Title
Competing Responses to a Single Stimulus Provides a Model for Decision-Making in Drosophila

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Publication Date
2011

Peer reviewed|Thesis/dissertation

Competing Responses to a Single Stimulus Provides a Model for Decision-Making in *Drosophila*

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Genetics

in the

GRADUATE DIVISION

[redacted]

Dedicated to my maternal grandparents, whose lives have always made me feel like my crazy interests and qualities are not so crazy after all.

Acknowledgements

I would like to begin my acknowledgements by thanking the members of the Heberlein laboratory, with whom I have had the immense pleasure to spend the past five years of my graduate career (give-or-take a rotation term). They have taught me countless valuable scientific lessons—every member of the laboratory has been immensely helpful and a true inspiration, and they all deserve numerous acknowledgements. I would like to thank Rachael F., who constantly answered my questions, which I would frequently yell through the wall that separated our desks, and provided me with much mentorship and advice pertaining to matters both inside and outside of the laboratory. I would also like to thank all the other laboratory members who helped me work out theoretical rationales, in particular Mark E. and Kim M., who as neighbors in the lab and fly room, respectively, bore the brunt of my tendency to think out loud. Karla K. and Reza A. merit special mention for helping me perfect both my data analysis and experimental techniques, while Ian K. was infinitely helpful with the development of my first publication, of which he was an author. Anita D. also deserves special credit, given her immeasurable help in instructing me on both topics and literature involving neuroscience—a subject with which I had very little experience when starting graduate school. In addition to being wonderful mentors and incredible scientists, the post-doctoral researchers and fellow graduate students have been amazing friends…between the huge, life altering-events like weddings and the birth of children, to the weekly traditions of coffee-breaks and lab-lunch, I have had the great fortune of spending my graduate years with the members of the Heberlein laboratory.

As an advisor and mentor, Ulrike Heberlein has been amazing in developing my skills as a scientist. Entering her office on the first day of my rotation, she recognized that I already had decent experience with *Drosophilia*. Rather than put me under the supervision of another lab member to instruct me on the basics of fly work, she proposed some ambitious projects that she personally would guide me through during my rotation. Her talent with recognizing interesting findings and pursuing unique scientific opportunities became especially apparent during my rotation project, when through her encouragement what started as a simple three month study on *Drosophila* preference became an in depth investigation that ultimately became the basis of my doctoral research. I am truly appreciative of her uncanny ability to balance my desire to work independently, but provide guidance at critical points when I was heading down a potentially dangerous scientific path. I am incredibly grateful for having the opportunity to learn from her example as a professor, researcher, and mentor.

Many acknowledgements are also merited to the members of my thesis committee, Nirao S. and Kaveh A., who have provided much helpful advice over the years. I am very thankful that they always kept their office doors open, and were more than happy to discuss new ideas about my project even when an official thesis meeting was in the distant future. Also within the UCSF community, I am incredibly indebted to the members of the Tetrad Department like Danny D., who has kept me focused on my graduate school objectives, and has provided much needed counseling and moral support. Both the faculty and staff in the UCSF Tetrad and Neuroscience Departments represent the best our field has to offer, and have truly been instrumental in the completion of my dissertation research.

Scientific endeavors are rarely restricted to a single institution, and I hold special thanks to Kristen S. of UC Berkeley, as well as Werner B. and Markus N. of the University

of Zürich. In addition to providing valuable tools and reagents for experiments, their constant advice and suggestions were essential to the completion of my various projects. I am incredibly grateful for their input, without which I would not have been able to answer many key investigative questions. Furthermore, I am very thankful to members of the Luo laboratory, as well as Melissa S., who characterized the expression patterns of the *GAL4* library that was indispensable in my studies. Additionally, I would like to thank Roth E. of San Francisco State University; it is not often that your best friend also ends up becoming a neuroscientist and colleague with whom you can have helpful scientific discussions about your research project and experimental designs.

Obtaining a Ph.D. is no easy task, and would not have been possible without the inspiration, love, and support of family and friends who accompanied my along the long journey. I would like to especially mention Alfred Balocca and Lee Balocca, who went above and beyond the role of loving grandparents, and were incredibly supportive of my academic pursuits. In particular, Alfred, or "Granka" always showed immense interest in what I was studying—even at times when I myself was not convinced over the importance of my project—and even took the time to download, read through, and understand my research article…no small task for any family member, much less a retired engineer of eighty plus years. Both Granka and his experiences have always been a source of inspiration during my graduate career, and have made me strive to be a careful, honest, and intelligent researcher. Similarly, I would also like to thank the members of Joseph family, notably my grandparents Louis Joseph and Patricia Joseph for teaching their children Rick, Kathy, and Chuck (my father) an incredible work-ethic, all of whom provided me with wonderful role models and

examples of what can be achieved through hard work and diligence…two traits that are absolutely necessary for the completion of a Ph.D.

I am also incredibly thankful for the support from Jorge Dubcovsky and Laura Dubcovsky. I am very fortunate to have such loving parents in-law who both have obtained a Ph.D., and are thus very familiar with the demands and rigors of obtaining an advanced degree (in their case, *numerous* advanced degrees). They have always provided a sympathetic ear during particularly rough times of graduate school, and have been more than willing to listen to me explain my experimental results and pitch my subsequent conclusions, and then offer questions that helped strengthen and improve my arguments.

I would not be anywhere—in both career and life in general—if it were not for my amazing parents, Chuck Joseph and Katie Joseph. They have always nurtured my scientific side, and encouraged me to pursue my academic interests to the best of my capabilities. I have had the great fortune of learning from their good examples and listening to their wise advice. Whether it be hearing me vent my stresses over the phone as I commute to work, or swinging by with my equally loving and supportive brother, Patrick Joseph, to rescue me from the laboratory and drive up to Portrero Hill for lunch and some much needed respite from graduate school, they have provided much needed love and moral support. Without them, receiving a Ph.D. would have been a far less enjoyable experience.

I would also like to acknowledge my son, Joaquín. Although I cannot say he necessarily helped complete my dissertation work, he definitely has been instrumental in giving me a more realistic perspective towards my scientific career. The happiness and joy he expresses is always wonderful to see after a long and difficult day in the laboratory, and his

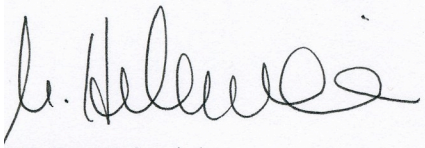
heartfelt love is always uplifting. Furthermore, his lack of immediate protest when I use him as a test subject in various two-choice assays is always appreciated.

Most importantly, I am indescribably grateful to the one person who made all this possible: Alejandra. Without your immense love and incredible patience, I do not believe either I or our marriage would have survived the intense first two years of graduate school. Your love has made me a much better person, and life would be much less happy and fun without you. Your intelligence as an academic has made me a much better scientist, and graduate school would have been much more difficult without your example. Your passion for living has made me a much more adventurous individual, and has allowed me to be the person I want to be. You deserve so many acknowledgements too numerous to list, and an infinite amount of gold stars. I am indebted to you, and incredibly grateful that I get to be with you and live life with you.

San Francisco, June 1st, 2011

To whom it concerns:
I hereby state that the work carried out by Ryan Joseph for the purpose of satisfying his Ph.D. requirement is comparable to dissertations typically awarded by the UCSF Tetrad Program (Biochemistry Department): Genetics Division. The work carried out in Chapter 2 was performed as described, with Anita Devineni conducting the neuroanatomical screen, Ian King developing the feeding assay, and Ryan Joseph performing the rest of the experiments. The work described in Chapter 2 was published in *Proceedings of the National Academy of Sciences*. Ryan Joseph carried out both the primary genetic screen and lobeline-based neuroanatomical screen in Chapter 3, while the secondary genetic screen was carried out by Peter Sohn, and the acetic acid-based neuroanatomical screen was performed by Anita Devineni. Ryan Joseph carried out the work described in Chapter 4 in its majority.

Sincerely,



Ulrike Heberlein,
Professor

Competing Responses to a Single Stimulus Provides a Model for Decision-Making in *Drosophila*

Ryan M. Joseph

Abstract

Proper decision-making is crucial to the day-to-day lives of all organisms, whether the situation requiring a choice be mundane and seemingly trivial, or critical and life-or-death. Simply defined, a "decision" occurs when an organism is presented with input stimuli that induce multiple alternative responses, but given the situation a single behavioral response can only be selected and executed as motor output. Given its importance, decision-making studies have been performed in numerous organisms, across numerous academic disciplines.

One advantage that *Drosophila melanogaster* possesses as a model organism is the vast array of neurogenetic tools available to answer biological questions. Given the experimental utility of the fruit fly, we set out to use *Drosophila* to study the behavioral mechanisms and neurophysiology underlying decision-making. We developed a two-choice assay that simultaneously measures the positional aversion and egg-laying attraction responses flies exhibit to a single environmental compound. Using our straightforward yet robust assay, we use acetic acid preferences to substantiate the *Drosophila* oviposition program as a model for choice-like behavior. Subsequently, we identified a number of genes

potentially important to the *Drosophila* oviposition program, and initially characterized the sensory systems and brain regions mediating these responses.

Next, to expand our acetic acid-based model for choice-like behavior into a more general paradigm of decision-making, and to perform more in-depth analysis of the neural circuitry governing this process, we modified our assay to employ bitter-tasting compounds like lobeline that induce contradictory positional and egg-laying responses. Subsequently, we identified the specific sensory neurons that mediate these preferences, and surprisingly find that the same gustatory receptor is required for both responses. We show *Drosophila* can employ tissue-specific activation of taste-neurons expressing this same receptor complex to elicit these very different and competing behavioral responses. Finally, we identified a higher-order brain structure, the mushroom body, which is an intersection point between the neural circuits governing the competing positional aversion and egg-laying attraction pathways, thereby offering suggestive evidence that the mushroom body is a candidate integration center in a true decision-making process that occurs within the *Drosophila* oviposition program.

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Introduction

INTRODUCTION

"Wisdom consists in being able to distinguish among dangers and make a choice of the least harmful."

- Niccolò Machiavelli, philosopher (1469 – 1527)

"Choose always the way that seems the best, however rough it may be. Custom will soon render it easy and agreeable."

- Pythagoras, philosopher (570 B.C. – 495 B.C.)

"You have chosen…wisely."

- Robert Eddison, *Indiana Jones and the Last Crusade* (Dir. Steven Spielberg, 1989)

"Our lives are a sum total of the choices we have made."

- Wayne Dyer, author (1940 – Present)

Decisions define the day-to-day lives of all organisms. As organisms proceed through their daily routine, they encounter different environmental stimuli that can lead to a multitude of behavioral responses. Since such behaviors cannot often be simultaneously executed (most animals have difficulty feeding and mating concurrently), a decision must be made about which response has priority given the particular environmental context. Many times, situations requiring a decision between different behavioral responses are critically important

to the survival of an organism. For example, when an insect is presented with a resource rich food patch that is also near predator nesting sites, a decision must be made between two behavioral responses that directly affect the livelihood of the organism: (1) remain in the food patch, at the risk of being eaten, or (2) leave the food patch, at the risk of starving to death. Other times, situations that necessitate a decision are seemingly trivial to the routine of an individual. For example, when a person selects the type of shoes he will wear for the day, the behavioral responses appear less dramatic: (1) wear running shoes to be more comfortable at work, or (2) choose dress shoes to make a good impression on superiors.

Regardless of the severity in the consequences associated with each situation, one goal is common to each decision: seek reward and avoid punishment (Cohen and Blum, 2002). In the first example, the reward and punishment associated with decision-making are obvious: the reward of beneficial nutrients with the risk of predation, versus the risk of starvation with the reward of safety from predation. However, the second example also possesses the same motivation for attainment of reward and avoidance of punishment, albeit with more subtle consequences: physical comfort with the risk of appearing not professional in the workplaces, versus physical discomfort with the reward of impressing those in charge of career advancement. In both cases, the organism involved in the decision-making event undergoes a common process stretching from reception of sensory input, assignment of value to perceived options, and selection of an optimal behavioral output that maximizes reward (Kristan, 2008; Kable and Glimcher, 2009). Using the aforementioned examples, the organisms detected cues as sensory input from their environment (i.e. there are nutrients and predators nearby; there are casual shoes and dress shoes available in the closet), assigned a value to the potential outcomes based on the received information (i.e. safety from predation

is more rewarding than nearby nutrients; career advancement is more rewarding than physical comfort), and selected an optimal response to execute as motor output (i.e. find a different food patch; wear dress shoes).

The fields that invoke the term "decision-making" are numerous and range from several distinct disciplines, including: economics, evolutionary ecology, neurobiology, computer science, and psychology (von Neumann and Morgenstern, 1944; Miller et al., 2011; Kristan, 2008; Sutton and Barto, 1998; Kahneman and Tversky, 1979). Given the broad fields that study this subject, cohesion in the definition of terms like decision, choice, and behavioral switch can often be lacking. For example, much of the original theoretical models of decision-making and choice-behavior are derived from theoretical models in economics (Kable and Glimcher, 2009), which seek to describe how people modulate their actions in the marketplace to maximize their personal profit, and hence reward. However, these theoretical models are rooted in observing the resulting actions of people being studied, and therefore any description of the mechanism are categorized as being "as if" models (Friedman, 1953). Basically, if a person behaves in a mathematically predictable fashion when presented with a set of different options, that person behaves "as if" their mind was a logical processor that (1) received information about the potential options, (2) next constructed a list ranking the options from most rewarding to least rewarding, and (3) then selected highest ordered option from the list that was readily available (Samuelson, 1937). Does this mean that the human mind is a logical processor? Not necessarily—the neurobiology that actually mediates this decision may not function like a logical processor, or at least in as clean a manner that the behavioral output suggests. Members of the economic field are readily explicit that their models are not necessarily intended to define the neural

mechanism of the choice behavior in economic transactions, but rather are intended to be utility theories that predict action and consequences of decisions in the marketplace.

