the approach of experimental evolution. In previous research done by Marcus et, al. (2018), they found that by manipulating variables surrounding learned and evolved preferences across generations of an organism in the lab can help researchers understand why some preferences are innate. Having the ability to learn the difference between stimuli or associations at a faster rate can be evolved (Mery & Kawecki 2002, Dunlap and Stephens 2009).

In the real world, there is often more than one simple choice to be made. When there is more than one stimulus present, the one that is the most reliable stimulus is typically learned. According to Rumbaugh et al (2007), organisms that are put into specific environments or natural situations repeatedly allow for relationships that are common to occur and become more salient over time. When an organism fails to learn a stimulus, or appears to ignore a stimulus, this may be due to a process known as overshadowing. Overshadowing is usually seen when there are two or more stimuli present simultaneously and one of those stimuli produces a stronger learning response than the other, thus overshadowing it. Overshadowing happens as a result of the differences between characteristics of a pair of stimuli, such as intensity, motivation, and the strength of any behavior such as an innate response. Implications of overshadowing are that not all stimuli are able to be learned equally when they are presented simultaneously. While overshadowing is a classic and well-known area of research into the psychology of learning (McIntosh, 1974), less is known about its function within more natural settings. We expect overshadowing to interact with how an animal's relationship to stimuli have evolved.

The main focus of this work is to test the hypothesis that an evolved preference for a stimulus interacts with learning. Does having an evolved preference affect learning? Specifically, will having an evolved preference for a stimulus make that stimulus more resistant to the psychological process of overshadowing by learned stimuli? With this experiment, the subjects of the experiments are the products of an experimental evolution study testing the effects of changing environments on the evolution of learning and unlearned preference. Populations of *Drosophila melanogaster* were previously experimentally evolved in our lab for female preference of laying eggs on either orange or pineapple following the procedures (Dunlap and Stephens in prep, following methods of Dunlap and Stephens 2009). We then used these populations to take a deeper look at these overshadowing effects and learning for oviposition. In this paradigm, a fly has the opportunity to learn about two substrates on which she can lay her eggs: one which is good, and one which is paired with quinine, a bitter substance which flies avoid. Within this aversion learning paradigm, we add the component of overshadowing and predict that evolved preference should be less susceptible to these effects. Specifically, flies with an evolved preference for orange will be less susceptible to potential effects of overshadowing of an orange-flavored substrate by a novel apple-flavored substrate than flies with an evolved preference for pineapple-flavored substrates. Similarly, the pineapple-preferring flies will be less susceptible to potential effects of overshadowing of the pineapple substrate by apple-flavor than orange-preferring flies. By analyzing this data, we have the opportunity to directly test how salient stimuli with a known evolutionary history can then induce or be resistant to overshadowing effects.

Methods

Flies and Husbandry. A total of 12 fly populations, experimentally evolved to prefer either orange or pineapple were used for this experiment. The fly populations were part of a larger experimental evolution study and are replicates of the preference flies described in Dunlap and Stephens (2009). Populations were labeled as A1 through A12, with the even or odd number signifying the evolved oviposition preference. Odd numbered lines were the orange preferred populations and even numbered lines were the pineapple preferred lines. All flies were reared in identical environments within standard narrow fly vials (1 1/4" diam × 4" H), on cornmeal-molasses food. To control for social developmental effects, flies were always reared at a density of about 80 eggs per vial. For a single day of testing, a total of 18 vials of eggs would be collected per line. After collection, the vials were housed in an environmental chamber at 24°C for 10 days until the adults enclose from their puparia. Adult flies were then moved into test cages in a similar set up to those shown in Figure 1, with every line having six cages each. Three vials of each fly line were added to each cage allowing for a population of about 240 adult flies per cage. Approx. 17,280 flies were tested in total. Test cages were similar to the size of a shoe box (30 cm length x 19 cm width x 10cm height). Each cage contained a removable tray (similar to a pull-out drawer) that could hold two 100mm petri dishes. Before testing began, flies were given two petri dishes with cornmeal and molasses media for three days. These days allowed flies to become accustomed to their environments, as well as allowing mating and females to reach high levels of fecundity.

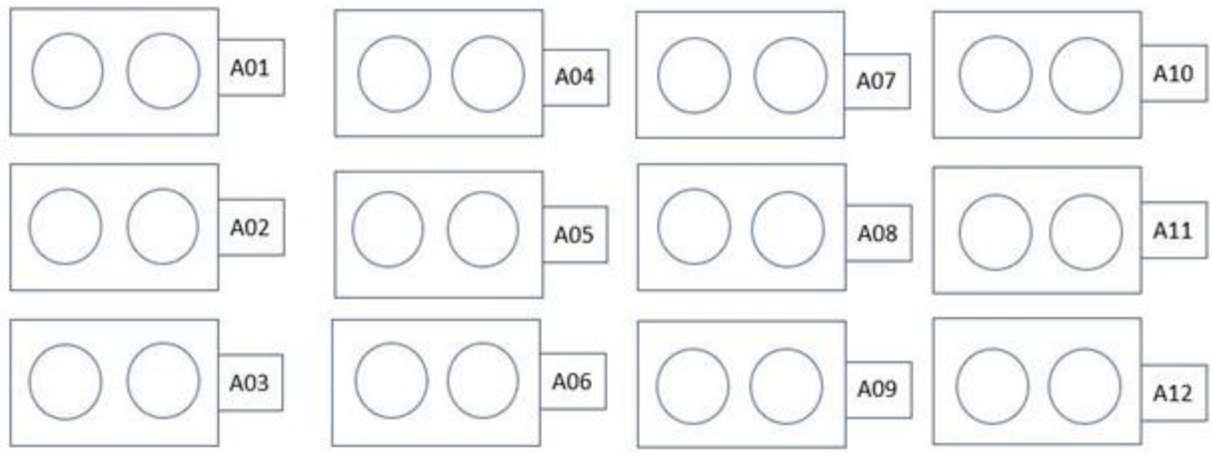

Figure 1. Overhead view of the experimental design of how selection cages were set positioned on a single shelf in an environmental chamber for testing purposes. The petri dish placement was randomized inside of each of the cages for both phases. Cage placement always ensured a spatial blocking of the evolutionary treatment types (denoted by odd versus even numbering of fly lines) within each shelf used for testing, thus controlling for potential microclimate and lighting effects.

Overshadowing and Selection of Fly Populations. Each test for a group of flies (a single cage) consisted of two phases, a learning (experience phase), in which flies were able to gain experience with the aversion learning pairing, and a test phase (consequence phase), in which we measured how that previous experience modified their choices. During the experience phase, flies were exposed to three flavors of a juice agar (made from frozen concentrated juice). Two of these flavors, orange, and pineapple, represented the choices the flies' mothers and grandmothers had made over many generations of experimental evolution. These populations had been selected to choose orange while ignoring pineapple, and vice versa. The remaining flavor, apple, was chosen as an arbitrary third option that the flies have not experienced in over 500 or more generations, which would be a fruit that flies would choose to lay eggs on, but not one they had recent evolutionary experience with.

We set up overshadowing tests according to the classic designs described in Chapter 1. Flies were given two petri dishes, each containing 10mL of one or a mixture of the juice agars for the experience phase that were placed on the removable tray at the bottom of each cage. One of the agars would include quinine at 4g/L to serve as the aversive stimulus. Learning flies should form associations such that they avoid the flavor(s) paired with quinine or the aversive option (S-) and choose to lay their eggs on the quinine-free agar or the rewarding option (S+). The petri dishes were placed on the sliding trays so that when they needed to be removed, it was easier to do so without having to remove any of the flies. After the three-hour experience phase, petri dishes were then removed, and the next phase would begin immediately. The second phase, a test phase, consisted of two petri dishes with 10ml of agar-based media, each with a single juice flavor, without quinine. Flies were then allowed to lay eggs for one hour. Females making choices consistent with learning the quinine association will lay eggs on the flavor not previously paired with quinine. The locations of the plates were randomized in both phases, meaning that the flavor associated with quinine placed in front during the learning face would now be in the back in the testing face and no longer associated with quinine and vice versa.

Treatments and Lines. Space and time constraints meant that a single replicate of every treatment for every population was not possible during a single day. Thus, during each day of testing, the 12 lines were randomly assigned to six of the eight treatments. There was a total of five selection periods, allowing each treatment type to have exactly four trials each. The experiment has two halves: one in which a single flavor was mixed with quinine, and thus the potential overshadowing would occur between the fruit stimulus, which have a positive valence. Thus, flies must contend with two stimuli where they should lay eggs. This follows classic overshadowing studies. In the second half of the treatments, quinine was mixed with two stimuli, and here flies are contending with two stimuli where they should avoid laying their eggs. Specifically, treatments A-D had quinine associated with a single flavor during the experience phase in one petri dish, while the other dish contained a mixture of two flavors without quinine. In the test phase, Treatments A-D had single flavors in each dish mixed with quinine. Treatments E-H had quinine associated with a mixture of two flavors during the experience phase in one petri dish, while the other dish contained a single flavor without quinine. Similar to Treatments A-D, in the test phase, Treatments E-H had single flavors in each dish. All treatments and predictions are depicted in Figure 2.