Neurophysiological models of decision-making are obviously more interested in explaining the mechanisms of how decisions are made at the molecular, cellular, and neural circuitry level of organism biology. Decision-making theories generated from such studies are sometimes referred to as "because" models, given that they often seek to explain the internal biological mechanisms that govern and constrain the ability of an organism to choose between different response output options. For example, male fruit flies undergo a series of stereotyped courtship behaviors "because" they have a set of male-specific *fruitless* neurons that are activated when they encounter a female (Manoli et al., 2005). The decision a male fruit fly makes between attempting mating or suppressing courtship can be influenced by a number of stimuli, including numerous pheromone signals, previous mating experiences, and receptivity of the female mate (Dickson, 2008). But ultimately, the decision is mechanistically dependent upon those male-specific *fruitless* neurons being activated. As a result, neurophysiological models of decision-making can be very predictive, but also can impose constraints on defining what constitutes a decision (Kable and Glimcher, 2009). Basically, if what appears to be a complex decision-making process in an "as if" model is reduced to a simple stimulus-response reaction in a "because" model, then can the process really be defined as a decision? For example, when the aforementioned insect chooses to flee a predator infested food patch, one could argue the insect acted "as if" it performed a series of complex valuations of all the potential outcomes to its actions, and therefore selected the best option available in a decision-making process. However, if a neurophysiological study revealed that the insect chooses to flee the food patch "because" a specific predator

pheromone activates a single sensory neuron X, thereby invoking a simple stimulus-response reaction to explain the behavior, one could argue not much of a decision-making process is involved.

Given the different methodologies of all the decision-making fields, a certain degree debate is expected in how to best describe what constitutes a decision. Since the results described in this dissertation deal with biological behaviors, neural circuitry, and genetic elements associated with choice-like responses, the emphasis of this thesis will be dealing with the neurophysiological mechanisms, and as such be rooted in "because" models of decision-making. However, the primary goal of this dissertation is to better characterize the neurons and genes involved in decision-making related behaviors in the model organism *Drosophila melanogaster*, rather than resolve components of the debate surrounding decision theory, which is beyond the scope of this dissertation. Thus, in order to adhere to the purposes of this study and simplify the interpretations from my experimental results, I begin Chapter 1 of this thesis by defining a "decision" using basic core concepts that are shared by most decision theories. Briefly, a decision-making process occurs when external input signals are presented to an organism such that multiple contradictory responses are possible, but given the nature of the situation the organism must select a single behavioral response and execute it as motor output. During this process, the organism undergoes (1) sensory *detection* of relevant input stimuli that lead to competing behavioral responses, (2) integrative *valuation* of possible responses based on their predicted reward outcomes, and (3) *selection* of the optimal behavioral response given the contextual relevance. This stepwise process of *detection-valuation-selection* between mutually exclusive behavioral response options is

common across different fields that study decision-making, and is therefore the basic description of a "decision" employed by this dissertation.

There are two primary goals of this thesis: (1) substantiate oviposition site-selection of *Drosophila melanogaster* as an effective model for simple decision-making, and (2) utilize the genetically tractable fruit fly to uncover the neural circuitry mediating the sensory detection, signal valuation, and response selection stages of the decision-making process. As such, I will continue Chapter 1 with brief explanations of the different decision-making models that can be generated from experiments using *Drosophila* oviposition (egg-laying) behavior as an experimental paradigm to create a situation where female flies must select between competing, mutually exclusive preference response outputs. Next, I will provide a short review of literature that supports *Drosophila* oviposition behavior as a model for simple decision-making, notably with regard to how the selection of ideal egg-laying substrates often requires that a female fruit fly choose between one of two mutually exclusive responses: (1) avoid the substrate and hold eggs, or (2) not avoid the substrate in order to lay eggs. Furthermore, since proper perception of the environment is essential for to make decisions necessary to both survival of individual animals and propagation of the species, I will provide a description of the primary *Drosophila* sensory systems employed during egg-laying behavior: the olfactory system and the gustatory system. Finally, I will provide concise summaries of the advantageous genetic and neurophysiological tools that are available in *Drosophila melanogaster*, which I employed during experiments pertaining to this dissertation.

Following Chapter 1, which establishes the terminology and tools required for the experiments that are the subject of this study, I will describe in Chapter 2 my characterization

of the competing positional aversion and egg-laying attraction responses that are induced by acetic acid, by using a novel two-choice assay specifically designed to simultaneously assay these mutually exclusive behavioral preferences that occur during the *Drosophila* oviposition program. Using acetic acid as a proof-of-principle compound that induces the process of *detection-valuation-selection* between mutually exclusive behavioral responses, I demonstrate that my two-choice model can be effectively used to identify neural circuitry involved in choice-like behavioral switches and decision-making processes. Subsequently, I describe in Chapter 3 the results of a forward genetic screen in which I employed acetic acid in my two-choice assay to uncover genes potentially important to the decision-making process that is employed by female *Drosophila* when selected optimal oviposition sites for their offspring. Next, I describe in Chapter 4 the expansion of this simple acetic acid-based model for choice-like behavior into a more general paradigm of decision-making by utilizing bitter compounds to induce contradictory positional aversion and egg-laying attraction responses in my two-choice assay. Additionally, I provide initial characterization of both the sensory systems and higher order brain regions that are responsible for mediating the selection between these two opposing preferences that operate within the *Drosophila* oviposition program. Furthermore, I describe novel findings about the *Drosophila* gustatory system, which arose from this expansion of the decision-making model. Notably, I demonstrate how the *Drosophila* gustatory system utilizes tissue-specific activation of taste-neurons that express the same receptor complex, but that are present in different anatomical locations, to elicit very different and often contradictory behavioral responses. Finally, I will discuss in Chapter 5 conclusions that can be drawn from the experiments detailed in this study, with regard to general principles of using *Drosophila* oviposition as a model for

simple decision-making, and to the future directions of investigation that are now available given the specific identification of sensory and central neurons that are involved in this important decision-making process.

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Chapter 1

Decision-Making Terminology and *Drosophila melanogaster* Techniques

Ryan M. Joseph

INTRODUCTION

As previously mentioned, the fields that study decision-making include several distinct disciplines like economics, evolutionary ecology, neurobiology, computer science, and psychology (von Neumann and Morgenstern, 1944; Miller et al., 2011; Kristan, 2008; Sutton and Barto, 1998; Kahneman and Tversky, 1979). As such, the terminology associated with decision-making can sometimes lack consensus, and often debate occurs over just how a decision should be classified (Kable and Glimcher, 2009). Should a particular choice of behavioral output be called a decision based solely on the behavioral output, regardless of the neural mechanisms used to select the response (Samuelson, 1937; Friedman, 1953)? Or does the classification of a decision-making event necessitate that the neurons mediating the selection process be identified, thereby providing biological evidence that input signals leading to distinct behavioral outputs are directly compared in the brain (Sugrue, 2005; Kristan, 2008)?

Resolving this debate is not the purpose of this dissertation. The nature of this study is neurophysiological in nature, with two primary goals: (1) substantiate the *Drosophila melanogaster* oviposition program as a simple decision-making process, specifically by characterizing the selection event that occurs between contradictory positional aversion and egg-laying attraction preference pathways within this behavioral program; (2) subsequently identify neural circuitry mediating this oviposition-related selection process in the genetically tractable fruit fly, such that the neural mechanisms behind decision-making can be better understood. Since the identifications of genes and neurons is the ultimate goal of this study, I have chosen to define a "decision" using basic concepts that are common to the decision theories from different academic disciplines. Subsequent interpretation of results will be

performed from this common denominator definition defined in the section below, to prevent confusion of results. I will subsequently describe additional terminology within the context of this definition, and review previous results that support why *Drosophila melanogaster* oviposition is an effective model for decision-making.

Section A: Decision Defined

Simply stated, a "decision" occurs when an organism is presented with input stimuli that induce multiple contradictory behavioral responses, but given the nature of the situation a single behavioral response must be selected and executed as motor output. In most theories, such a situation must normally be presented to qualify a selection between response alternatives as a decision (Schall, 2005; Kristan, 2008; Kable and Glimcher, 2009). Furthermore, the decision-making process in these theories shares common steps: (1) sensory *detection* of input stimuli that induce distinct responses, (2) *valuation* of the possible responses based their predicted reward or punishment outcomes, and (3) *selection* of a behavioral response for execution through motor output. This stepwise process of *detection-valuation-selection* between mutually exclusive behavioral response options is therefore the basic description of a decision-making process employed by this thesis.

A distinction is sometimes made about the difference between a decision and a choice. Although both terms can be used when an organism is confronted with a situation that requires selection between alternative courses of action, the term *decision* normally implies a higher level of evaluation in which the organism dynamically perceives and "thinks about" potentially rewarding outcomes (Kristan, 2008), whereas the term *choice* does not necessarily require that the organism is aware that such an evaluation of potentially

rewarding outcomes is occurring (Schall, 2005). Given the fact that recent behavioral studies have described the selection of optimal egg-laying sites by *Drosophila melanogaster* as decision-making (Yang et al., 2008; Miller et al., 2011), this dissertation will also use the term *decision* when discussing selection events involved in the *Drosophila* oviposition program, in order to be consistent. Furthermore, a decision is often referred to as an "overt act of choosing" (Schall, 2005); with this description in mind, when mentioned in the text of this dissertation the term *choice* can be thought of as a component of the overall decision-making process involved in the *Drosophila* oviposition behavior.

Section B: Classification of Competitive Behavioral Responses

In order for a decision to be made, an organism must be confronted by a situation where at least two alternative responses are available, and each course of action cannot be performed simultaneously. For example, when a male fly encounters a mixed population of both other males and receptive females, the male fly in question has at least two options with regard to behavioral output: (1) court females or (2) fight males. These responses are competitive and contradictory, since the male cannot execute both simultaneously, and must select a single course of action (Dickson, 2008). As such, the identification of behavioral responses that directly compete and mutually exclude each other is crucial for substantiating a particular situation as a decision-making process. In fact, much of the experimental characterization conducted in Chapter 2 of this dissertation is dedicated to demonstrating that the overall *Drosophila* oviposition program contains *directly* competing positional aversion and egg-laying attraction responses for compounds like acetic acid, thereby establishing a decision-like situation in our two-choice experimental paradigm.

However, behavioral responses can compete at different levels, notably at either the level of neural signal integration or motor response output. Since the selection between competitive and mutually exclusive behavioral responses is essential for classifying a process as decision-making, a general description of the different models for these types of competition is recommended, and offered below. For the following examples, I will describe the competition between two contradictory behavioral responses, simply labeled as attraction or repulsion, which can be approximated as the organism in question making a decision to either approach or avoid an environmental stimulus.

i.) Parallel Pathway Model

In the simplest model for competition between behavioral outputs, relevant sensory input is received by the organism and induces the neural circuitry governing the individual attraction and repulsion responses. Sensory input signals are then translated into motor response outputs by independent and parallel pathways that do not intersect nor interact at the neuroanatomical level within the brain (Figure 1; blue lines for repulsion, red lines for attraction). As such, both behavioral outputs are executed, but one eventually overrides the other, based on the intensity of the response that is elicited by each respective input signal. In other words, both behaviors are attempted, but a single output response is selected simply because the activation of one particular neural circuit is stronger than the other. The competition between the two responses only occurs at the level of behavioral output, without any integrative comparison or complex evaluation of input signals within the brain (Figure 1; ↔ labels competition in only behavioral output box).

Of note, any observed competition between opposing behavioral responses in the *parallel pathway model* is a result of behavioral logistics: an organism cannot simultaneously avoid and approach a particular environmental stimulus, and one response must therefore win. This relationship between contradictory responses suggests that the choice between attraction and repulsion reflects more of a "tug-of-war" between independent preference pathways, rather than a truly dynamic decision-making process. For example, given the description of decision-making as containing *detection-valuation-selection* stages, the *valuation* step is noticeably minimal, in that there is no combined comparison of the potential rewards and/or punishments associated with the attractive and repulsive responses. Thus, although competitive behavioral preferences are present and selection between responses occurs, the parallel pathway model does not perfectly fit the criteria for a dynamic decision-making, because it can be reduced to a behavioral output balance of stimulus-response reactions. In fact, much fuel to the debate over what constitutes an actual decision stems from neurophysiological arguments that there must be shared neurons between the different neural circuits that mediate competing responses, thereby suggesting that some concurrent evaluation (i.e. the *valuation* stage) of input signals is occurring at the neuronal level (Kristan, 2008; Kable and Glimcher, 2009).

ii.) Intersecting Pathway Model

A more complex model for competition between behavioral outputs involves the intersection of neural circuitry mediating the separate attraction and repulsion. Signal input is received by the relevant sensory systems of the organism, and information is relayed to the corresponding neural circuits for each behavior (Figure 2; blue lines for repulsion, red lines

for attraction). However, the neurons relaying information from both preference pathways then interact at the neuroanatomical level within the brain. This intersection between the competing neural circuits leaves the possibility that *direct* comparison of the competing signals occurs between neurons common to both circuits, thereby providing a more compelling argument that the organism is indeed conducting the *valuation* step in the *detection-valuation-selection* process. For example, in *Caenorhabditis elegans* sensory neurons that receive input for the attractive response to diacetyl odorant and the aversive response to Cu^{2+} converge on shared interneurons that are essential to the execution of both preferences, suggesting that there is comparison of input information at the cellular level before a motor output is selected (Shinkai et al., 2011). After signals converge on common neurons, either an attractive or repulsive output is selected. Unlike the simple balance between stimulus-response reflexes in the *parallel pathway model*, the selection of behavioral response in the *intersecting pathway model* reflects a combinatorial process, in which the selection is made after the organism evaluates the reward and/or punishment associated with one response in the context of the other response, before subsequent motor output is executed (Figure 2; purple lines highlight combined selection between two behaviors).

Although intersection between neurons of the competing behavioral response pathways provides strong argument for the presence of a decision-making and choice-like process in a given environmental situation and/or experimental paradigm, it does not necessarily suggest that the organism is aware of the valuation and comparison being performed between both behavioral responses. For example, the interaction between neural circuits could reflect a simple cross-inhibition of pathways; sensory neurons from the

avoidance pathway could intersect presynaptically on pattern-generating neurons from the attraction pathway, such that any activation of the avoidance pathway causes a global lateral inhibition of the opposing attraction pathway (Kristan, 2008). Indeed, the suppression of tactile behavioral responses by activation of feeding behavior in the *Hirudo* leech (Misell et al., 1998; Gaudry et al., 2009) as well as the suppression of mechanosensory responses by activation of pain-associated reactions in mammals (Fields, 2007) both function in this manner. However, one could argue that these situations do not reflect dynamic decision making either, since the *selection* step in the *detection-valuation-selection* process is now in question. Notably, does the choice between attraction and repulsion truly reflect a decision when the selection event is determined by an indiscriminate "all-or-none" lateral inhibition of one pathway by the other pathway?

iii.) Central Integration Model

The most complex model described in this dissertation for competition between contradictory behavioral outputs involves the convergence of neural circuits mediating the attraction and repulsion responses in shared higher-order brain regions that are capable of integrating signals and performing complex evaluation processes. Input is again received by the relevant sensory systems of the organism and information is relayed through the corresponding neural circuits for each behavior (Figure 3; blue lines for repulsion, red lines for attraction). Signals from attraction and repulsion pathways then converge in central integration centers that both conduct *valuation* of the response options and *select* which response option is ideal given the circumstances (Figure 3; purple lines highlight combinatorial selection of a single output after competing outputs have been directly

compared within the higher-order brain region). In the *central integration model*, the interaction of competing signals in brain structures capable of complex cognitive processes provides the strongest neurophysiological argument that the organism is indeed performing decision-making.

Indeed, numerous neurophysiological studies of decision-making have identified brain regions that are likely integration centers for multiple competing behavioral pathways. In primate studies, dynamic levels of synaptic activity can be observed in the parietal cortex neurons when animals are choosing between two alternative responses in a visual-based selection test (Platt and Glimcher, 1999), while the prefrontal cortex has been shown to perform valuation of multiple external stimuli and reward assignment of competing behavioral responses during decision-making tasks (Shidara and Richmond, 2002; Sugrue et al. 2005). Similarly, in zebrafish the selection of activation or suppression of an innate light-avoidance response can be isolated to the dorsal telencephalic region, which is the anatomical homolog to the mammalian amygdala (Lau et. al, 2011). Finally, in *Drosophila melanogaster* the decision to fly towards one of two alternative visual cues is modulated by neural activity of the mushroom body, a higher-order neuronal structure present in the *Drosophila* brain (Tang and Guo, 2001). These studies support that the *central integration model* as a plausible description of the competition between mutually exclusive behavioral responses in decision-making processes, even in *Drosophila melanogaster,* the model organism used in this study.

iv) Combinatorial Model

When characterizing the selection between opposing behavioral outputs, the identification of neural circuitry and brain regions common to both preference pathways can

therefore provide evidence that the selection event being studied is a decision-making process. As such, during the experiments detailed in Chapter 2, Chapter 3, and Chapter 4, we sought to uncover genes and neurons that, when mutated or silenced, caused a disruption in both repulsive positional or attractive egg-laying responses to a particular compound. The identification of a single neuronal structure responsible for both of the competing behavioral responses would support the *central integration model*, thereby strongly substantiating the selection female *Drosophila* make between positional aversion and egg-laying attraction as a decision-making process.

However, interpretation of results involving the competition between mutually exclusive behavioral outputs can often be ambiguous. In fact, even the aforementioned lateral inhibition of tactile behavioral responses by activation of feeding behavior in the *Hirudo*, which strongly supports the *intersection pathway model*, is not completely clear. An additional study demonstrates that suppression of tactile behaviors in *Hirudo* can be modulated by additional neurons, and as such a simple "all-or-none" lateral inhibition cannot be the sole source of neuronal interaction between pathways (Briggman et al., 2005). In addition, complex neural networks rarely possess a single intersection point, especially given that the sensory systems that detect signal inputs for different behavioral pathways are often shared. Furthermore, competition between opposing responses can still occur at the behavioral output level, even if the responses are not mediated by completely independent and parallel neural circuitry. As such, one can envision the actual neural circuit map defining the competition between mutually exclusive responses in a decision-making process to be a combination of the *parallel pathway, intersection pathway, and central integration* models.

Section C: Rationale for *Drosophila* as a Model Organism for Decision-Making

As demonstrated by the numerous examples described above, decision-making has been studied in numerous organisms, ranging from the free-living nematode *Caenorhabditis elegans* (Shinkai et al., 2011), the fruit fly *Drosophila melanogaster* (Dickson, 2008), the leech *Hirudo medicinalis* (Gaudry et al., 2009), the zebrafish *Danio rerio* (Lau et. al., 2011), laboratory strains of mice and rats (Balleine and O'Doherty, 2010; Dalley et al., 2011), numerous species of monkeys (Sugrue et al., 2005), and humans (Kahnt et. al 2011). The differences and similarities between each organism are too numerous to discuss; needless to say, each organism has advantages and disadvantages with regard to studying decision-making processes. For example, organisms with more complex brain neuroanatomy have a wider assortment of behavioral responses that can be presented as alternatives in a decision-making assay that adheres to the *detection-valuation-selection* definition previously described; however, the complexity of the nervous system in these organisms makes isolation of important neural circuit interactions relatively difficult. In contrast, organisms with simpler brain neuroanatomy often have fewer behavioral responses that can be presented in true decision-making contexts; however, if an experimental paradigm can be established in these organisms that constitutes a *detection-valuation-selection* decision-making process, then identifying and characterizing important neural circuitry in substantially easier.

In either case, an essential problem in any experimental organism is identifying mutually exclusive behaviors that can be simultaneously presented and alternatively selected in a true decision-making paradigm. Furthermore, once contradictory behavioral responses have been identified, the experimental protocols required to establish the selection situation or observe neurophysiological responses can be rather difficult. For example, in primate

studies the insertion of electrodes into brain regions responsible for decision-making requires highly technical surgeries into complex anatomy, which must be conducted after a lengthy developmental period (Platt and Glimcher, 1999). Even in *Drosophila*, which possesses comparatively simpler neuroanatomy, the technical requirements for establishing certain decision-making paradigms, such as the selection between opposing visual cues in the *Drosophila* flight simulator assay, can be quite demanding (Zhang et al., 2007; Xi et al., 2008). As such, finding relatively straightforward competing responses that can be utilized in two-choice assays for simpler model organisms can be particularly advantageous for studying decision-making processes.

Oviposition, or egg-laying site selection in *Drosophila melanogaster* provides a particularly attractive model for simple decision-making, as detailed by Chapter 2 of this dissertation (published as Joseph et. al., 209) and additional studies (Yang et. al. 2008; Miller et. al. 2011), notably because competing responses within the *Drosophila* oviposition program can be generated in relatively simple experimental assays. Although substantiation of the *Drosophila* oviposition program as a decision-making model will be a primary subject of Chapter 2, a brief description of the properties that initially made *Drosophila* egg-laying behavior an attractive candidate to study choice-like processes is relevant, in order to provide rationale for the line of investigation taken in subsequent sections of this dissertation.

i.) The Drosophila Oviposition Program

The oviposition program provides a robust yet straightforward method for monitoring numerous different types of preference behaviors in *Drosophila melanogaster*, since a deposited egg represents a biomarker for where female flies have spent their time. Previous

studies of different sister-species of the *Drosophila* genus have used egg-laying as a readout for conditions advantageous to progeny development (van Delden and Kamping, 1990; Ruiz-Dubreuil, 1994), and have demonstrated that oviposition preferences can be used to effectively separate larva of different sibling species of *Drosophila* (Richmond and Gerking, 1979; Chess and Ringo, 1985). Oviposition preferences have also been used to study behavioral aversion towards compounds that are either toxic or non-appetitive for both larvae and adults (Moreteau et al., 1994; Possidente et al. 1999; Mery and Kawecki; 2002). Furthermore, numerous studies have employed patterns of oviposition to distinguish subtle differences in attraction for different host plants and environmental resources, which have provided insights into resource requirements and ecological behaviors of different *Drosophila* species (Barker et al., 1994; Amlou et al., 1998; Matsuo et al., 2007; Harada et al., 2008). Thus, previous studies have observed both attractive preferences and repulsive responses within the overall *Drosophila* oviposition program.

As such, when initially designing a two-choice assay for studying choice-like processes, we reasoned that the overall *Drosophila* oviposition program was a good candidate to establish a decision-making situation, since we could potentially find two competing, mutually exclusive response alternatives to present to our experimental flies. Indeed, findings from previous studies hinted that competition between competitive responses occurred within the *Drosophila* oviposition program. For example, in certain experimental contexts *Drosophila melanogaster* finds the smell of acetic acid aversive (Fuyama, 1976), but in other assays female fruit flies attractively deposit eggs on media containing acetic acid (Eisses, 1997). Similarly, *Drosophila* females have been shown to lay eggs on substrates that are not necessarily optimal for larval or adult fitness, suggesting that

competition from additional preference pathways likely influence egg-laying behavioral responses (Jaenike, 1982). Indeed, we successfully induced a selection between contradictory behavioral responses by constructing our two-choice assay for monitoring decision-making by first using acetic acid (Chapter 2) and then bitter-tasting lobeline (Chapter 4) to simultaneously induce competitive positional aversion and egg-laying attraction responses in female *Drosophila.* Furthermore, in contrast to more complex decision-making paradigms in *Drosophila*, the employment of oviposition behavior to setup a two-choice selection situation was relatively simple, thereby facilitating experimental design, data analysis, and rapid genetic screening (Chapter 3).

ii.) An Extensive Neurogenetic Toolkit

Drosophila melanogaster has been the subject of genetic study for over 100 years (Kohler, 1994), and has remained an incredibly useful model organism for over a century because of numerous characteristics. Fruit flies are small, inexpensive to rear, and have a rapid generation time of only 10-days, allowing for quick production of thousands of testable progeny (St. Johnston, 2002). The *Drosophila* genome was one of the first to be annotated (Adams et al., 2000), and has been extensively studied and categorized into numerous databases open to researchers. *Drosophila* are particularly amenable to genetic screening, and countless mutants can be generated in a relatively short time-frame using either ethyl methane sulfonate (EMS) to produce nucleotide substitutions and small deletion, or the mobilization of transposable sequences called P-elements to disrupt the expression of genes (Engels, 1983). P-element transposition is particularly useful, since the genomic location of the insertion can be easily identified using inverse-PCR for known DNA sequences in the P-

element, and when used in conjunction with the aforementioned genome databases, the mutated gene can be rapidly identified without the need for chromosomal mapping. Genetic crosses in *Drosophila* are also fairly straightforward, notably because fruit flies possess only four homologous chromosomes, and because researchers have developed visibly marked balancer chromosomes that impede homologous recombination (Greenspan, 1997). In addition to forward-genetic techniques, reverse-genetics techniques can also be performed by using targeted mutagenesis with homologous recombination (Rong and Goli, 2000) or by degrading specific mRNA sequences with double-stranded RNA molecules designed to induce an endogenous RNA interference response (Carthew, 2001; Kalidas and Smith, 2002).

Genome comparisons have revealed a large number of genes in *Drosophila* that share homology with important genes in other organisms, including mammals, demonstrating that investigations in *Drosophila* can have broad implications for understanding evolutionarily conserved biological processes in higher organisms like humans. Importantly, this conservation is not limited to developmental or cellular processes, but also extends to areas of behavioral neuroscience. Indeed, *Drosophila melanogaster* is capable of numerous complex behaviors, many of which have similar correlates in other organisms (Sokolowski, 2001; Busto et al., 2010), like mating, taste-related responses, olfactory learning/memory, and foraging behavior. When compared to more complex mammalian organisms, the structure and organization of the fruit fly nervous system is relatively simple and tractable, but is also sufficiently complex for the identification of neuronal brain structures that are homologous to their mammalian counterparts (Olsen and Wilson, 2008).