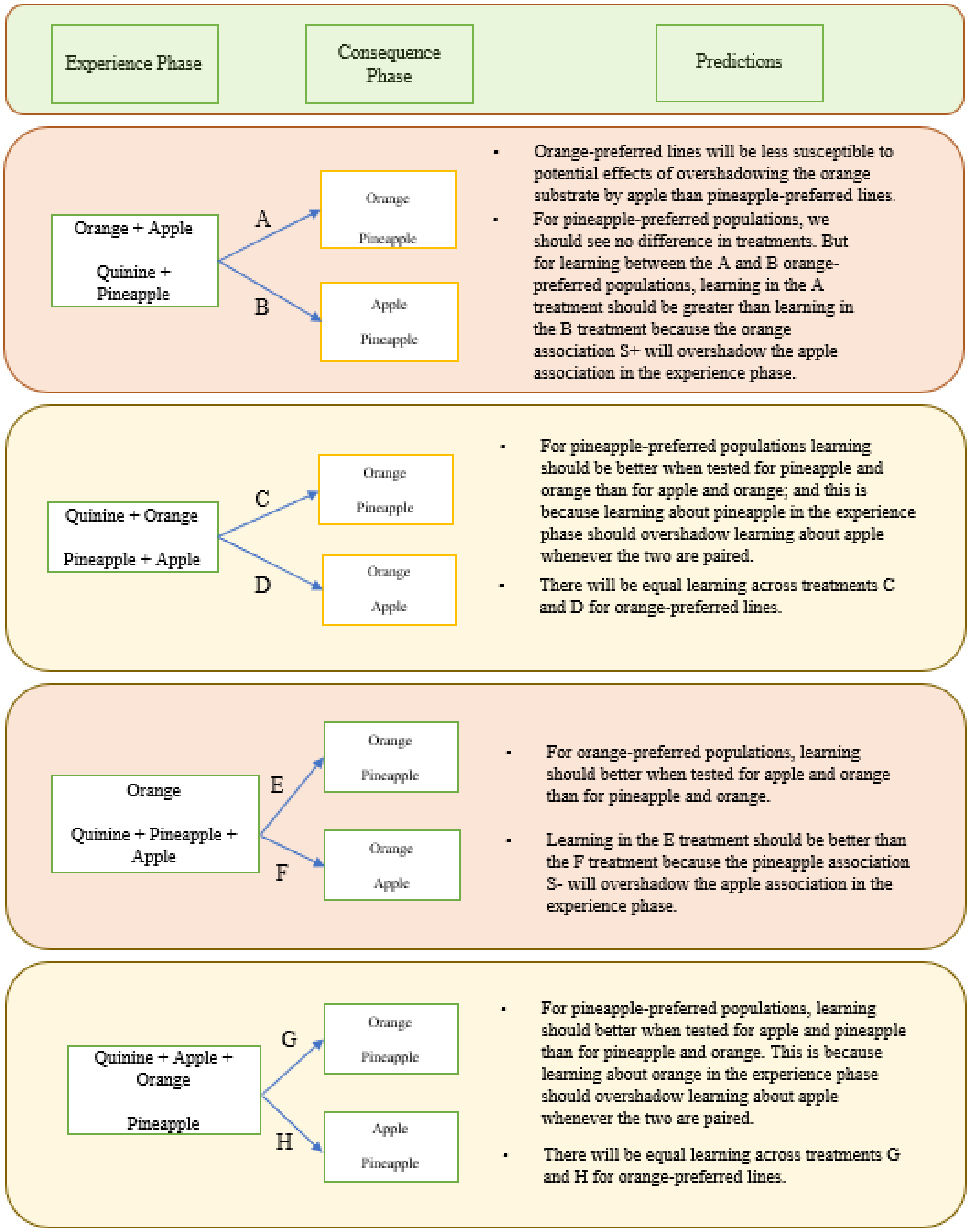

Figure 2. Experimental design for squares on the left show the experience phases for all 8 treatments. Middle squares show test phases for all 8 treatments. Right side of the figure explains the predictions for each of the 8 test phases. In the experience, treatments A-D had a choice of quinine associated with a single flavor or a mix of two flavors without quinine. In the test phase,

treatments A-D had a choice of two single flavors. In the experience phase, treatments E-H had a choice of quinine associated with a mix of two flavors or a single flavor without quinine. In the test phase, treatments E-H had a choice of two single flavors.

Data Collection. All petri dishes were labeled with a red serial number that was used to identify the agar-based media used in each treatment, as well as the replicate cage. The serial numbers were unique to each petri dish. Placement of the petri dishes were randomized. Red markers were used to number the bottom of the petri dish due to the fact that flies do not have red photoreceptors and cannot see the color red. Numbers were recorded when each petri dish was initially placed into cages for testing of each treatment type. After every testing day, high resolution photos were taken of each of the petri dishes individually using a DSLR camera with a macro lens. The camera was attached to a copy stand to take pictures of the petri dishes from the above view. The staging table was illuminated underneath so that each egg could be counted more easily. Photo files were numbered to match the petri dish serial numbers. Egg density was counted using the iPad Apple store application Visual Counter. Images of the media plates were open through the app and each egg was counted individually. Egg counts were recorded by date, treatment, and petri dish serial number. Treatments were unknown while counting eggs in order not to introduce bias while counting eggs. From these values, a preference index was calculated for each cage replicate. Preference index was calculated by the number of eggs laid on media flavor one minus the number of eggs laid on media flavor two divided by the number of eggs laid on media flavor one plus the number of eggs laid on media flavor two.

Statistical Analysis

A factorial analysis of variance (ANOVA) was performed with the outcome variable of the proportion of eggs laid on the target media for each replicate, which for each treatment was the correct choice defined by what would be consistent with learned behavior (laying eggs on the substrate not previously paired with quinine). Each evolved population of flies was treated as a random effect, to account for repeated measures of each population, and each of the populations was nested within their evolutionary background (evolved to prefer orange or evolved to prefer pineapple). We tested main effects of the Overshadowing Target, abbreviated as OS Target (orange or pineapple), the Overshadowing Type, abbreviated as OS Type (whether the compound stimuli were the S+ (rewarding option) or the S- (punishing option)), Test Option, abbreviated as TO (whether an evolved stimulus, or the novel apple stimulus), and Evolutionary Background (orange or pineapple), and then the full interactions of these effects. We are specifically predicting that the evolutionary background will interact with the assigned OS Target as well as with OS Type.

The specific model is:

Model: (Intercept), OSTarget, OSType, TO, EvolBackground, OSTarget * OSType, OSTarget * TO, OSTarget * EvolBackground, OSType * TO, OSType * EvolBackground, TO * EvolBackground, OSTarget * OSType * TO, OSTarget * OSType * EvolBackground, OSTarget * TO * EvolBackground, OSType * TO * EvolBackground, OSTarget * OSType * TO * EvolBackground, Line (EvolBackground)

Results

In these results, I first present a general overview of the data, split in two-treatment comparisons as in the predictions. I present the results from a statistical analysis of the entire experiment, going through each significant main effect and the interactions among effects. I then focus on the basic differences between populations and treatments, as well as the variability among them. I then present the results from a statistical analysis of the entire experiment, going through each significant main effect and the interactions among effects.

Treatments A-D were testing overshadowing and learning where to oviposit. For overshadowing, the two substrates from the flies' evolutionary history: orange and pineapple. Our prediction for treatments A and B stated that flies with an evolved bias for orange should show more overshadowing here than flies without an evolved preference for orange. Orange-preferred populations were less susceptible to potential effects of overshadowing the orange substrate by apple than the pineapple-preferred lines for treatments A and B. For pineapple-preferred populations, we saw no difference in treatments. While flies were in the consequence phase for the A and B treatments, orange-preferred populations laid more eggs in the A treatment than the B treatment. Fly populations learned that in the B treatment the orange association S+ (rewarding option) overshadowed the apple (novel) association in the experience phase. Our prediction for treatments C and D stated that flies with an evolved bias for pineapple should show more overshadowing here than flies without an evolved preference for pineapple. Pineapple-preferred populations saw more eggs laid in the C treatment than the D treatment while flies were in the consequence phase. Fly populations learned that in the D treatment the pineapple association S+ (rewarding option) overshadowed the apple (novel) association in the experience phase when the two were paired. For orange-preferred populations, we saw no difference in treatments.

Treatments A-D were testing overshadowing and learning where not to oviposit. Our prediction for treatments E and F stated that with an evolved bias for orange should show more overshadowing here than flies without an evolved preference for orange. While flies were in the consequence phase for the E and F treatments, orange-preferred populations laid more eggs in the E treatment than the F treatment. The pineapple association S- (aversive option) overshadowed the apple association in the experience phase. Pineapple-preferred populations saw more eggs laid in the H treatment than the G treatment while flies were in the consequence phase. Fly populations learned that in the H treatment more eggs should be laid when apple and pineapple are in the test rather than pineapple and orange in the experience phase whenever the two are paired. For orange-preferred populations, we saw no difference in treatments across treatments G and H.

The effect of the overshadowing target is showing very strong evidence of statistical significance (X^2 =34.759, $p < 0.000$; Figure 3). Overshadowing target is looking at whether the compound stimulus (the one paired with apple) contained orange or pineapple. Across the entire experiment, flies are learning better when orange is paired with apple and thus has the potential to overshadow. The effect of the overshowing type is showing very strong statistical significance ($X^{2\,=}$144.627, $p < 0.000$; Figure 4). Specifically, we are assessing the effect of the compound stimulus on learning, depending on whether it was the rewarding option (S+) or punishing option (S-). It makes a difference, and it does not matter which stimuli they are tested with (orange or pineapple), having the compound stimulus being aversive is more effective later for learning. The effect of the overshadowing target and overshadowing type is showing very strong statistical significance ($X^{2\,=}$32.085, $p < 0.000$; Figure 5). The interaction of overshadowing target and overshadowing type shows which compound stimulus is paired with apple and whether the compound stimulus is paired as punishing or rewarding. Evolutionary background has no effect on this interaction. S- (punishing option) had more eggs laid when the S- included pineapple. S+

(rewarding option) had more eggs laid when the S+ included orange. The effect of the test option is showing good evidence of statistical significance ($X^{2} = 4.687$, $p < 0.030$; Figure 6). Test Option is looking at whether there was orange and pineapple for the test or one of those options plus apple. More eggs were laid when a novel stimulus is being offered in the test against a stimulus they evolved with. The effect of overshadowing type and test option is showing very strong evidence of statistical significance ($X^{2} = 10.840$, $p < 0.001$; Figure 7). The interaction of overshadowing type and test option shows whether the compound stimulus is paired as aversive or rewarding with a novel or evolved stimulus. When S- trials were tested with apple, flies laid more eggs than if that option was the other half of the S- pairing (orange or pineapple). This pattern is reversed for the S+ treatments. The interaction also shows that overall, the learning is better for S- than S+ and the novel (apple) test trials is what is really driving this. The effect of overshadowing target, overshowing type and test option is showing good evidence of statistical significance ($X^{2} = 4.202$, $p < 0.040$; Figure 8). This is a three-way significant interaction that shows that these important treatment factors in the previous interactions are also interacting in this bigger way. There is not a significant effect of evolutionary background ($X^{2} = .312$, $p < 0.577$; Figure 9). Evolutionary Background is looking at the odd lines (orange preferred) versus the even lines (pineapple preferred). Nothing here is statistically significant and we wouldn't predict it to be. These differences should emerge as interactions with the treatment conditions. Our specific prediction is that this background should become apparent as an interaction between overshadowing target and the overshadowing type. The effect of overshadowing target, overshadowing type, and evolutionary background ($X^{2} = 6.014$, $p < 0.014$; Figure 10). This three-way interaction is the only interaction where evolutionary background has a significant effect. This interaction is looking at the overall effect of evolutionary background, the odd lines (orange) versus the even lines (pineapple), there was nothing statistically significant there. The differences only emerged as interactions with the treatment conditions (Figures 11 and 12). When comparing the orange and pineapple background, the effects are actually quite small, which is interesting.