In addition to having a relatively simpler yet sufficiently complex nervous system and behavioral repertoire, the generation of transgenic *Drosophila* strains is remarkably facile

when compared to other mammalian systems (Spradling and Rubin, 1982; Rubin and Spradling, 1982). With regard to the subjects discussed in this thesis, this allows for the development of incredibly useful tools for manipulating neuronal signaling and hence behavioral processes in transgenic fruit flies. In particular, the implementation of the yeast *UAS/GAL4* binary system in *Drosophila* (Brand and Perrimon, 1993; Brand et al., 1994) has been immeasurably important to this field of research. The *UAS/GAL4* system allows researchers to express the yeast transcriptional activator, *GAL4*, in restricted subsets of cells—including neurons—within the fly. *GAL4* directly binds to *UAS* nucleotide sequences and signals transcriptional activation of open reading frames downstream from the binding site. By fusing transgenes of interest to the *UAS* sequence and introducing the construct in flies expressing the *GAL4* activator in neuronal subsets of interest, one can perform numerous transgenic manipulations within these subsets of cells and observe any phenotypic consequences in behavior and/or development. For example, with regard to the specific experiments in this thesis, the *GAL4/UAS* expression of temperature-sensitive dynamin, $Shibire^{ts}$ (Kitamoto, 2001) or tetanus toxin light chain, *TeTxLC* (also called *TNT*) (Sweeney et al., 1995) can be used to disrupt synaptic vesicle endocytosis and exocytosis, respectively, which subsequently silences signaling in neurons expressing *GAL4*. Ectopic activation of neurons with the *GAL4/UAS* system can also be performed using *UAS* fusions to: *channelrhodopsin-2*, a light-activated cation channel (Schroll, 2006); *Drosophila TRPA1*, a thermally activated and voltage-gated cation channel (Hamada, 2008; Shang, 2008); *ether-à-go-go (eag)*, an inward rectifying potassium channel that hyperactivates neurons when ectopically expressed, to name a few transgenic manipulations.

With regard to temporal-specific and tissue-specific manipulations, even further tweaking of the *GAL4/UAS* system can be performed for increased experimental resolution. Notably, the TARGET system utilizes a temperature-sensitive inhibitor of *GAL4*, called $GAL80^{ts}$, to repress the *GAL4/UAS* system when flies are placed at the permissible 18°C temperature and to activate the *GAL4/UAS* system when flies are placed at the restrictive 29°C temperature (McGuire et al. 2004). The TARGET system therefore allows researchers to control when transgenes are expressed, and thus observe their effects at different times during development. Another modification to the *GAL4/UAS* system involves using a *GAL80* transgene flanked by *FRT* recombination sequences to generate genetic mosaic animals; stochastic induction of recombination between the *FRT*-sites causes the *GAL80* transgene to be removed from a nearby promoter, which subsequently stops *GAL80* expression and activates *GAL4/UAS* expression. By varying the level of recombination frequency, a highly restricted subset of neurons within a particular *GAL4* expression pattern can be generated for analysis (Gordon and Scott, 2009), allowing the identification of single neurons that are critical to behavioral responses.

Several advanced techniques have been recently established in *Drosophila melanogaster,* which will likely aid in producing even more refined investigations into the *Drosophila* nervous system. Namely, the recent implementation of high-resolution, multi-color labeling techniques that track neuronal lineage development (Brainbow-1 System: Hampel et al., 2011) and the development of a second binary transcriptional system that operates independently of *GAL4/UAS* (LexA System: Lai and Lee, 2006) should have broad applications in the future studies relating to behavioral neuroscience. In summary,

Drosophila melanogaster therefore provides a powerful neurogenetic tool for studying behaviors pertaining to decision-making.

Section D: Sensory Perception

Given the large amount of neurogenetic tools available in *Drosophila*, it is no surprise that the fruit fly has been used to extensively study sensory perception. Surprisingly, although proper sensory perception is a critical part of the *detection-valuation-selection* process of decision-making, much emphasis in decision theories has been placed on the *valuation* and *selection* steps, while the *detection* stage has been relegated to the fields of neuroscience. Since a primary goal of this dissertation is to identify neural circuitry mediating decision-making behavior in the *Drosophila* oviposition program, we felt that mapping sensory systems was a critical part of understanding this process. Thus, a brief contrast and comparison of the *Drosophila* olfactory and gustatory systems is provided below. Of note, the following sections are offered as a brief overview of olfaction and gustation in *Drosophila*, and are not meant to be an exhaustive review (See Hallem, Dahanukar, and Carlson, 2006; Masse et al., 2009 for reviews of the olfactory system. See Isono and Morita, 2010; Miyazaki and Ito, 2010 for reviews of the gustatory system). Rather, the descriptions are provided to situate novel findings in this study within the context of the overall field relating to sensory perception:

i.) Drosophila Olfactory System

Olfactory sensory neurons (OSNs) are housed in bristles of three different morphologies that populate antenna and maxillary palps, the primary olfactory organs of

Drosophila (Stocker, 1994; Shanbhag, 1999). Each olfactory bristle contains 2-4 OSNs that extend their dendrites into an aqueous-filled lumen, through which volatile odorants diffuse to bind to olfactory receptors (ORs) that are expressed on the membranes of OSNs (Hallem et. al., 2004; Masse et al., 2009). ORs comprise a group of 60 highly diverse, seven transmembrane domain genes (Clyne et al., 1999), which were originally thought to be G-coupled receptors but later turned out to have inverted morphology when compared to these proteins (Benton et al., 2006). Approximately 45 ORs express in the antenna and maxillary palps (Couto et al., 2005); each individual OSN expresses a single, unique OR that confers its molecular and cellular identity, with regard to the odorants that the particular OSN detects (Hallem et al., 2006). Furthermore, approximately 70-80% of OSNs also express the *Or83b* co-receptor, which is involved in trafficking its specific OR partner to the synaptic membrane and is likely involved proper signal transduction for behavioral responses (Larsson et al., 2004; Neuhaus et al., 2005, Sato et al., 2008; Wicher et al., 2008).

Drosophila OSNs then project their axons to the antennal lobe, which is the fruit fly equivalent of the mammalian olfactory bulb (Masse et al., 2009). The antennal lobe is a primary processing center of sensory input, where processing of olfactory signals occurs after being received by OSNs and before being relayed to higher-order centers (Miyazaki and Ito, 2010). The antennal lobe is divided into discrete neuronal structures called glomeruli, which receive projections from OSNs expressing one type of *OR/Or83b* pair (Fishilevich and Vosshall, 2005). The processing and transformation of input in the antennal lobe can be modulated by local interneurons that establish connections between the glomeruli (Olsen et al., 2007), and from neurons that project back to the antennal lobe from higher-order structures further downstream in the signaling pathway (Masse et al., 2009). Given the

stereotyped projection patterns of specific ORs to identifiable glomeruli, olfactory sensory circuit maps have been constructed (Vosshall et al., 2000). Furthermore, when these olfactory sensory circuit maps are used in conjunction with electrophysiological studies defining what odorants each specific OR can detect (de Bruyne, 2001; Hallem et al., 2006), researchers can generate an "odor space" to predict which chemical compounds synaptically stimulate specific ORs, leading to stereotyped activation patterns of glomeruli in the antennal lobe, and subsequent behavioral outputs (Masse et al., 2009).

After signals have been transformed in the antennal lobe, input is then relayed to higher-order structures like the mushroom body and lateral horn for additional processing and subsequent execution of behavioral output (de Belle and Heisenberg, 1994; Connolly et al., 1996; Heimbeck et al. 2001). Although information undergoes substantial transformation during the sequential circuit stages of the olfactory system, a certain degree of the sensory map that is produced in the antennal lobe by OR neurons is maintained in higher-order structures; notably, projection neurons from specific antennal lobe glomeruli project to specific regions of the lateral horn and mushroom body (Wong et al., 2002; Tanaka et al., 2004). Given the importance of the mushroom body for learning/memory (Krashes et al., 2007), reward-association (Strube-Bloss et al., 2011), and decision-making (Zhang et al., 2007; Brembs, 2009; Wu and Guo, 2011), it seems likely that olfactory responses pertaining to the *Drosophila* oviposition program would be involved in a decision-making process.

ii.) Drosophila Gustatory System

Similar to OSNs in the olfactory system, *Drosophila* gustatory sensory neurons (GSNs) are housed in bristles with an aqueous-filled lumen (Stocker and Schorderet, 1981;

Stocker, 1994); these bristles are also broken down into three different morphological classes, at least on the primary *Drosophila* taste organ: the labellum (Hiroi et al., 2002). Furthermore, each bristle contains either 2 or 4 individual GSNs that contain gustatory receptors (GRs) that confer sensitivity to one of the following taste-modalities: (*i*) sweet-tasting sugars, (*ii*) bitter-tasting compounds and high concentrations of salt, (*iii*) low concentrations of salt, and (*iv*) water and low osmolarity (Weiss et al. 2011). In contrast to the *Drosophila* olfactory system—which has only two primary sensory organs that express OSN-containing sensilla—GSN-containing sensilla are located in many more diverse tissues of the fly, including: the labellum (Dunipace, et al., 2001; Weiss et al., 2011); the pharynx (Stocker and Schorderet, 1981; Gendre et al., 2003); the legs (Mitri et al., 2009), the wings (Dunipace, et al., 2001); ovipositor and vaginal plate (Taylor, 1989); the abdomen (Thorne and Amrein, 2008; Shimono et al., 2009).

One key difference of the *Drosophila* gustatory system is that unlike OSNs, which typically express a unique *OR/Or83b* pair that defines its identity and makes circuit mapping relatively straightforward, *Drosophila* gustatory neurons co-express multiple gustatory receptors (Lee et al., 2009; Isono and Morita, 2010; Weiss et al., 2011; Jiao et al. 2008; Thorne et al., 2004; Wang et al. 2004). This co-expression of multiple GRs is particularly dramatic in bitter-sensing OSNs that express *Gr66a* (Weiss et al., 2011; Marella et al., 2006), which causes challenges in constructing neuronal maps that explain how single GSNs with overlapping GR-profiles can induce diverse behavioral responses to specific compounds. This overlapping expression of GRs is also observed in sweet-sensing OSNs that express *Gr5a*, which have been shown to express different isoforms of *Gr64* in order to specifically sense different sugars (Dahanukar et al., 2007; Jiao et al., 2008).

Axons from GSNs project from taste bristles to the subesophageal ganglion (SOG), which has been hypothesized to be a primary processing center similar to the antennal lobe (Miyazaki and Ito, 2010), where signals undergo transformation and are sent to higher-order structures for further integration. Although bitter-sensing *Gr66a* neurons and sweet-sensing *Gr5a* neurons project to non-overlapping regions of the SOG (Thorne et al., 2004; Wang et al., 2004), the construction of neural maps within the SOG has been limited due to the lack of obvious compartmentalization of projections into discrete glomerular structures. However, a recent high-resolution imaging study that concurrently utilizes *GAL4/UAS* and *LexA* systems demonstrates that GSNs do indeed project to discrete regions of the SOG, in a predictable manner based—to some extent—on the types the GR-expression profile and anatomical origin of a GSN (Miyazaki and Ito, 2010).

The aforementioned study demonstrates the potential for constructing neural circuit maps for the gustatory system. However, unlike the *Drosophila* olfactory system, investigations into the higher-order structures that mediate behaviors in the gustatory system have only recently been initiated (Melcher and Pankratz, 2005; Chatterjee and Hardin, 2010l Chapter 2 and Chapter 4). Furthermore, second-order neurons directly connecting the SOG to higher-order brain regions have yet to be identified in *Drosophila melanogaster*, but such neural links have been identified in other insects (Schroter and Menzel, 2003). One brain structure potentially downstream of the SOG is the mushroom body, which has been implicated in the integration of multiple sensory inputs (Xi et al., 2008) and formation of appetitive memories of food-associated odors (Krashes et al., 2009). Thus, although characterization of the central processing centers has not been as extensive as studies pertaining to the olfactory system, future investigations into the primary, secondary, and

higher-order neural circuitry involved in the *Drosophila* gustatory system appear promising. Subsequently, if higher-order structures involved in *Drosophila* olfaction are also identified to be important in *Drosophila* gustatory behaviors, potential investigative inroads into studying decision-making processes could become readily available.

DISCUSSION

In summary, with regards to this dissertation, a "decision" occurs when an organism is presented with input stimuli that induce multiple contradictory behavioral responses, but given the nature of the situation a single behavioral response must be selected and executed as motor output. The decision-making process involves (1) *detection* of relevant sensory input, (2) *valuation* and direct comparison of the potential rewards associated with the different behavioral response alternatives, and (3) *selection* of a single behavioral response output. In order to establish a situation that requires a decision be made by an organism, competing and mutually exclusive behavioral responses must be identified and simultaneously presented as options to the organism. As such, three models for behavioral response competition are presented above: (*i*) the *parallel pathway model*, (*ii*) the *intersecting pathway model*, and (*iii*) the *central integration model*, with the third model representing a situation that best satisfies the aforementioned definition of a decision. Given the potential for *Drosophila* females to exhibit both attractive and aversive preferences while searching for an appropriate site to deposit eggs, and the extensive neurogenetic toolkit available in the fruit fly, we selected the *Drosophila* oviposition program to investigate decision-making. Specifically, the two primary goals of study for this dissertation are: (1) substantiate the *Drosophila melanogaster* oviposition program as a model for simple

decision-making; (2) identify sensory and central neurons that mediate oviposition-related selection processes, such that the neural mechanisms behind decision-making can be better understood.

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FIGURE LEGENDS

Figure 1: Parallel Pathway Model for Competing Behavioral Responses

In the *parallel pathway model* for the selection of contradictory behavioral responses, input signals for both the avoidance and attraction responses enter into independent neural circuits and proceed through parallel pathways in order to elicit corresponding output responses. There is no intersection of neural circuitry governing each response, and therefore there is no direct interaction of signals within the brain, suggesting that a true *valuation* step in the *detection-valuation-selection* process of decision-making may not be present. Instead, any observation of competition between the two behavioral responses is a result of logistics at the level of motor output execution: an organism cannot simultaneously avoid and approach a particular environmental stimulus, and one behavioral response is selected simply by being more strongly induced by its respective signal input intensity. Blue lines/labels refer to the avoidance behavioral response. Red lines/labels refer to the attraction behavioral response. Gray boxes of increasing shading refer to the different stages in neural circuit relay that occur between sensory input and execution of a behavioral response as motor output. The ↔ symbol labels competition between responses at the level of behavioral output.

Figure 2: Intersecting Pathway Model for Competing Behavioral Responses

In the *intersecting pathway model* for the selection of contradictory behavioral responses, input signals for both the avoidance and attraction responses enter into respective neural circuits. The neurons relaying information from both preference pathways then physically

interact at the neuroanatomical level within the brain, such that direct interaction between the signals from the competing preference pathways is possible. This interaction can be as simple a cross-inhibition between the two neural circuits (i.e. detection of avoidance input causes a synaptic inhibition of neurons in the attractive response pathway), or as complex as simultaneous comparison of opposing inputs in a common neurons (i.e. sensory neurons converge on a single neuron where intracellular evaluation and transformation of both signal input occurs). Regardless of the complexity of the neural wiring, an important aspect of this model is that a behavioral response is generated after intersecting neural circuitry evaluates one input stimuli while receiving signal information from the competing pathway. Subsequently, a combinatorial behavioral response is selected *before* motor output is executed, as demonstrated by the purple lines/labels in Figure 2. Blue lines/labels refer to the avoidance behavioral response. Red lines/labels refer to the attraction behavioral response. Purple lines/labels refer to the combined output response generated after neural circuitry and input signals from opposing preference pathways interact. Gray boxes of increasing shading refer to the different stages in neural circuit relay that occur between sensory input and execution of a behavioral response as motor output.

Figure 3: Central Integration Model for Competing Behavioral Responses

In the *central integration model* for the selection of contradictory behavioral responses, input signals for both the avoidance and attraction responses enter into respective neural circuits. Information is then sent through relay neurons that converge on higher order brain structures capable of integrating signals and performing complex evaluation processes. In these integration centers, signals from both pathways are likely evaluated and ranked for their

potential reward and/or punishment properties in the *valuation* step of decision-making. After directly comparing the alternative responses, one response is *selected* by the integration center for execution as behavioral motor output. In this model, the convergence of competing signals in brain structures capable of complex cognitive processes provides the strongest neurophysiological argument that the organism is indeed performing decision-making. Blue lines/labels refer to the avoidance behavioral response. Red lines/labels refer to the attraction behavioral response. Purple lines/labels refer to the combined output response generated after neural circuitry and input signals from opposing preference pathways interact. Gray boxes of increasing shading refer to the different stages in neural circuit relay that occur between sensory input and execution of a behavioral response as motor output.

Figure 1: Parallel Pathway Model for Competing Behavioral Responses

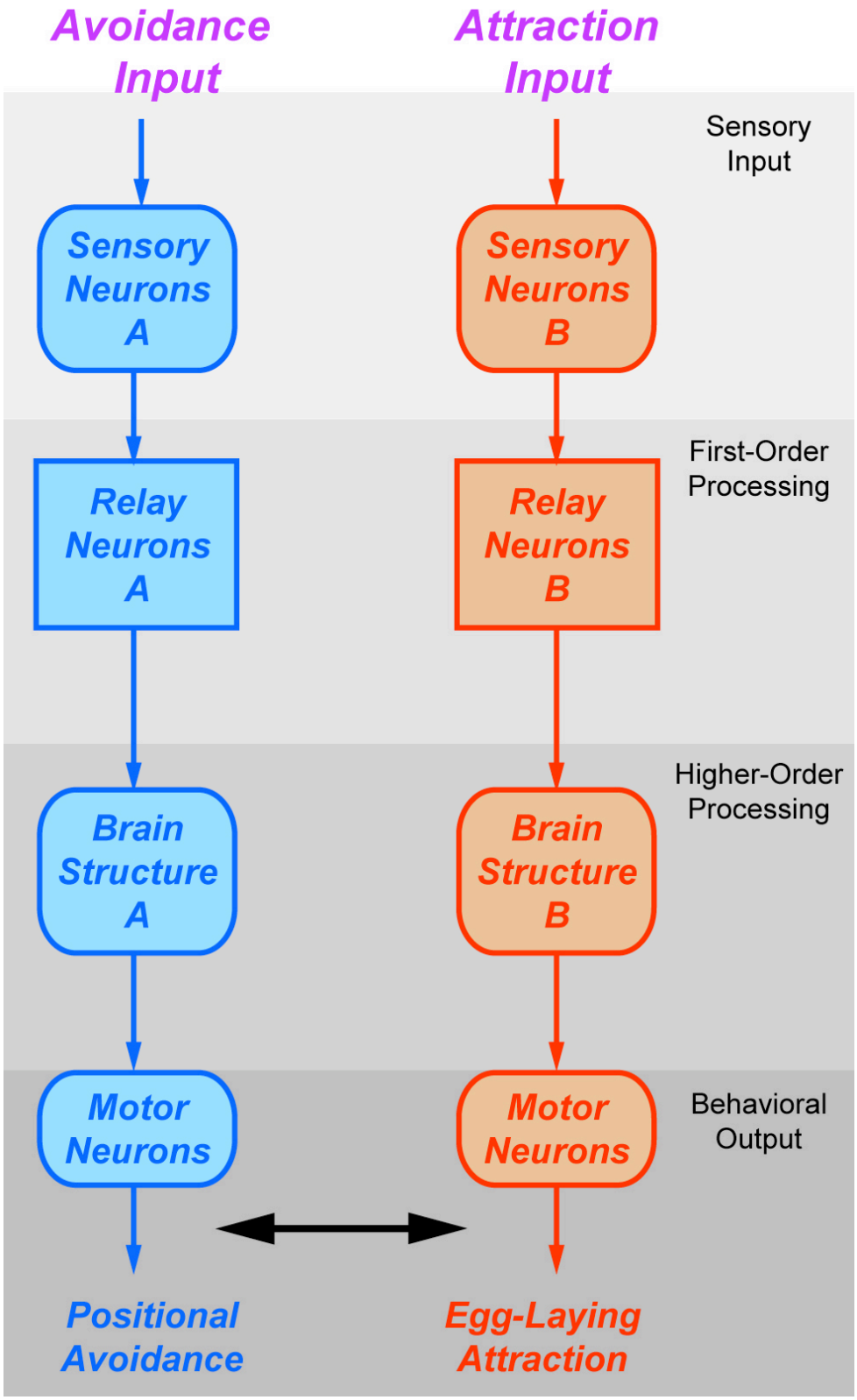


Figure 2: Intersecting Pathway Model for Competing Behavioral Responses

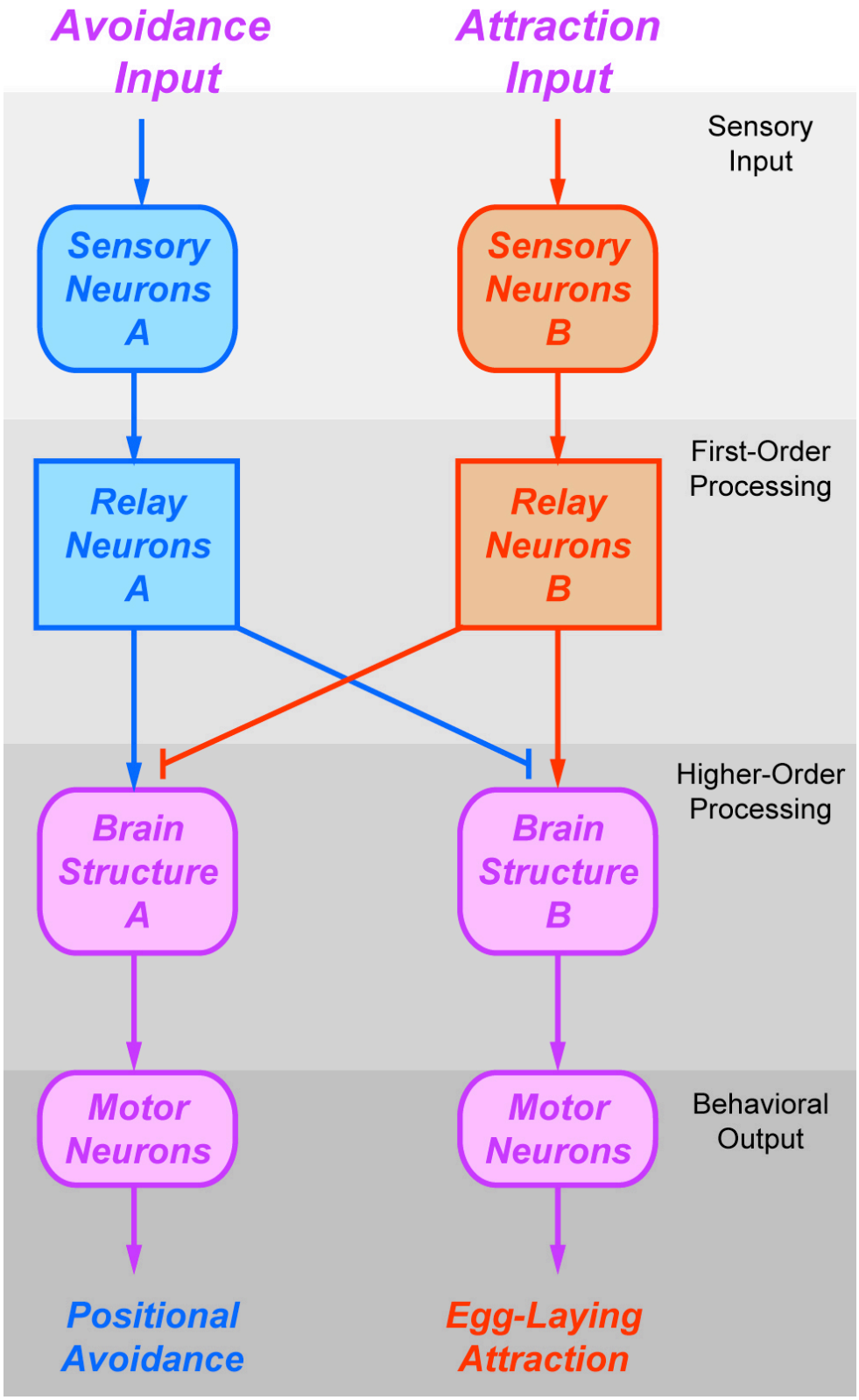
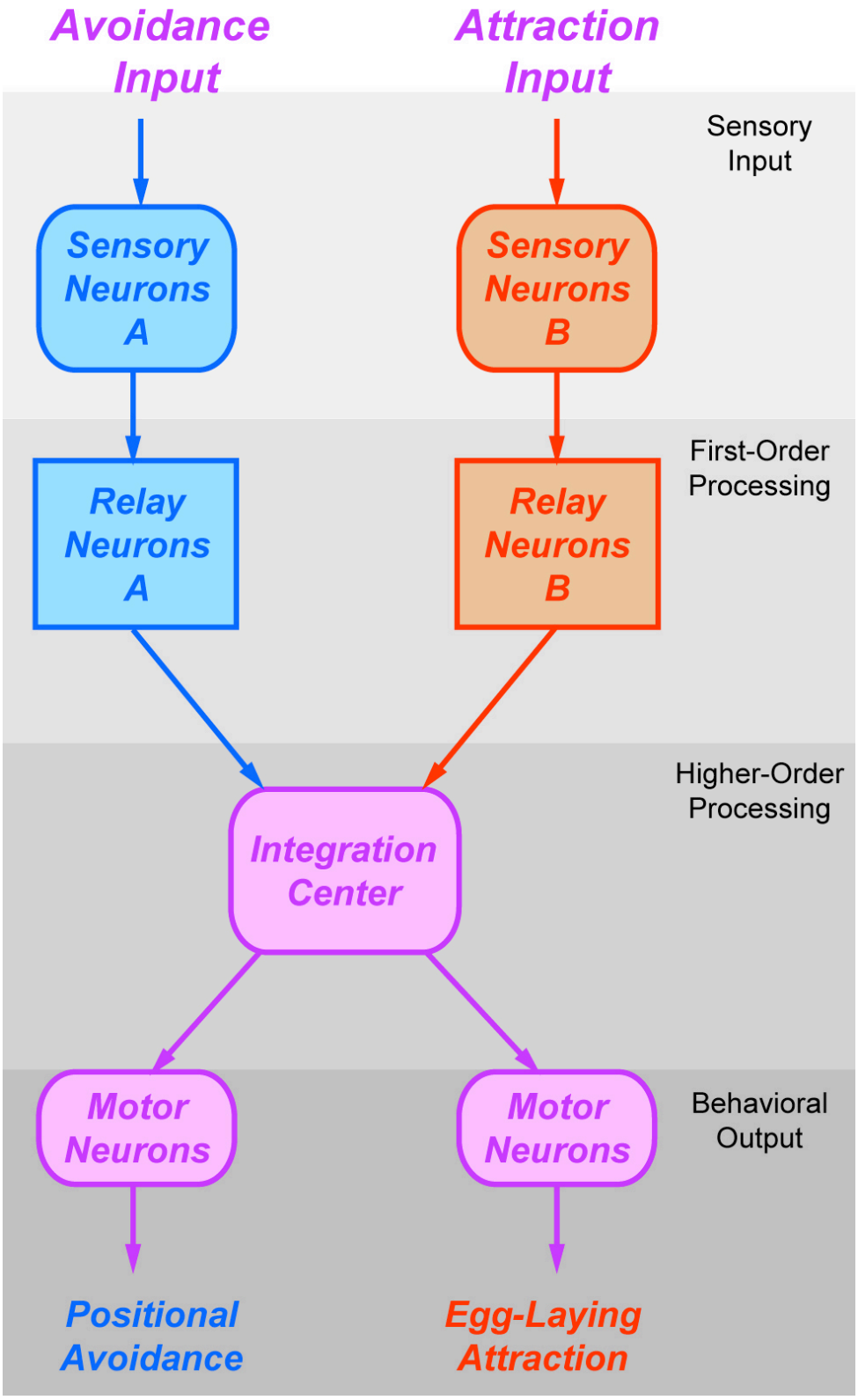