The main results of this analysis can be found in Table 1.

Table 1. Results of Factorial ANOVA for the full experiment.

Parameter	B	SE	95% Wald CI		Hypothesis Test		
			Lower	Upper	Wald X^2	df	Sig.
(Intercept)	.279	.0388	.203	.355	51.668	1	.000
Overshadowing Target (Orange versus Pineapple)	.254	.0430	.169	.338	34.759	1	.000
Overshadowing Type (S- versus S+)	.518	.0430	.433	.602	144.627	1	.000
Test Option (Evolved versus Novel)	.093	.0430	.009	.178	4.687	1	.030
Evolved Background (orange versus pineapple)	-.031	.0549	-.138	.077	.312	1	.577
Overshadowing Target x Overshadowing Type	-.345	.0609	-.464	-.225	32.085	1	.000
Overshadowing Target x Test Option	.060	.0609	-.060	.179	.958	1	.328
Overshadowing Target x Evolved Background (orange versus pineapple)	.113	.0609	-.007	.232	3.430	1	.064
Overshadowing Type (S- versus S+) x Test Option	-.200	.0609	-.320	-.081	10.840	1	.001
Overshadowing Type x Evolved Background (orange versus pineapple)]	.125	.0609	.006	.244	4.214	1	.040
Test Option (Evolved versus Novel) x Evolved Background (orange versus pineapple)	.010	.0609	-.109	.129	.027	1	.870
Overshadowing Target (orange versus pineapple) x Overshadowing Type (Evolved versus Novel) x Test Option (S- versus S+)	-.176	.0861	-.345	-.008	4.202	1	.040
Overshadowing Target (orange versus pineapple) x * Overshadowing Type (Evolved versus Novel) x Evolved Background (orange versus pineapple)	-.211	.0861	-.380	-.042	6.014	1	.014
Overshadowing Target (orange versus pineapple) x Test Option (S- versus S+) x Evolved Background (orange versus pineapple)	.014	.0861	-.155	.183	.026	1	.872
Overshadowing Type (S- versus S+) x Test Option (Evolved versus Novel) x Evolved Background (orange versus pineapple)	-.053	.0861	-.222	.116	.380	1	.538
Overshadowing Target (orange versus pineapple) x Overshadowing Type (S- versus S+) x Test Option (Evolved vs. Novel) x Evolved Background (orange versus pineapple)	-.049	.1217	-.288	.189	.163	1	.686
(Scale)	.022[b]	.0016	.019	.026			

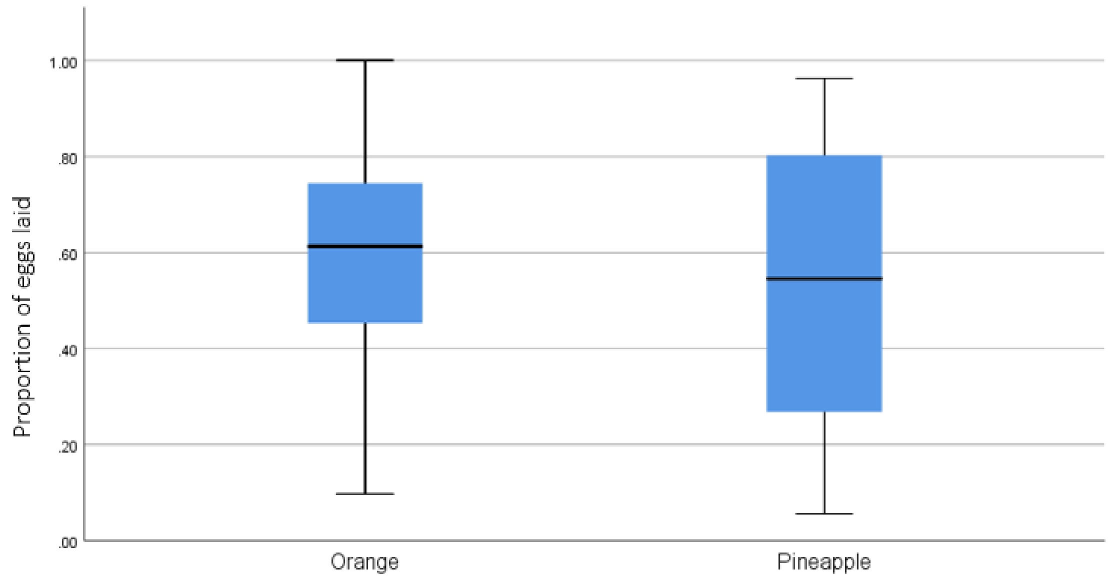

Figure 3. Difference between oviposition response when paired with the compound stimulus apple. The compound stimulus effect is manipulated to get the overshadowing effect. Across all the treatments, lines, etc. flies are learning slightly better when orange was a part of that paring.

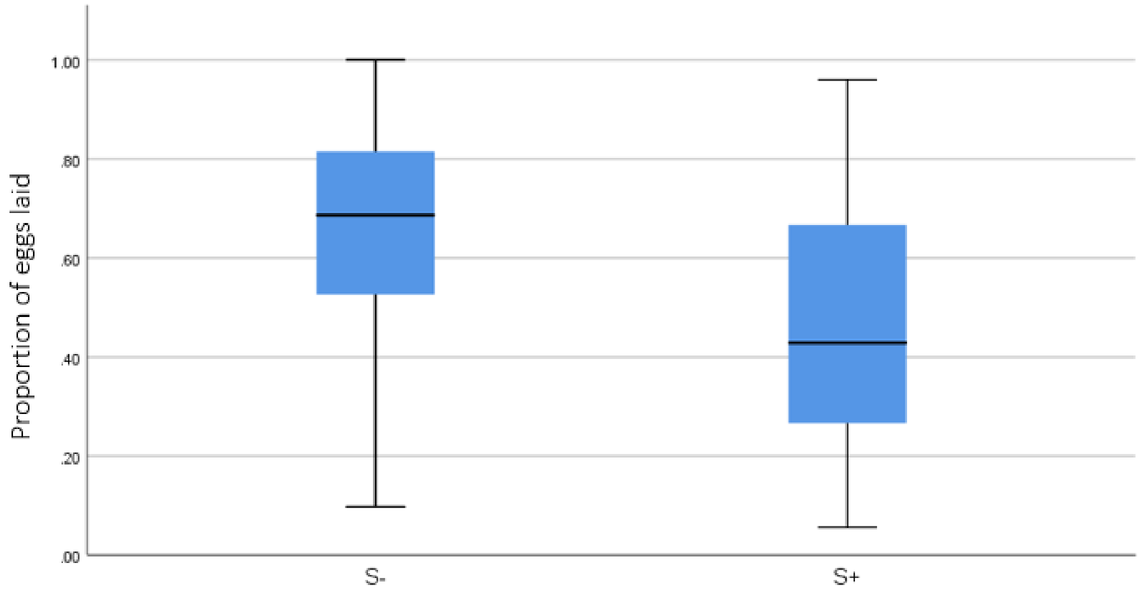

Figure 4. Difference of oviposition preference learning when compound stimulus apple is aversive versus non-aversive. Regardless of which stimuli the compound stimulus is tested with, learning is better overall when paired with the aversive taste.

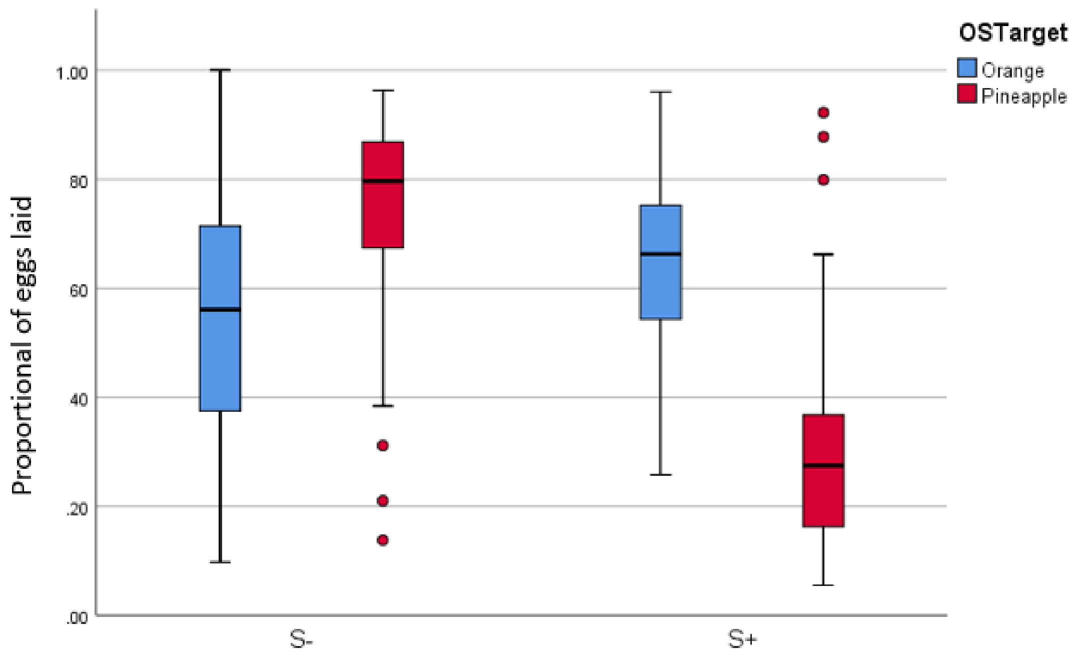

Figure 5. The interaction between overshadowing target with overshadowing type. This is the interaction between S+/S- pairing (Treatments A-D versus E-H) and the paired target (orange or pineapple with apple).