Figure 3: Central Integration Model for Competing Behavioral Responses



Chapter 2

Oviposition Preference For and Positional Avoidance of Acetic Acid Provide a Model for Competing Behavioral Drives in *Drosophila*

Ryan M. Joseph, Anita V. Devineni, Ian F.G. King

ABSTRACT

Selection of appropriate oviposition sites is essential for progeny survival and fitness in generalist insect species, such as *Drosophila melanogaster*, yet little is known about the mechanisms regulating how environmental conditions and innate adult preferences are evaluated and balanced to yield the final substrate choice for egg-deposition. Female *Drosophila melanogaster* are attracted to food containing acetic acid (AA) as an oviposition substrate. However, our observations reveal that this egg-laying preference is a complex process, as it directly opposes an otherwise strong, default behavior of positional avoidance for the same food. We show that two distinct sensory modalities detect AA. Attraction to AA-containing food for the purpose of egg-laying relies on the gustatory system, while positional repulsion depends primarily on the olfactory system. Similarly, distinct central brain regions are involved in AA attraction and repulsion. Given this unique situation, in which a single environmental stimulus yields two opposing behavioral outputs, we propose that the interaction of egg-laying attraction and positional aversion for AA provides a powerful model for studying how organisms balance competing behavioral drives and integrate signals involved in choice-like and decision-making processes.

INTRODUCTION

Oviposition provides a powerful yet simple means for monitoring preference behavior in *Drosophila melanogaster*, since a laid egg represents a marker for female position. Past studies have used egg-laying as a readout for conditions advantageous to progeny development (Richmond and Gerking, 1979; Chess and Ringo, 1985), in which oviposition preference effectively separates larva of different sibling species of *Drosophila.* Egg-laying

has also been used to detect aversion towards compounds toxic to both larvae and adults (Possidente et al., 1999; Moreteau et al., 1994). Furthermore, numerous studies have employed patterns of oviposition to distinguish subtle differences in host plant preferences, which have provided insights into resource requirements and ecological behaviors of different *Drosophila* species (Amlou et al., 1998; Barker et al., 1994).

Despite numerous studies utilizing oviposition site selection as a behavioral readout, direct study of the relevant sensory circuits and the actual oviposition program itself have been initiated only recently in *Drosophila melanogaster* (Matsuo et al, 2007; Yang et al., 2008). To investigate the genetic mechanisms and neural circuits regulating this important behavioral choice in *Drosophila*, we developed a simple yet robust two-choice assay that utilizes acetic acid (AA), a naturally occurring product of fruit fermentation, as an egg-laying attractant (Laudien et al., 1977; Eisses, 1997). However, in addition to verifying a strong egg–laying preference for AA, we surprisingly observed *Drosophila* show a strong positional aversion to the same AA-containing food. We demonstrate that when sampling for oviposition sites, females integrate input from distinct sensory modalities to evaluate and choose a particular behavioral output from two competing options: egg-laying attraction for AA and positional repulsion to AA. Egg-laying preference is primarily relayed through gustatory neurons, while positional aversion is relayed through the olfactory system. We also map some of the central brain regions mediating these competing behaviors. Taken together, the process by which females integrate sensory information to execute these competing and interacting behavioral drives provides a tractable model for studying choice-like behavior in *Drosophila melanogaster*.

RESULTS

Note: Ian F.G. King designed and perfected the novel feeding assay described in Chapter 2, and wrote the protocol that was subsequently published with the results detailed in this study. Anita V. Devineni conducted the initial *GAL4* neuroanatomy screen that isolated mushroom body and ellipsoid body lines important to egg-laying and positional preferences to AA, respectively, and aided in imaging of the *5-98*^{GAL4} line; Ryan M. Joseph performed subsequent characterization of behavioral responses associated with these brain regions. Unless otherwise stated, Ryan M. Joseph conducted all other experiments and analysis.

Egg–laying preference for and positional aversion to AA-containing food

To investigate the mechanisms involved in *D. melanogaster* egg-laying preference, we devised a simple apparatus in which females are allowed the choice to lay eggs on regular food or food containing various concentrations of AA (Figure 1A). Similar to previous observations (Laudien and Iken, 1977; Eisses, 1997), mated females laid approximately 91% of their eggs on food containing 5% AA (Figure 1B, D; +AA) as compared to regular food (Figure 1B, D; –AA), with an oviposition index (OI) of +0.82. It has been postulated that *D. melanogaster* may utilize AA as an energy source (Parsons, 1982), such that oviposition preference would result from an attraction to AA-containing media as a feeding source. To test this hypothesis, we first observed the physical location of flies during the 3–h oviposition assay. Surprisingly, females avoided food containing 5% AA (the concentration found naturally in vinegar), with a position index (PI) of –0.33 (Figure 1B, E). To test for feeding preferences, we employed a modified two–choice assay in which different food dyes were mixed into the halves of the dish. After a sampling period, gut contents were analyzed by thin

layer chromatography to quantify the relative ingestion of each dye. Flies ingested essentially equal amounts of food containing or lacking AA (Figure 1C). Thus, oviposition site selection does not reflect innate positional or feeding preferences, and may be in direct conflict with positional preference under ecologically relevant conditions. Recent studies show similar decoupling between adult taste and egg-laying preferences (Matsuo et al., 2007; Harada et al., 2008).

Interestingly, positional repulsion for AA-containing food was stronger in virgin females and males (Figure 1B). Since virgin females lay fewer eggs than mated females (Figure S1A), they likely search for egg-laying substrates less frequently, and may therefore have less incentive to overcome their innate positional aversion to AA-containing food. Males explore AA-containing food even less frequently than virgin females. Thus, the positional aversion to AA grows as the need to lay eggs is either diminished in virgin females or eliminated in males, implying that the attractive oviposition and repulsive positional drives are in competition. However, mated and virgin females showed equivalently high OI values in response to AA-containing food (Figure 1B). These results suggest that attraction to AA as an oviposition substrate is an innate preference that is not affected by post–mating behavioral modifications (Kubli, 2003).

To determine if oviposition preference and positional aversion were specific to AA or elicited by the acidity of AA-containing food, we analyzed these behaviors on foods containing acetic, hydrochloric (HCl), or sulfuric (H_2SO_4) acids, titrated to equivalent pH values. At pH 3.5 (5% AA), females showed negligible oviposition preference for foods with HCl or H_2SO_4, while preference for AA-containing food was high (Figure 2A). Likewise, the positional aversion observed with 5% AA was eliminated when food was acidified with HCl

or H_2SO_4 (Figure 2A); similar positional responses were observed in males (Figure S2). Additionally, HCl and H_2SO_4 did not suppress egg-laying (Figure 2B). Thus, egg–laying preference for 5% AA cannot be solely explained by the food's acidity at this pH.

However, with regard to egg-laying preference, when acidity was increased even further, foods with HCl or H_2SO_4 became attractive, while AA became aversive (Figure 2C and S3A). This aversion to lay eggs on higher concentrations of AA was accompanied with increased positional repulsion (Figure 2D and S3B), suggesting that repulsion overrides the attraction to lay eggs on higher AA concentrations. Positional repulsion appeared to be specific for high AA concentrations, rather than for the increased acidity of the food, as flies showed no positional aversion to HCl or H_2SO_4 at lower pH values (Figure 2D). Thus, females show a specific attraction for AA as an oviposition substrate that cannot be explained by increased food acidity. Moreover, these data show that egg-laying and positional preferences are in competition when tested with AA, but not other acids, such that decreases in oviposition preference are accompanied with increases in positional aversion.

To explore the idea that the choice of egg–laying substrate reflects an active sampling and evaluation process, rather than a simple reflex, we assayed flies in additional experimental contexts. When tested in a "stripe assay" (Figure S4A), in which females sequentially encounter alternating segments of control food and food with increasing concentrations of AA (see Suppl. Methods), they showed a high preference for 5% AA, despite sampling the 0.25% AA stripe first. Thus, flies explored their environment prior to selecting a preferred oviposition site. Further evidence for exploration of AA-containing food is shown using single–fly locomotor traces (Figure S4B).

The olfactory system mediates positional aversion to AA

Although the sensory inputs and genetic pathways involved in *D. melanogaster* oviposition preference are relatively uncharacterized, the role of taste and olfaction in egg–laying of other insects has been investigated (Richmond and Gerking, 1979; Barker, et al., 1994; Higa and Fuyama, 1993; Mitchell et al., 1999). In addition, AA can be aversive to *D. melanogaster* in certain olfactory assays (Amlou et al., 1998; Fuyama, 1976). We therefore analyzed the behavior of flies with impaired or enhanced olfaction. To impair olfaction, we surgically removed the primary olfactory organs, the third antennal segments (de Bruyne et al., 2001; Keller and Vosshall, 2007). Antennaectomized females, while normal for egg–laying preference (Figure 3A), lost their positional aversion to 5% AA (Figure 3B). Thus, olfaction is essential for positional aversion to AA, but is not for oviposition preference. Consistent with these data is our observation that silencing of antennal projection neurons disrupted positional aversion (Table S2). Males lacking antennae also showed diminished aversion to 5% AA (Figure S5C).

To analyze the effect of enhanced olfactory input, we tested mutant flies with an increased sense of smell and wild–type flies exposed to higher AA concentrations. Mutations in *white rabbit* (*whir*) show an elevated olfactory startle response to ethanol and other odorants (Rothenfluh et al., 2006) and are suspected to possess an enhanced sense of smell. Consistent with this hypothesis, positional aversion to AA was increased in $whir^{1}$ females, an effect that was significantly diminished by antennal removal (Figure 3B). Furthermore, the increased positional repulsion exhibited by $whir^{1}$ females was accompanied by egg–laying aversion for AA-containing food; this effect was also strongly ameliorated by antennaectomy (Figure 3A). Similarly, removing the antenna of $whir^{1}$ males reduced their excessive

positional repulsion to AA (Figure S5C). We next tested responses to a high concentration (10% AA), which normally eliminates oviposition preference (Figure S5A) and enhances positional aversion (Figure S5B). Removing antennae restored egg–laying preference to nearly normal levels (Figure S5A) and normalized positional aversion (Figure S5B). Positional aversion was not completely eliminated in antenaectomized *whir*^{1} females and wild–type flies exposed to 10% AA (Figure 3B and S5B-C), suggesting that either olfactory neurons on the maxillary palps or other sensory modalities are engaged at high AA concentrations. Despite this caveat, our data show the olfactory neurons in the third antennal segment produce the primary sensory input that induces positional aversion to AA.

To further show that oviposition and positional preferences are competing drives, we asked if reduced olfactory input would increase egg–laying preference for AA. Since oviposition preference approaches saturation at 5% AA (Figure 3A and S3A), an increase in OI would be concealed by a "ceiling effect". We therefore analyzed responses to 0.25% AA, a concentration that yielded a moderately attractive egg–laying response and no positional avoidance (OI=+0.34, PI=-0.03; Figure S5D). Antennaectomized females exhibited increased egg–laying preference and a small but significant shift to a more positive positional preference (OI=+0.55, PI=+0.10; Figure S5D). Thus, even low AA concentrations are detected by the olfactory system and perceived as slightly repulsive, and further support the hypothesis that olfactory-based aversion competes with egg–laying attraction for AA.

The gustatory system mediates oviposition attraction to AA

Our data indicated that a sensory modality other than olfaction mediates egg–laying preference; a likely candidate was the gustatory system. Gustatory bristles are present on the

primary taste structures: the labellum, front legs, wing margins and the ovipositor (Stocker, 1994). To test if gustatory neurons mediate egg–laying preference, we assayed *pox-neuro* (*poxn*) mutants, in which taste bristles are transformed into mechanosensory bristles that lack gustatory receptors (Awasaki and Kimura, 1997; Clyne et al., 2000); null mutants also have defects in the central nervous system (Boll and Noll, 2002). Homozygous $poxn^{\Delta M22\text{-}B5}$ females showed reduced egg–laying preference (OI=+0.28; Figure 4A) when compared to wild–type, $poxn^{\Delta M22\text{-}B5}$ heterozygous, and $poxn^{\Delta M22\text{-}B5}$ homozygous flies carrying the *SuperA* transgenic construct (Boll and Noll, 2002) that rescues all *poxn* defects. These data implicate taste receptors in the egg-laying attraction for AA. However, positional AA aversion was also reduced in homozygous $poxn^{\Delta M22\text{-}B5}$ females (PI=-0.09; Figure 4B), likely due to abnormalities in olfactory processing centers in the mutant (Boll and Noll, 2002).