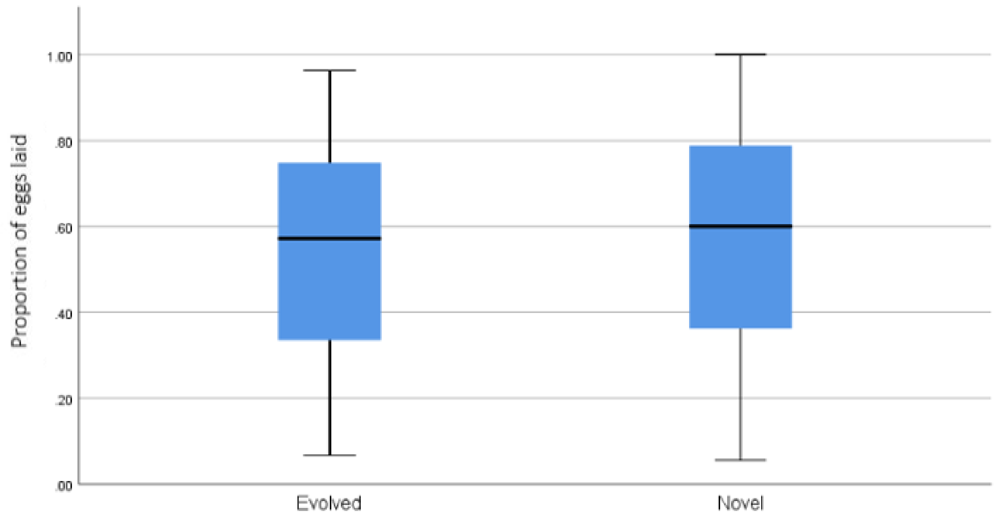

Figure 6. Difference of oviposition preference learning when the test option is orange and pineapple for the test or one of those options plus apple. Learning is better when a novel stimulus is being offered in the test against a stimulus they evolved with.

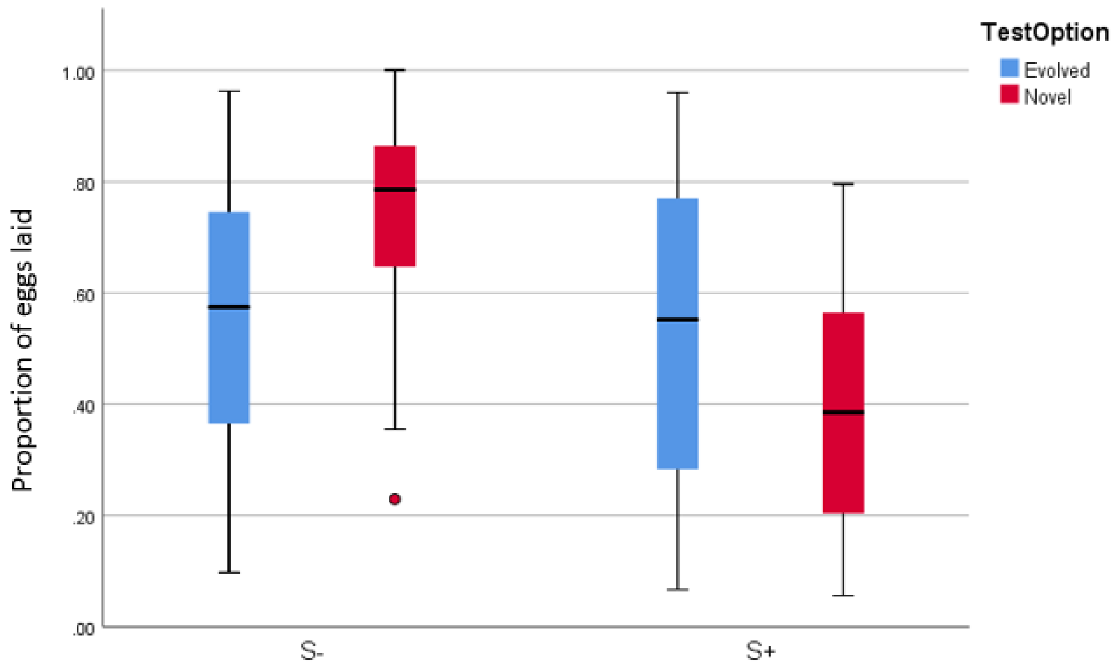

Figure 7. The interaction of overshadowing type and test option looking at how the aversive (S-) or rewarding option (S+) does with the kind of test being offered, whether it is orange versus pineapple (evolved) or one of those versus apple (novel).

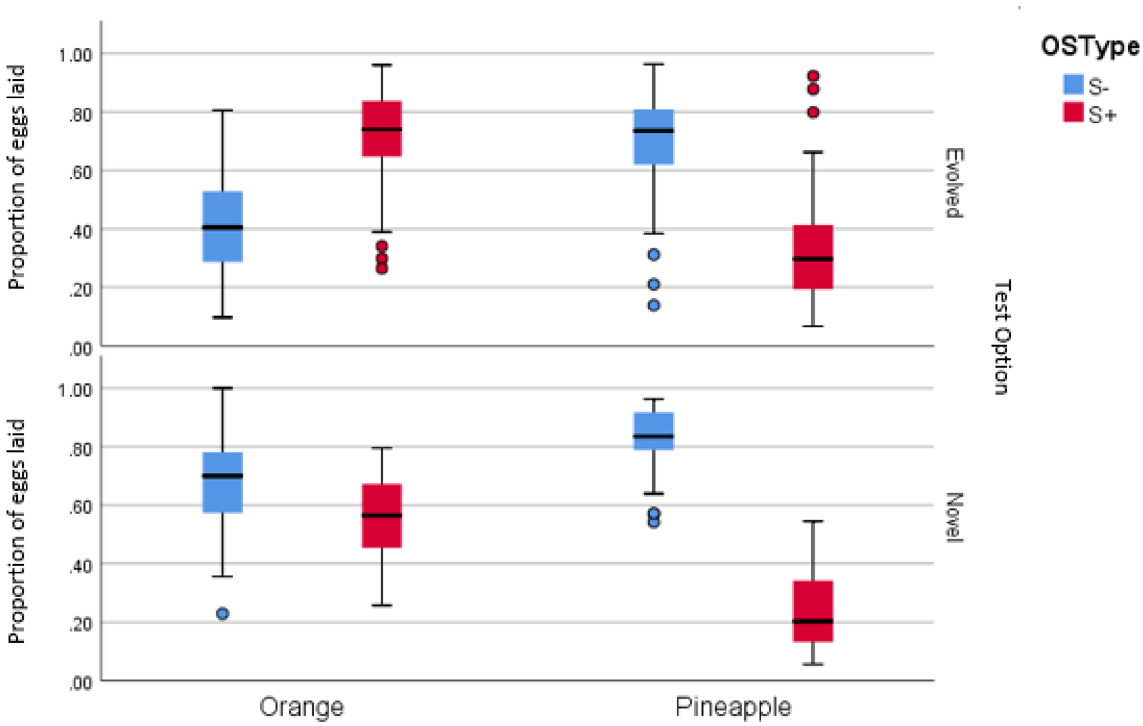

Figure 8. Three-way interaction in oviposition preference between overshadowing type (S-/S+), overshadowing target (orange versus pineapple), and test option (evolved vs novel).

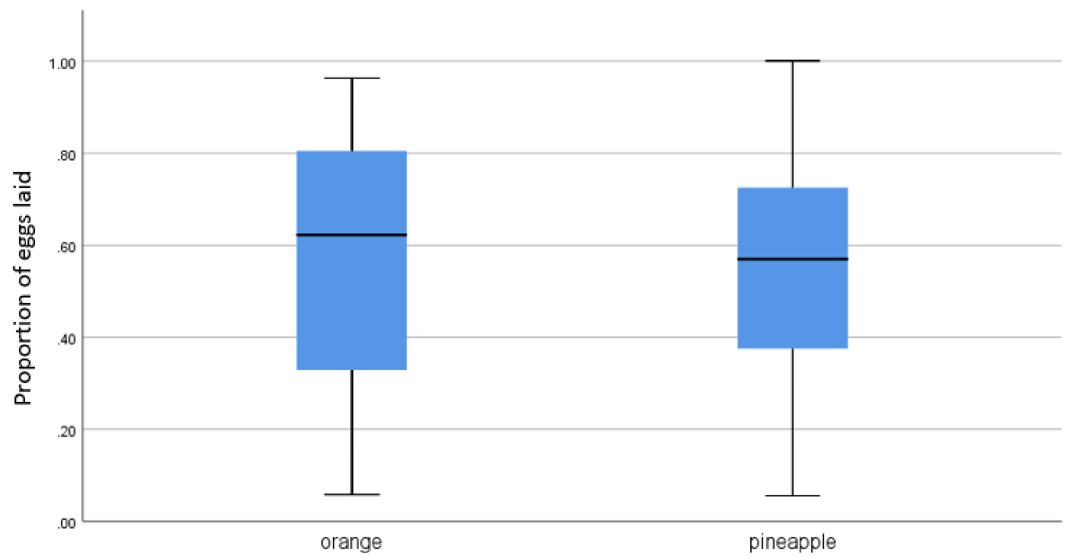

Figure 9. Response of oviposition substrate preference based on evolutionary background. Evolutionary preference for orange vs. evolutionary preference for pineapple.

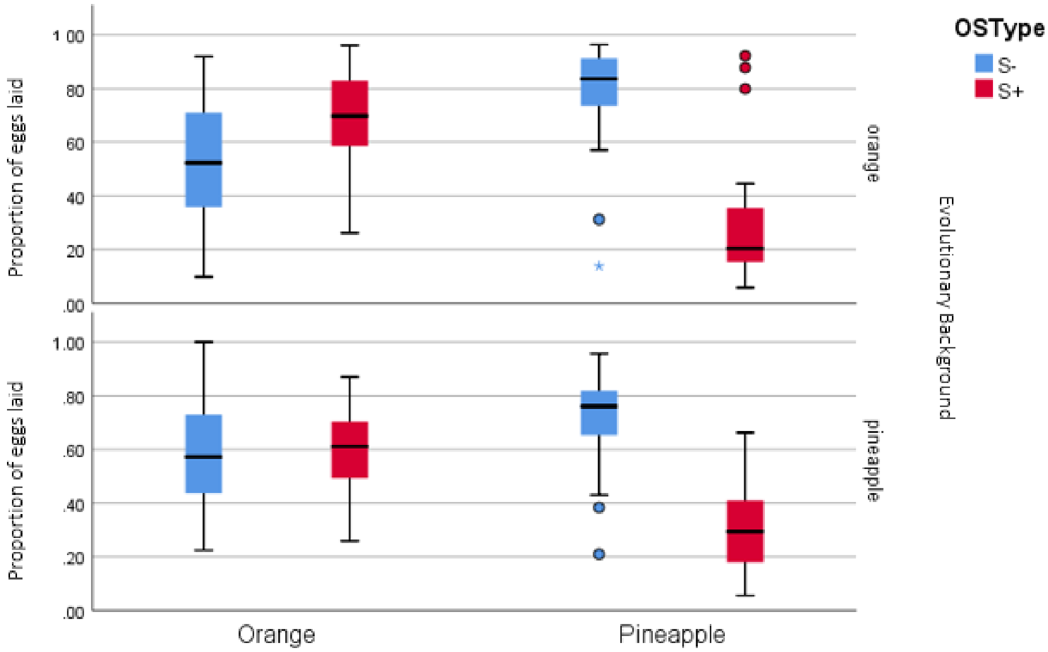

Figure 10. The interaction between overshadowing target (orange versus pineapple), overshadowing type (S-/S+) and evolutionary background (orange preferred versus pineapple preferred) on oviposition preference.

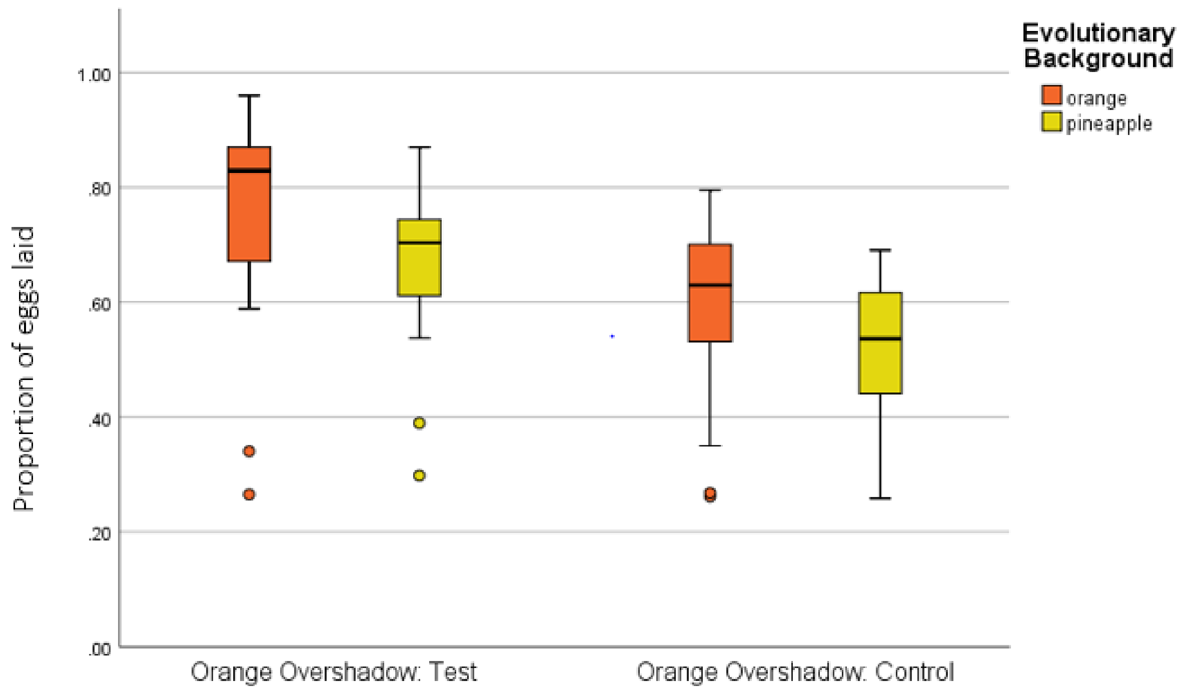

Figure 11. Response of oviposition substrate preference conditioning where orange overshadows learning apple for treatments A-D.

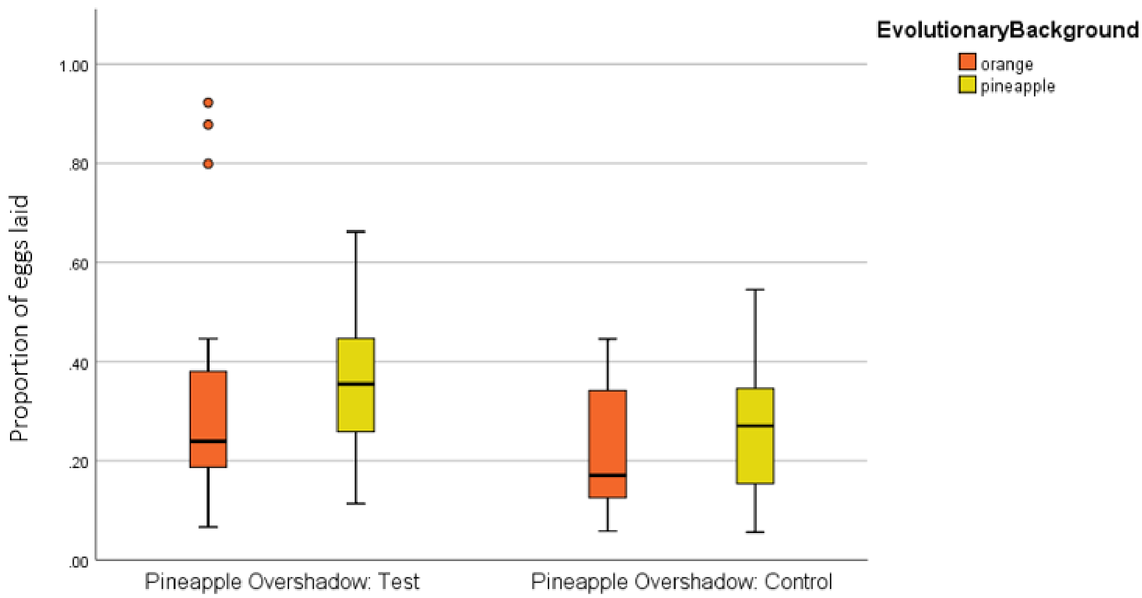

Figure 12. Response of oviposition substrate preference conditioning where pineapple overshadows learning apple for treatments E-H.

Discussion

We tested flies with an innate bias in a classic paradigm from animal learning, overshadowing, where salience of the stimuli can affect what is learned and what is not learned. Salience is usually defined as a stimulus that is seen as salient because it overshadows another stimulus. Because the recent evolutionary history is known for our flies, we are directly testing this major assumption about salience in animal learning. By experimentally evolving oviposition bias in a scenario where the value of that information is controlled, we can then look at this important interaction with learning. We find that evolutionary history or having an evolved innate bias does affect learning in an overshadowing paradigm. With this, we find that the effects are quite subtle and not as apparent as we thought would be seen. As predicted, we see both an effect of treatment and of evolutionary background. Flies learned better in the test condition then the control condition, and flies evolved for orange preference are learning better than those with pineapple preference. The evolutionary history of the flies being tested, interacts with a series of main effects in our experiment. Evolutionary background does matter, but only in the context of the interactions. We found that the flavor orange overshadows learning about apple while testing the role of overshadowing for orange in treatments A versus B. In the test trials, we look at orange versus pineapple, and in the control trials we look at apple versus pineapple. Flies laid more eggs in the test condition than the control condition, and flies evolved for orange preference learning better than those with pineapple preference. However, the predicted interaction is not significant. This means that flies responded in similar directions in response to the overshadowing treatments, regardless of evolutionary background. Flies with the orange preference are learning better as a whole, making the orange-apple S+ (rewarding option) association to both stimuli better than pineapple preference flies, and of that compound stimulus, they are learning orange better than pineapple, but not in a statistically significant way.