To eliminate issues related to abnormal olfactory brain centers, we tested transgenic strains in which *poxn* expression was restored in a tissue–specific manner. The *full-1* and *-152* transgenes restore normal brain morphology and chemosensory bristles to $poxn^{\Delta M22\text{-}B5}$ flies, with the exception of taste organs found on the labellum (Boll and Noll, 2002). $poxn^{\Delta M22\text{-}B5}$ females carrying the *full-1* or *full-152* transgene showed diminished AA egg-laying preference (OI=+0.12, +0.23, respectively; Figure 4A), but still maintained a robust positional aversion to 5% AA (PI=-0.47, -0.42, respectively; Figure 4B). In fact, positional aversion to 5% AA was enhanced when compared to control strains. To confirm that gustatory and not olfactory pathways mediate egg–laying responses to AA, we removed the third antennal segments from the *poxn*-rescue lines. As expected, antennaectomized flies showed reduced positional aversion to AA, while oviposition indices remained similar to their baseline values (Figure 4). Overall, these data show that females use taste neurons on

the labellum to recognize AA as an egg-laying attractant, and that reduced egg–laying preference leads to a compensatory increase in positional repulsion.

Brain centers involved in egg-laying and positional preferences for AA

Thus far, our data has identified peripheral sensory systems that induce egg–laying and positional responses to AA, and shown that behavioral outputs of the two preference pathways are in competition. To identify higher–order brain regions that mediate and integrate signals from these competing pathways, we silenced specific neuronal populations by expressing a temperature-sensitive *Shibire* transgene, *UAS-Shi^{ts}* (Kitamoto, 2001), under the control of various GAL4 lines. 58 GAL4–expressing lines were crossed to *UAS-Shi^{ts}*, and their progeny were assayed for egg–laying and positional preferences at the permissive (23°C) and restrictive (30°C) temperatures (Table S2).

Three GAL4 lines with highly selective expression in the mushroom body (MB) lost egg–laying preference for 5% AA. Two representative lines, *GAL4$^{5\text{-}120}$* and *GAL4$^{5\text{-}98}$* showed strongly reduced oviposition preference at 30°C in the presence of *UAS-Shi^{ts}* (Figure 5A and S6A). Meanwhile, positional aversion to 5% AA was unaffected in experimental and control flies (Figure 5A and S6A), providing the first evidence for dissociation between the competing behavioral choices towards AA. Expression of GAL4 in both the *GAL4$^{5\text{-}120}$* and *GAL4$^{5\text{-}98}$* lines, visualized with a *UAS-GFP* transgene (Marin et al., 2002), was preferentially found in the MB, some lateral neurons (LNs), and a few scattered cells in the brain (Figs. 5B and S6B). Assays conducted with *pdf-GAL4/UAS-Shi^{ts}* flies, which express GAL4 specifically in LNs, did not affect egg–laying or positional responses (Figure S7). Furthermore, we did not detect GFP expression in olfactory and gustatory neurons of *GAL4$^{5\text{-}}$*

^{120} and *GAL4*$^{5-98}$ lines (Ryan M. Joseph, unpublished observations), excluding the possibility that the observed phenotypes were due to silencing of sensory systems.

We also identified four lines with highly specific expression in the ellipsoid body (EB) ring neurons that exhibited disrupted positional aversion to 5% AA. Two representative lines, *GAL4*$^{4-67}$ and *GAL4*$^{2-72}$ showed reductions in positional aversion to 5% AA in the presence of *UAS-Shi*^{ts} at 30°C (Figure 5C and S6C) when compared to the singly transgenic controls. Egg–laying preference in the experimental flies was essentially unchanged (Figs. 5C and S6C). *GAL4*$^{4-67}$ and *GAL4*$^{2-72}$ lines express GAL4 primarily in the cell bodies of the EB ring neurons, (Figs. 5D and S6D); peripheral sensory structures revealed no GFP expression (Ryan M. Joseph, unpublished observations). Of note, females showed increased positional aversion to 5% AA at 30ºC (Figure 5, S6, 7), likely due to enhanced olfactory input caused by higher volatility of AA at 30°C; this effect was consistent across all genotypes and thus did not confound data interpretation. *GAL4*$^{4-67}$ and *GAL4*$^{2-72}$ also showed disrupted positional aversion in the presence of *UAS-Shi*^{ts} at 23ºC (Figs. 5C and S6C), an effect likely caused by residual function of the *UAS-Shi*^{ts} transgene in neurons that are particularly sensitive to synaptic silencing (Kimura et al., 2008). We were unable to determine if the disruption in positional aversion seen upon silencing EB neurons was associated with an increase in egg–laying preference, as the latter was nearly maximal at 5% AA. Attempts to carry out these tests at lower AA concentrations were unsuccessful, as positional responses were too subtle for definitive conclusions.

To further investigate if the MB and EB function in separate or interconnected pathways, we simultaneously silenced both regions in "double-GAL4" flies carrying *GAL4*$^{5-120}$, *GAL4*$^{4-67}$ and *UAS-Shi*^{ts}. Cross-talk between the two circuits could manifest as non-

additive (synergistic or epistatic) effects on the behavioral choices. Compared to the respective single *GAL4/UAS-Shi^{ts}* lines, double-GAL4 females showed disruptions of oviposition and positional preference that were essentially the sum of those seen with the individual GAL4 lines (Figure S8), which suggests the MB and EB function in largely separate pathways to affect egg-laying attraction and positional repulsion to 5% AA, respectively.

DISCUSSION

Our data provide a neurobehavioral model in which AA, a single ecologically-relevant input, is detected by separate sensory systems to generate two distinct behavioral outputs: gustatory–based egg–laying attraction and olfactory–based positional repulsion (Figure 6). We postulate *D. melanogaster* has an innate positional repulsion to the smell of AA. However, when needing to lay eggs, the attraction for AA overrides this positional repulsion, thereby allowing females to deposit their eggs on AA-containing food. Other studies have revealed opposing behavioral responses to a single compound; when detected as carbonation by the gustatory system, CO_2 is attractive (Fischler et al., 2007), but when detected as an odorant, it is aversive (Suh et al., 2004). However, our experimental setup is unique in that opposing behavioral responses to a single stimulus (AA) are concurrently induced and assayed, affording direct observation of the competition between the two behavioral drives. Thus, we are able to resolve compensatory shifts between the behavioral responses, in which increasing or decreasing sensory input for one pathway causes an inversely correlated change in the behavioral output of the competing preference pathway.

Several models can be invoked to explain the data surrounding these competing

drives. In one extreme model, information gathered by the olfactory and gustatory systems would interact and be processed in common neurons inside the brain, where concurrent evaluation of sensory input from both pathways would result in the selection of either repulsion or attraction before a final motor program for each behavior is executed (Figure 6, #1). This model requires that neurons within the brain, particularly in centers involved in complex processing, simultaneously integrate sensory inputs to drive either egg–laying or positional behaviors. In the alternative extreme model, gustatory and olfactory signals would be independently processed by parallel neural circuits, such that attraction and repulsion only compete at the behavioral output level, after motor-program selection, since a female fly can only be in one place at a given time (Figure 6, #2). Combinatorial models invoking both central integration and competition of behavioral outputs are also possible (Figure 7, #3-5).

Models that involve competition through central integration, as described by the *central integration model* in Chapter 1 of this dissertation, imply that signals converge on common neurons in higher brain centers, and therefore silencing these neurons would be expected to disrupt both egg–laying attraction and positional repulsion. In our limited *UAS-Shi*^{ts} screen, we did not identify such a region. However, we did find higher–order structures that regulate each individual preference pathway. The MB appears to mediate taste–based attraction to AA for egg–laying purposes. Given the role of the MB in olfactory learning and memory (Zars, 2000; Heisenberg, 2003), it was surprising that it regulates taste–based behavior in our paradigm. However, a neural connection between the subesophageal ganglion (SOG), which receives gustatory input, and the MB has been described recently in honeybees (Schroter and Menzel, 2003), suggesting that there is an as yet undiscovered neuroanatomical link between the SOG and MB. Meanwhile, the EB (likely the R1 and R4

ring neurons) plays a role in the olfactory–based positional repulsion to AA. Our data is consistent with studies showing the EB plays a role in olfactory-related tasks (Heisenberg, 2003; Schroter and Menzel, 2003) and spatial memory (Neuser et al., 2003).

Exactly how and where the MB and EB function in the neural circuits that regulate AA responses remains to be determined. However, the results obtained with MB and/or EB silencing allow us to draw important conclusions regarding the models presented in Figure 6. Experiments with *poxn* flies showed that abrogating gustatory input upstream of potential central integration in the brain not only impaired egg-laying preference for AA, but also caused a concomitant enhancement of positional aversion. In contrast, synaptic silencing of the MB, while also causing a robust decrease of egg-laying preference, did not result in a compensatory increase in positional aversion, suggesting that competition between mutually exclusive responses does not occur solely at the level of behavioral output. In other words, the degree of positional aversion exhibited by the female may have already been "set" before the execution of motor output, and therefore altering the level of attractive behavioral output therefore does not change the level of positional aversion that the female exhibits.

These data argue against the *parallel pathway model* for competition of behavioral outputs, and support the *intersecting pathways model*, in which some cross-talk and/or intersection between olfactory and gustatory inputs must occur within the brain, as described in Chapter 1 of this dissertation. Furthermore, these results suggests that when relaying AA-based signal input, the MB functions downstream of such cross-talk and/or intersection, after a positional response has been chosen, as its silencing affects only the motor program involved in egg-laying preference, without altering the magnitude of positional aversion. With regards to the EB, our double–GAL4 experiments (Figure S7) revealed an additive

effect, leading us to hypothesize that the EB functions in parallel to the MB to control the motor program involved in positional aversion. Thus, potential cross-talk between the two circuits could also occur upstream from the EB.

Our data clearly show that disrupting peripheral sensory input causes compensatory shifts in egg–laying attraction and positional aversion (Figure 3, 4, S5). Thus, despite evidence for central integration, competition between behavioral outputs also contributes to the overall response of flies when choosing a substrate for oviposition. Such competition arises from a logistical issue; flies lay equal numbers of eggs on regular food or food supplemented with 5% AA when not given a choice, but when provided with the choice of both oviposition substrates, they lay approximately 90% of their eggs on AA-containing food. Since laying an egg takes time (Yang et al., 2008), and females cannot be in two places at the same time, the OI and PI values must be at least partially correlated. Thus, our data supports a combinatorial model that invokes both the *central integration model* and *parallel pathway models*, where both central integration and competition of behavioral outputs mediate the choice-like behavior elicited when females encounter different oviposition substrate options (Figure 6, #3).

Of note , although the reverse-genetic screen for neural circuitry important to egg-laying attraction and positional aversion responses to 5% AA revealed numerous brain regions important to each response (Table S1), we still did not identify a discrete brain region responsible for *both* behaviors, and subsequently cannot draw direct conclusions regarding possible integration centers that perform the *valuation* or *selection* steps of a decision-making process between positional aversion and egg-laying attraction responses. Although we have identified two mutually exclusive behavioral pathways that directly competed, the possibility

remained that a common integration center might be difficult to find using AA in our candidate neuroanatomy screen, since the competing pathways proceed through pathways governed by different sensory modalities: olfaction and gustation. As such, there may be too much non-overlapping neural circuitry to cause a complete disruption in both the smell-based positional aversion and taste-based egg-laying attraction responses, even if a central integration center was in fact knocked out by our silencing techniques. We postulate that, if we can identify competing responses that are induced by sensory circuits and relay neurons within a single sensory modality—such as gustation—then there would be a higher likelihood that the neural circuits of the competing preference pathways converge in common centers within the brain. Subsequently, there would be a higher possibility that silencing these common centers could simultaneously disrupt *both* the positional aversion and egg-laying attraction responses, thereby implicating these brain regions as possible decision-making centers. Testing of this hypothesis using a representative bitter-tasting compound, lobeline, is described in Chapter 4 of this dissertation.

Taken together, our results allow us to suggest that our paradigm can be employed as a simple model for choice-like behavior in *D. melanogaster*. Supporting this possibility, a recent study by Yang et al., 2008 employs a different setup as a readout for simple decision-making, in which females use their gustatory system to evaluate bitter and sweet egg-laying substrates. Our model differs in that it uses a single compound to stimulate competing drives via two distinct sensory modalities. Both systems provide powerful new paradigms to study the molecular and neural bases of simple decision-making in *D. melanogaster*.