We found that the flavor pineapple overshadows learning about apple while testing the role of overshadowing for pineapple in treatments C and D. In the test trials, we look at pineapple versus orange, and in the control trials we look at apple versus orange. Flies learned better in the test condition than the control condition, and flies evolved for pineapple preference learning better than those with orange preference. However, the predicted interaction is not significant. Flies responded in similar directions in response to the overshadowing treatments, regardless of evolutionary background. This could mean that all of the flies have evolved, prior to this experiment, an enhanced preference for pineapple. Pineapple is showing an effect of overshadowing of apple throughout. Flies with the pineapple preference are learning better as a whole, making the pineapple-apple S+ (rewarding option) association to both stimuli better than orange preference flies, and that compound stimulus, they are learning pineapple better than orange, but not in a statistically significant way.

For treatments E and F, our focus is on the evolved, orange-preferred populations. In the test trials, we look at orange versus pineapple, and in the control trials we look at apple versus orange. Flies learned better in the test condition than the control condition, and flies evolved for apple and orange than for pineapple and orange. Learning in the E treatment was greater than learning in the F treatment because the pineapple association S- (aversive option) overshadowed the apple association in the experience phase.

For treatments G and H, we test the opposite pairing from above. Here our focus is on the evolved pineapple-preference populations. In the test trials, we look at orange versus pineapple, and in the control trails we look at apple and pineapple. For these populations, learning was better when tested for apple and pineapple than for pineapple and orange; and this is because learning about orange in the experience phase overshadowed learning about apple whenever the two were paired. This was not the case for evolved, orange-preferred lines, where there was in fact equal learning across treatments G and H.

Overshadowing is stronger, across the whole experiment, when the compound stimuli are paired with quinine. Learning to avoid a stimulus is stronger than learning to go towards a stimulus. But this learning is also affected by which evolved stimulus is part of the compound stimulus (being paired with the novel apple). Learning is better when a novel stimulus is on offer in the test than when it is a choice between only the evolved stimuli.

We have evidence of salience of orange as a stimulus that extends beyond the experimental evolution, and this is consistent with other tests of those lines in our lab. Flies, generally, responded in similar directions in response to the overshadowing treatments, regardless of evolutionary background. We see evidence of evolutionary history beyond our evolved history in the lab, in terms of the slight bias for orange across the entire experiment. This is consistent with other studies of preference in the lab and work from Dweck and colleagues. This may explain the subtle effects we find for evolutionary background, as well as the strong interactions between the test option and overshadowing target (the placement of orange and pineapple within the learning and testing phases). There are experiences that affect behavior more than others. We find evidence that evolutionary history, both recent and in the deep past affects choice. We also find that this preference can be modified by learning and influence how learning happens when multiple stimuli are present.

With the compound stimulus, across all the treatments, lines, etc. flies are learning slightly better when orange was a part of that pairing. This may have to do with the evolved preference for citrus flavoring. Across all the treatments and fly lines, flies are learning slightly better when orange was a part of that pairing. Looking at the overall effect of evolutionary background of the odd lines (orange preferred) versus the even lines (pineapple preferred), there was not a statistically significant difference. The compound stimulus has a huge effect on learning for the flies. Apple being aversive was better for learning no matter if the flies were a pineapple or orange preferred line. The interaction between the S+/S- pairing in treatments A-D versus E-H and the paired target whether orange or pineapple were in the compound stimulus with apple, was really strong.

When looking at the compound stimulus (the one paired with apple) containing orange or pineapple, across all of the treatments, lines, etc. flies were learning slightly better when orange was a part of that pairing. The effect of the compound stimulus on learning, depending on whether it was with the S+ (rewarding option) or S- (punishing option), definitely made a difference. The basic paradigm of taste aversion is very interesting to experiment with. Discrimination learning with a rewarding option (S+) and a punishing option (S-), gave the flies the opportunity to learn about two substrates. Figuring out which is good or rewarding, and which is paired with quinine was affected by evolution of the species. The compound stimulus has a large effect on learning, depending on whether it was the S+ or S-. There is a difference, and regardless of which stimuli they are testing with, having that compound stimulus being aversive is more effective for later learning. In this experiment, we found that in the S- (punishing option) treatments, learning is better when that S- includes pineapple. The opposite is true for the S+ (rewarding option) treatments, where learning is better when that S+ contains orange.

There is a two-way interaction between overshadowing type (S+ or S-) and how it interacts with the kind of test being offered, orange versus pineapple (evolved) or one of those versus apple (novel). When S- trials were tested with apple, flies showed better learning than of that option was the other half of the S- pairing (the orange or pineapple). This is reversed for the S+ treatments. Overall, it seems that learning was better for S- than S+ and this interaction is showing that the novel stimulus trials is the driving force behind this interaction. If looking specifically at the novel stimulus results, there is a big drop between S- and S+ treatment. We can ask about the effect of how evolutionary background interacts with this, but our model effects table tells us that it is not significant.

Looking specifically at the test option, if orange and pineapple were given for the test, or one of those plus apple, the flies learned better when a novel stimulus was offered. Personally, I do not believe that apple was a special flavor. I think that if for example grape was used as the novel flavor, the results would have been the same. Having another flavor present in general would cause some effect with overshadowing. I don t think there has to be one specific thing that could affect overshadowing. Even in human examples, any stimuli that appears could affect learning in multiple ways. In this experiment, the flies did better when a novel stimulus was offered in the test.

There were also three-way interactions that are significant in this experiment. Looking at the test option (evolved or novel) provided, flies learned better when orange was the S+ for the evolved flavor preference and when pineapple was the S-. When both flavors were S- with the novel test option, flies learned better overall. Looking at the evolutionary background (orange or pineapple) preferred lines, flies learned better when orange was associated with S+ (rewarding option) while pineapple was associated with S- (punishing option).

The overall effect of evolutionary background (the odd lines versus the even lines) was not statistically significant and it was not predicted to be either. The differences should emerge as interactions with the treatment conditions. Overall, if a fly line had a previously evolved preference for a flavor, this preference did affect how the flies learned even if there was a novel stimulus available. Having an evolutionary background of preferring orange was very prevalent in the results that we found. Flies did learn slightly better when orange was a part of the pairing presented.

For future experiments, I would like to see if adding a consolidation period in between the experience and consequence phases. Would this have an effect on learning and memory if the flies had to wait a certain amount of time before being tested? For this experiment, the flies entered directly into the consequence phase. Also, I would like to see if adding in a different stimulus such as an additional substrate would change results. The novel stimuli of apple had not been encountered in over 500 generations of the experimentally evolved flies tested. Would adding in grape affect what is learned in a future experiment? This specific experiment was mainly focused on overshadowing and preference. If an experiment was run with blocking as the main focus rather than overshadowing, would we see similar results? I believe that it might be a possibility that the results will be similar, but we would not know for sure until it is actually tested. Blocking and overshadowing seem to have some similarities and it is quite possible that similar results could happen. If this research should continue in the future, these are two aspects that I would like to see incorporated and results generated.

References

Burnham, Terence C., et al. “Experimental Evolution and Economics.” *SAGE Open*, Oct. 2015, doi:10.1177/2158244015612524.

Cassaday, Helen Joan. “Blocking, Overshadowing and Related Concepts.” *Encyclopedia of Psychopharmacology*, 2014, pp.1-9

Dunlap, Aimee S., and David W. Stephens. "Components of Change in the Evolution of Learning and Unlearned Preference." *Proceedings of the Royal Society. B, Biological Sciences*, vol. 276, no. 1670, 2009, pp. 3201-3208.

Dunlap, Aimee S., and David W. Stephens. "Reliability, Uncertainty, and Costs in the Evolution of Animal Learning." *Current Opinion in Behavioral Sciences*, vol. 12, 2016, pp. 73-79.

Dunlap, Aimee S., Matthew W. Austin, and Andreia Figueiredo. "Components of Change and the Evolution of Learning in Theory and Experiment." *Animal Behaviour*, vol. 147, 2019, pp. 157-166.

Dweck, Hany K M., et al. "Olfactory Preference for Egg Laying on Citrus Substrates in Drosophila." *Current Biology*, vol. 23, no. 24, 2013, pp. 2472-2480.

Garcia, J., R. A. Koelling, and Harvard Medical School, Boston. Massachusetts General Hospital, Boston. "A Comparison of Aversions Induced by X Rays, Toxins, and Drugs in the Rat." *Radiation Research. Supplement*, vol. 7, 1967, pp. 439-450.

Kahneman, Daniel., et al. "Judgement Under Uncertainty: Heuristics and Biases." Cambridge University Press, Cambridge; New York, 1982

Kamin, L. J. "Predictability, surprise, attention, and conditioning." *B. Campbell & R. Church*

(Eds.), Punishment and aversive behavior. New York: Appleton-Century-Crofts, 1969.

Macario, Alessandro, et al. "Male phenotypic diversity experienced during ontogeny mediates female mate choice in guppies." *Behavioral Ecology*, vol. 30.2, 2019, pp. 465–473

Mackintosh, N.J. "Overshadowing and Stimulus Intensity". *Animal Learning & Behavior*, vol. 4, no. 2, 1976, pp.186-192.

Mansourian, Suzan, et al. "Wild African Drosophila Melanogaster are Seasonal Specialists on Marula Fruit." *Current Biology*, vol. 28, no. 24, 2018, pp. 3960-3968.e3

Mansourian, Suzan, and Marcus C. Stensmyr. "The Chemical Ecology of the Fly." *Current Opinion in Neurobiology*, vol. 34, 2015, pp. 95-102.

Marcus, Mellissa, et al. "Experimental Evolution of Color Preference for Oviposition in Drosophila Melanogaster." *Journal of Bioeconomics*, vol 20.1, 2018, pp. 125-40.

Mery, Frederic, and Tadeusz J. Kawecki. "Experimental Evolution of Learning Ability in Fruit Flies." *Proceedings of the National Academy of Sciences - PNAS*, vol. 99, no. 22, 2002, pp. 14274-14279.

Mery, F. and Kawecki, T.J. (2004), The effect of Learning on Experimental Evolution of resource preference in Drosophila Melanogaster. Evolution, 58: 757-767.

Rumbaugh, Duane M., et al. "A Salience Theory of Learning and Behavior: With Perspectives on Neurobiology and Cognition." *International Journal of Primatology*, vol. 28, no. 5, 2007, pp. 973-996.

Ryan, Michael J., and William E. Wagner. “Asymmetries in Mating Preferences between Species: Female Swordtails Prefer Heterospecific Males.” *Science*, vol. 236, no. 4801, 1987, pp. 595–97.

Appendix 1

Detailed Description of Results and Variability Within Populations

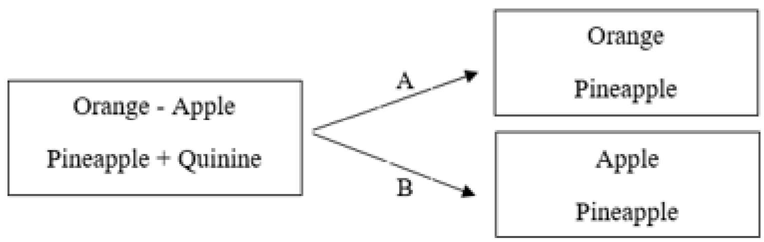

Figure 13. The experience and test phase for both treatments A and B.

We should see a difference in learning between orange- and pineapple-preferred evolved populations. Namely, that orange-preferred flies will be less susceptible to potential effects of overshadowing the orange substrate by apple than pineapple-preferred flies. And an evolved preference for a specific substrate stimulus should result in that stimulus overshadowing other stimuli.

In other words, for pineapple-preferred populations, we should see no difference in treatments. But for learning between the A and B orange-preferred populations, learning in the A treatment should be greater than learning in the B treatment because the orange association S+ will overshadow the apple association in the experience phase.

A)

B)

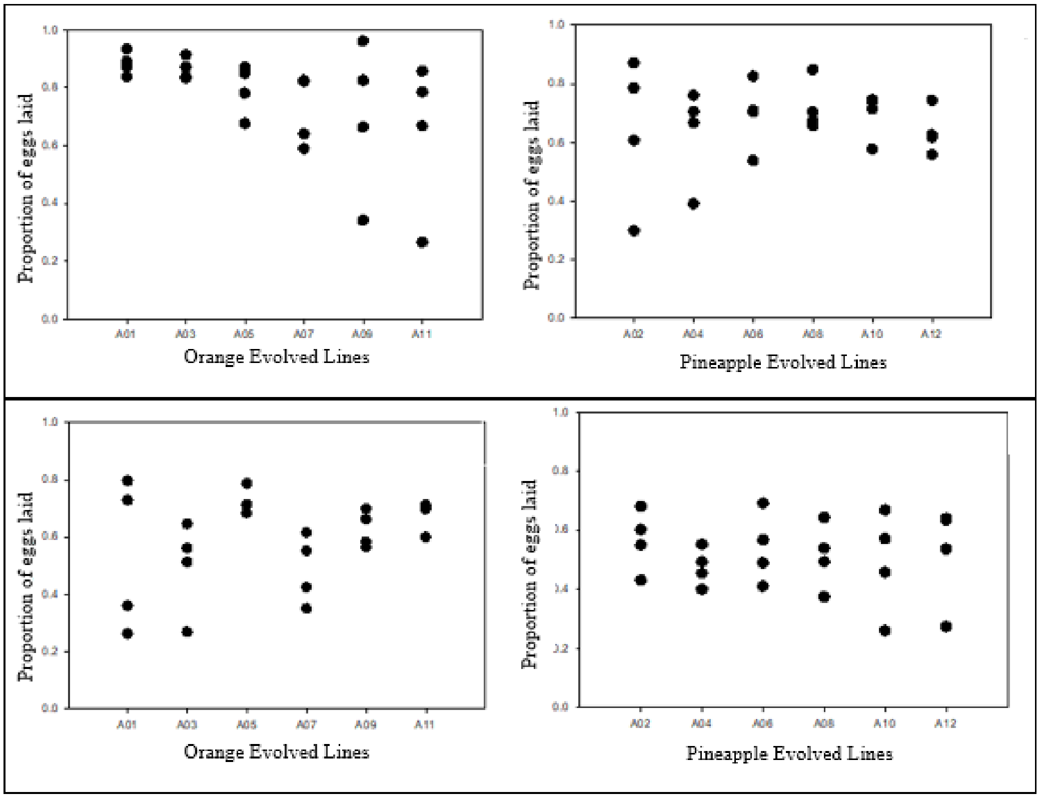

Figure 14. Each panel depicts the Proportion of eggs laid value calculated for the four replicates of each fly line for treatment types A and B. The lines represent the replicate populations for each treatment and show individual variability. Performance across treatments is shown for a) learning phase consisting of orange-apple (S+), pine + Q (S-) with a testing phase of orange and pineapple. These lines were experimentally evolved to have a preference for orange. Learning phase consisting of orange-apple (S+), pine + Q (S-) with a testing phase of orange and pineapple. These lines were experimentally evolved to have a preference for pineapple. B) learning phase consisting of orange-apple (S+), pine + Q (S-) with a testing phase of apple and pineapple. These lines were experimentally evolved to have a preference for orange. Learning phase consisting of orange-apple (S+), pine + Q (S-) with a testing phase of apple and pineapple. These lines were experimentally evolved to have a preference for pineapple.

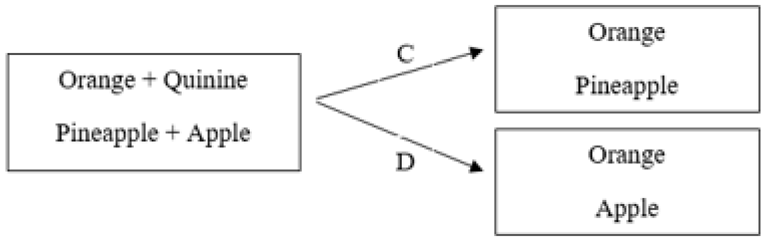

Figure 15. The experience and test phases for both treatments C and D.

In these treatments, we test the opposite pairing from above. Here our focus is on the evolved pineapple-preference populations. For these populations, learning should be better when tested for pineapple and orange than for apple and orange; and this is because learning about pineapple in the experience phase should overshadow learning about apple whenever the two are paired. This will not be the case for evolved, orange-preferred lines, where we predict equal learning across treatments C and D.

C)

D)

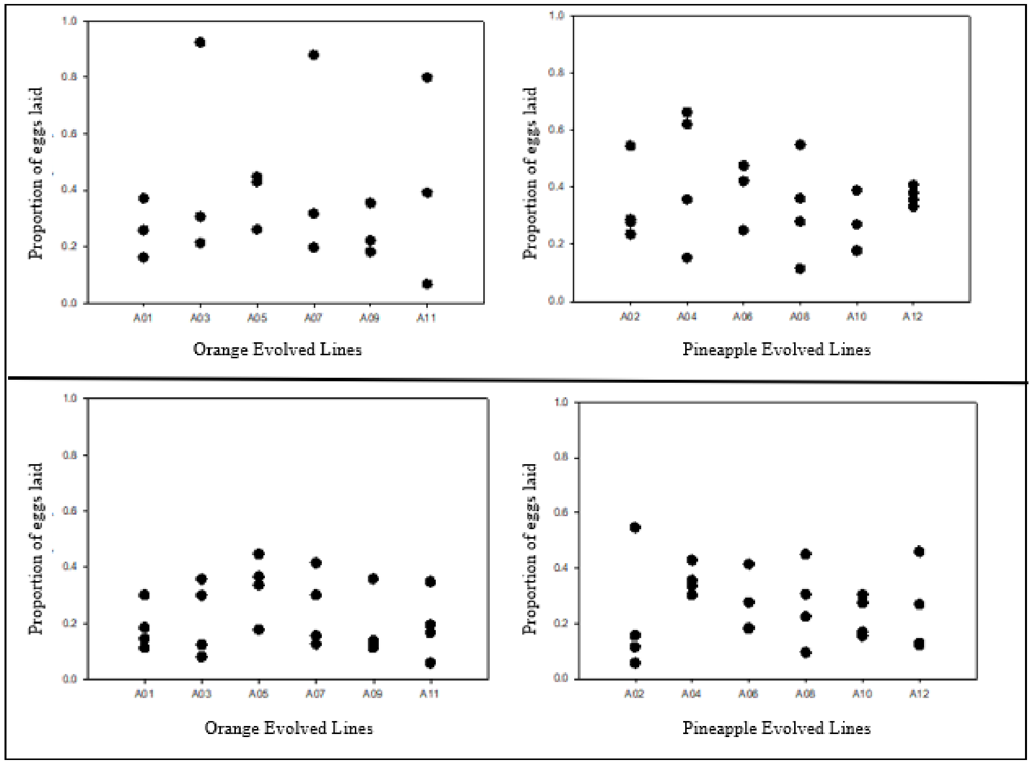

Figure 16. Each panel depicts the proportion of eggs laid value calculated for the four replicates of each fly line for treatment types C and D. The lines represent the replicate populations for each treatment. Performance across treatments is shown for a) learning phase consisting of pineapple-apple (S+), orange + Q (S-) with a testing phase of pineapple and orange. These lines were experimentally evolved to prefer orange. B) learning phase consisting of pineapple-apple (S+), orange + Q (S-) with a testing phase of pineapple and orange. These lines were experimentally evolved to prefer pineapple. C) learning phase consisting of pineapple-apple (S+), orange + Q (S-) with a testing phase of apple and orange. These lines were experimentally evolved to prefer orange. D) learning phase consisting of pineapple-apple (S+), orange + Q (S-) with a testing phase of apple and orange. These lines were experimentally evolved to have a preference for pineapple.

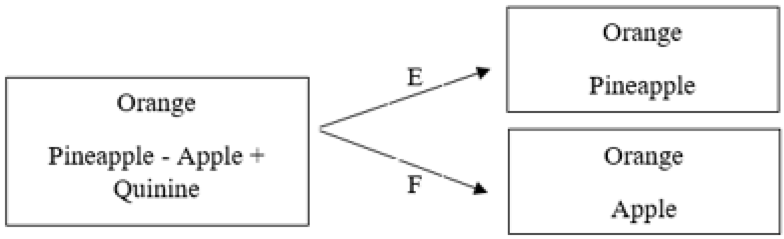

Figure 17. The experience and test phases for both treatments E and F.

For treatments E and F, we are looking at orange versus pineapple, and in the control trials we look at apple versus orange. Flies learned better in the test condition than the control condition, and flies evolved for apple and orange than for pineapple and orange. Learning in the E treatment was greater than learning in the F treatment because the pineapple association S- overshadowed the apple association in the experience phase.

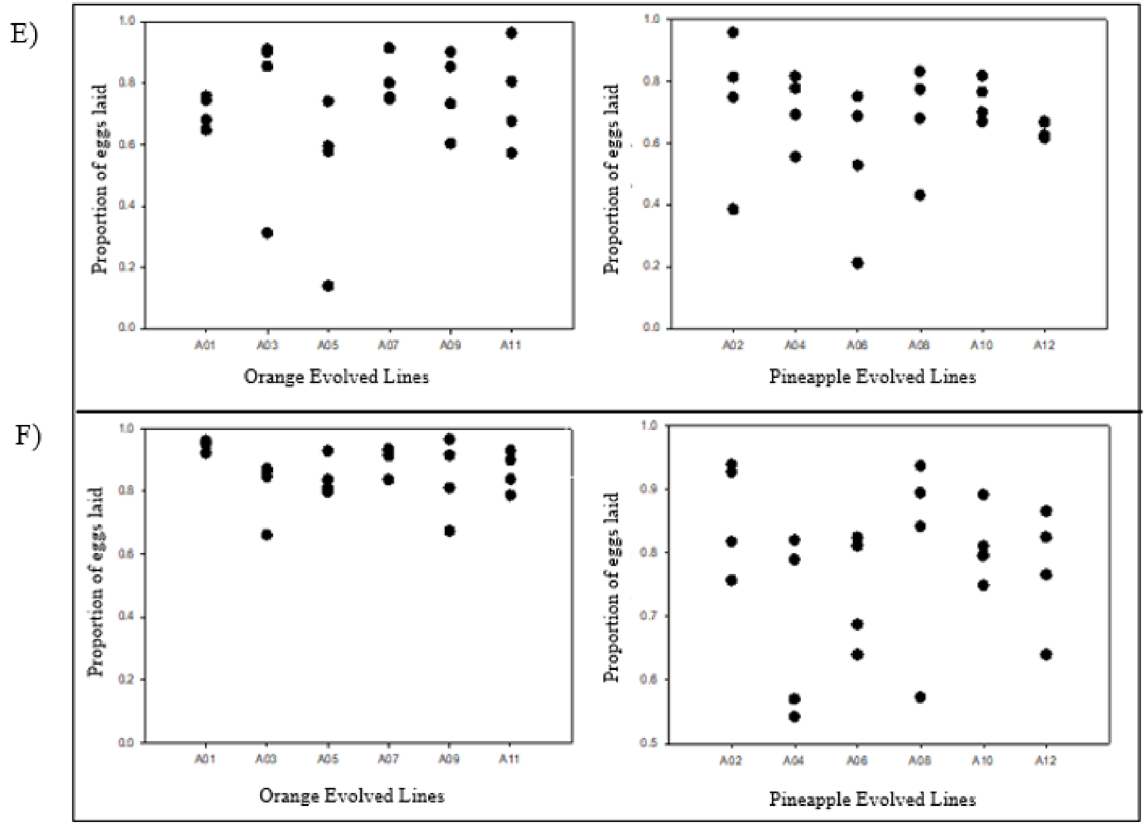

Figure 18. Each panel depicts the P (Learn) value calculated for the four replicates of each fly line for treatment types E and F. The lines represent the replicate populations for each treatment. Performance across treatments is shown for a) learning phase consisting of orange (S+), pineapple-apple + Q (S-) with a testing phase of orange and pineapple. These lines were experimentally evolved to have a preference for orange. B) learning phase consisting of orange (S+), pineapple-apple + Q (S-) with a testing phase of orange and pineapple. These lines were experimentally evolved to have a preference for pineapple. C) learning phase consisting of orange (S+), pineapple-apple + Q (S-) with a testing phase of orange and apple. These lines were experimentally evolved to have a preference for orange. D) learning phase consisting of orange (S+), pineapple-apple + Q (S-) with a testing phase of orange and apple. These lines were experimentally evolved to have a preference for pineapple.

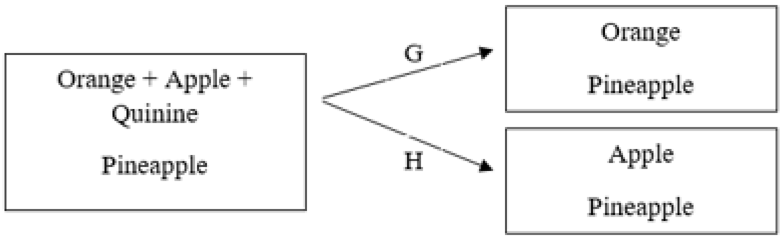

Figure 19. The experience and test phases for both treatments G and H.

For treatments G and H, we test the opposite pairing from above. Here our focus is on the evolved pineapple-preference populations. In the test trials, we look at orange versus pineapple, and in the control trails we look at apple and pineapple. For these populations, learning was better when tested for apple and pineapple than for pineapple and orange; and this is because learning about orange in the experience phase overshadowed learning about apple whenever the two were paired. This was not the case for evolved, orange-preferred lines, where there was in fact equal learning across treatments G and H.

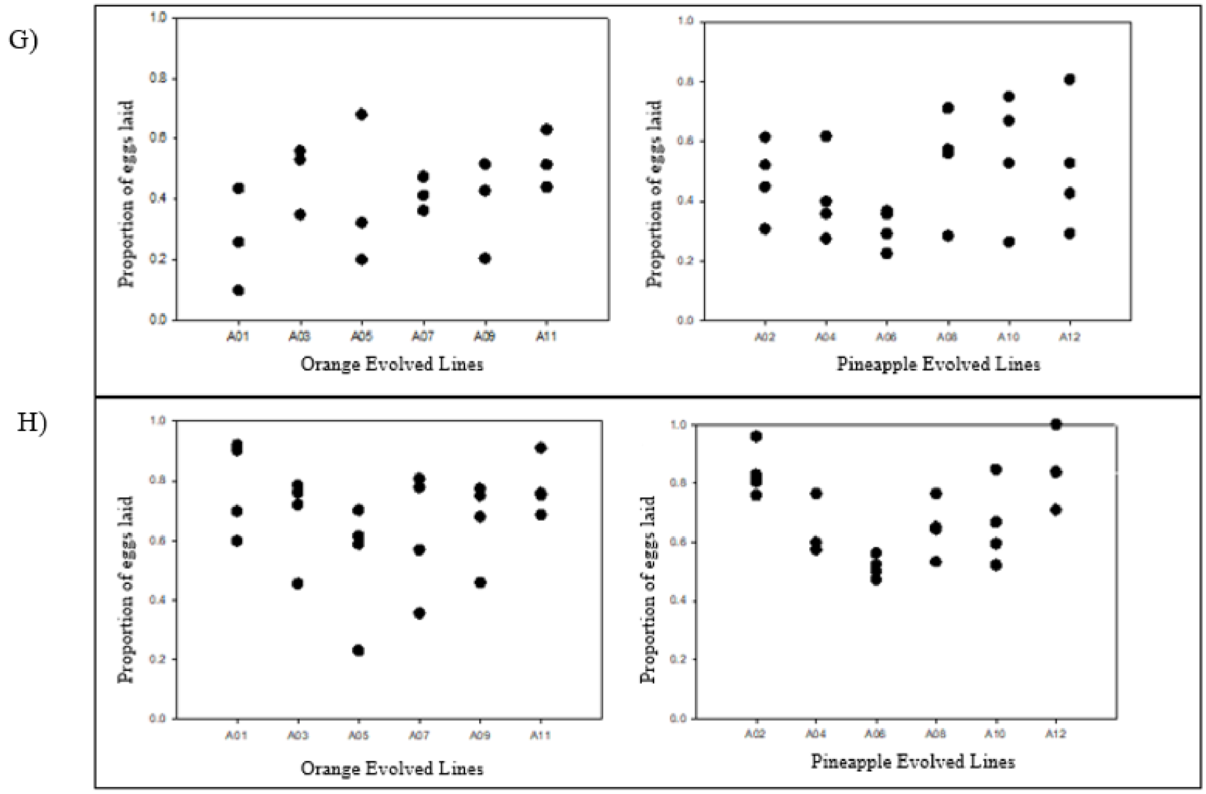

Figure 20. Each panel depicts the P (Learn) value calculated for the four replicates of each fly line for treatment types G and H. The lines represent the replicate populations for each treatment. Performance across treatments is shown for a) learning phase consisting of pineapple (S+), orange-apple + Q (S-) with a testing phase of orange and pineapple. These lines were experimentally evolved to prefer orange. Learning phase consisting of pineapple (S+), orange-apple + Q (S-) with a testing phase of orange and pineapple. These lines were experimentally evolved to prefer pineapple. C) learning phase consisting of pineapple (S+), orange-apple + Q (S-) with a testing phase of apple and pineapple. These lines were experimentally evolved to prefer orange. D) learning phase consisting of pineapple (S+), orange-apple + Q (S-) with a testing phase of apple and pineapple. These lines were experimentally evolved to prefer pineapple.