MATERIALS AND METHODS

Fly Stocks

Behavioral analysis, the genetic screen for egg-laying preference mutants, and *white rabbit* (*whir*^{1}) experiments were performed in *w*^{1118} *Berlin* genetic background. *poxn*$^{\Delta M22\text{-}B5}$ deficiency (*poxn*$^{\Delta M22\text{-}B5\text{-}/\text{-}}$) and *SuperA*^{158}, *full-1*, and *full-152* transgenic lines were a generous gift from W. Boll and M. Noll (Boll and Noll, 2002). The *poxn*$^{\Delta M22\text{-}B5}$ lines used were a mixed *w Berlin* background, in which flies contained the original *poxn*$^{\Delta M22\text{-}B5}$ second chromosome, but all other chromosomes were from the *w*^{1118} *Berlin* strain. Comparisons between *w Berlin*, *poxn*$^{\Delta M22\text{-}B5}$ heterozygotes, and *poxn*$^{\Delta M22\text{-}B5}$ homozygotes carrying the *SuperA* transgene revealed no statistical differences between these control lines for either oviposition or positional behaviors, with or without antenna (+ant/-ant, Figure 5; $P>0.05$; one-way ANOVA; $n\geq12$), demonstrating that the mixed genetic background for the second chromosome has minimal effects on oviposition and position preference behaviors.

UAS-Shibire^{ts} transgenic flies employed in our neuronal silencing experiments (Figure 6, 7, S5, S6) contain two insertions of the transgene in a *w*^{1118} *Canton S* background (Kitamoto, 2001). *w Berlin, w Berlin/UAS-Shibire*^{ts}, and *w Berlin/GAL4* (in *w Berlin* background), exhibited similar behaviors at both 23°C and 30°C, Occasionally, the *w Berlin/UAS-shibire*^{ts} control would trend towards slightly less repulsion. However, when observed, this effect was sufficiently small to not confound interpretation of the data. Unless otherwise noted, flies were reared in constant light at 25°C and 70% humidity on cornmeal/molasses/yeast fly food.

Protocol for two-choice assay of egg-laying and positional preference

The apparatus to simultaneously assay egg-laying and position was assembled as follows. The base of plastic 6-ounce round bottom stock bottles (E & K Scientific, Santa Clara, CA) was cut off using a razor blade. A transparent 60-mm Petri dish lid was inserted into the removed portion of the bottle and secured with double-sided tape, thereby allowing fly position to be easily ascertained during the assay. Food-substrates were made by mixing the appropriate volume of experimental compound (AA, HCl, H_2SO_4 or H_2O) into molten fly food (standard cornmeal, molasses, agar medium), at temperatures below the boiling point of the particular experimental compound being tested. Two-choice dishes were made by dividing a 35-mm Petri dish (Becton Dickinson Labware, Franklin Lakes NJ) lid with a razor blade, and pouring two samples of food-substrate into each half. Each dish was therefore divided into two halves with: (i) an experimental sample with fly food mixed with a candidate compound, and (ii) a control sample with fly food mixed with an equivalent volume of distilled water, to control for changes in texture associated with dilution of the food. The razor blade was removed after the food hardened (approximately 10-15 min).

For each test, 15-20 recently-eclosed females were collected and allowed to mate for 2-3 days before being assayed. Flies were gently knocked into a bottle without anesthesia to eliminate $C0_2$-based behavioral artifacts. The bottle was then capped with the 35mm two-choice dish and inverted, such that the two-choice dish was at the bottom of the apparatus. Flies were allowed to sample for 3-h. To determine oviposition preference, the amount of eggs on each half of the two-choice dish was counted, and a preference index, or oviposition index (OI) for that particular compound was calculated [OI = (# eggs laid on experimental food - # eggs laid on control food) / # total eggs laid]. For positional preference, the number

of flies on each half of the dish was counted at 15-min intervals for 3 h. Values were totaled and a position index (PI) for the three hour time period was calculated [PI = (flies on experimental food flies on control food) / (flies on experimental food + flies on control food). OIs and PIs did not significantly change throughout the 3-hour experiment. It should be noted that in our assay flies are not constrained to be physically on the food, as they can climb the walls of the observation chamber (Figure 1A). We therefore always monitored the fraction of flies on the dish, and subsequently scored only females engaged in behaviors during which they are in direct contact with the food (which comprised roughly 60% of the females' time; Figure S1B). Thus, our indices measure preference only when flies are actively receiving close–range sensory inputs from the food. It is interesting to note that when not given a choice, females laid essentially the same number of eggs on regular food and food containing 5% AA (Figure 2B). The strongly positive OIs for AA are therefore not simply due to an AA-induced increase in egg–laying, but rather, an active choice female flies appear to make for a preferred substrate.

Protocol for two-choice feeding assay

To simultaneously assay egg-laying and feeding preferences, the food mixing protocol was modified such that either Erioglaucine (FD&C Blue #1) or Fast Green FCF dye (Green #3) (Sigma-Aldrich, St. Louis MO) were mixed into the experimental 5% acetic acid (AA) or 5% H2O food. The following two-choice dishes were made: (*i*) -AA/-AA in which flies selected between 5% H_2O + 0.4% Blue and 5% H_2O + 0.4% Green; (*ii*) +AA/-AA in which flies selected between 5% H_2O + 0.4% Blue and 5% AA + 0.4% Green; (*iii*) -AA/+AA in which flies selected between 5% AA + 0.4% Blue and 5% H_2O + 0.4%

Green; (*iv*) no-choice control dishes with 0.4% Blue dye + 0.4% Green dye. 30 mated females were allowed to sample and feed for 4 h, after which they were frozen. Flies were then homogenized in ethanol and centrifuged to remove insoluble material. Dyes in the supernatant were separated by Thin Layer Chromatography (TLC) using 0.1M NaCl, 20% methanol solution on silica gel TLC plates (Selecto Scientific, Suwanee, GA). An Epson 2400 Photo Scanner (Epson America Inc., Long Beach CA) was used to digitally image the TLC plates, and the optical densities (OD) of each dye-spot were determined using ImageJ (version 1.38x; http://rsb.info.nih.gov/ij/). OD values were then converted into Blue OD ratios (Blue OD/Total OD) and Green OD ratios (Green OD/Total OD) for preference comparisons.

Blue #1 and Green #3 dyes migrate and adhere on silica plates differently. To normalize for differences in OD attributed solely to dye properties, the gut contents of flies given two-choice experimental dishes were concurrently analyzed with gut contents from flies given no-choice control dishes (*iv:* 0.4% Blue + 0.4% Green in both halves of the dish). To calculate appropriate correction factors, OD ratios for no-choice dye-spots were obtained as described above. The correction factors x and y were then determined by solving x(no-choice Green OD ratio) = y(no-choice Blue OD ratio). Subsequently, feeding preferences for the two-choice experiments were measured as a feeding index (FI). FI = [x(Green-AA OD ratio) – y(Blue-AA ratio)] for Green-AA vs. Blue+AA experiments and Green-AA vs. Blue-AA controls. FI = [y(Blue+AA OD ratio) – x(Green-AA ratio)] for Blue-AA vs. Green-AA experiments.

Titrating food-substrate to equivalent pH values using different acids

Fly food is a heterogeneous mixture of ingredients, and subsequently has a complex buffering capacity. Therefore, we conducted acid titrations and empirically measured the concentrations of acetic acid (AA), hydrochloric acid (HCl), and sulfuric acid (H_2SO_4) that yielded food-substrate mixtures with equivalent pH values between 2.0 and 4.5. Appropriate amounts of distilled water were added to the control side of the dish to equalize the dilution of the fly food between samples, controlling for volumetric changes in food softness and texture. The pH of the food surface was measured using colorpHast pH indicator strips (EMD Chemicals, Gibbstown, NJ), once the food cooled. pH values were estimated within the nearest half-unit. To verify our direct measurement estimates, hardened food was re-heated, diluted in distilled water (1:10 dilution factor), and the pH of the resulting solution was measured using a calibrated pH meter. Acid concentrations that yielded equivalent pH values are listed in Table S1.

Single fly tracking

A modified food-substrate mixture was employed to facilitate filming during tracking assays. To produce sufficient contrast between the single fly and the oviposition substrate, a semi-transparent food mixture was made with either 5% AA or 5% H_2O mixed into molten 1.0% agarose + 20% grape juice (Welch's 100% Grape Juice, Concord MA), by volume. The base of a 35-mm Petri dish was divided with a trimmed razor blade, and 5% AA or 5% H_2O media was poured into each half of the dish. After cooling, the razor blade was removed and fluon (Northern Products, Inc., Woonsocket RI) was brushed onto the sides of the dish to prevent females from crawling off the food and onto the Petri dish lid. A single 3-4 day old

mated female was carefully trapped in the 35-mm Petri dish, without prior anesthesia. The dish was placed on a light box and filmed with a digital video camera (Sony DCR-TRV900) for 3 h. Single fly sampling behavior was captured in QuickTime movie (Apple Inc., Cupertino, CA) format at 10 frames/sec, using an Apple G4 PowerPC running Adobe Premiere (Adobe Systems Inc., San Jose, CA). To facilitate appropriate processing, the film was divided into three files approximately 1 h in length. Dynamic Image Analysis System (DIAS, Solltech, Oakdale, IA) was used to mark the position of the single fly during active substrate sampling, using a protocol adapted from previous studies in our laboratory (Wolf et al., 2002). Traces in Figure S4B represent 1-h of filming; each dot represents the position of the single fly during each frame. Although individual females did not lay sufficient number of eggs during the one-hour time frame to calculate reliable OI values, females were left in the dish overnight for approximately 12hours to assay their egg-laying preference. Under these conditions using the modified food substrate, the average egg-laying preference for the +AA half of the dish was attractive with an OI =+0.47±0.15). Of particular not, we have observed that agarose-based media is a less attractive oviposition substrate than regular yeast/molasses food, thereby explaining the reduced OI value when using the modified food-substrate mixture.

Egg-laying stripe assay

Molten stock fly food was diluted with 10% H_2O. 100 ml of 10% H_2O was then poured into a 90-mm x 70mm glass box and allowed to cool. Using a spatula, lines were cut into the layer of 10% H2O food at approximately every 13-mm, dividing the layer into seven stripes. The first, third, fifth, and seventh food stripe were carefully removed, and the

following molten mixtures were added from left-to-right to the empty spaces between the remaining food stripes: 2% agarose, 0.25% AA+9.75% H2O, 5% AA+5% H_2O, and 10% AA, by volume. Food samples were therefore diluted equally by 10% volume from their corresponding combinations of AA and water, to control for volume-related changes in food softness and texture. The 2% agarose substrate was employed as the "starting stripe" to provide a firm and unattractive egg-laying substrate. To ensure females walked across the stripes of food from their starting position on the 2% agarose, glass boxes with a small volume were selected to impede flying, and fluon (Northern Products, Inc., Woonsocket RI) was brushed onto the sides of box to prevent flies from crawling on the glass. 50 females were allowed to mate for 2-3 days prior to testing, after which they were anesthetized with a quick $C0_2$ pulse and placed on the 2% agarose stripe. Glass boxes were placed in dark conditions overnight, after which the number of eggs on each stripe was counted. The amount of eggs laid on each stripe was measured as the percent of the total eggs laid during the assay.

Surgeries

Surgeries were performed to remove the third antennal segment, the primary olfactory organ in *D. melanogaster*. Females were anesthetized with $C0_2$, and the third antennal segment was removed with a set of sharp forceps. Females were allowed to recover for 2 days before testing, during their mating period.

Candidate screen of brain regions involved in egg-laying and positional preference

Previous characterization of the GAL4 expression patterns in our P{GawB} insertion library (E. Marin, L. Luo, U.H., unpublished) allowed us to select lines with GAL4 expression in spatially restricted neuronal subsets of the adult fly brain. We selected 58 lines with discrete expression in several brain structures, including the mushroom body, ellipsoid body, sand subesophageal ganglion. We also tested the GH146 GAL4 line, which drives expression in the antennal lobe projection neurons, and $GAL4^{pdf}$, which drives expression in the PDF^{+} lateral neurons (Taghert et al., 2001). Lines were crossed to flies carrying *UAS-Shibire*^{ts} transgenes *(UAS-Shi*^{ts}*)* to conditionally silence synapses in the GAL4-expressing cells at the non-permissive temperature (30°C). Egg-laying and positional preference assays were performed as described above, with the following modifications: duplicate populations of *GAL4/UAS-Shi*^{ts}*, GAL4/*+, and *UAS-Shi*^{ts}/+ adult flies were placed at room temperature (23°C) or in an incubator raised to non-permissive temperature (30ºC). Mated females were allowed to equilibrate to their respective temperatures for 30 min, after which the number of flies on each half of the two-choice dish was counted at 10-min intervals. After eight time points (t=70 min.), both the 23°C and 30°C experiments were switched to dark conditions for the remainder of the assay for optimal egg-laying. PI values were calculated by averaging the values recorded during the eight time points, and OI values were calculated after a total of 3 h had elapsed from the time when the flies were initially introduced into the apparatuses, using the formulas for OI and PI described above. We were unable to measure positional preference at temperatures lower than room temperature (23°C) as we did not have access to an appropriately cooled room. As a result, the relatively strong expression of *UAS-Shibire*^{ts} transgene double-insertion likely resulted in residual dynamin disruption explaining the

behavioral effects seen in *GAL4/UAS-Shi^{ts}* experimental flies at 23°C. Similar effects have been described in other recent studies (Laissue et al., 1999).

Immunohistochemistry

Immunohistochemistry of *GAL4/UAS-CD8.GFP* fly brains was performed with antibodies against GFP and nc82, which labels neuropil. Stained brains were imaged as described (Marin et al., 2002). Briefly, brains were dissected from 2 to 3-day old females in 1X PBS and fixed in 4% formaldehyde. After washing, primary antibody staining was performed with a 1:200 dilution of rabbit anti-GFP antibody (Clontech, Mountain View, CA) and a 1:10 dilution of mouse nc82 antibody (Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA). Secondary antibody staining was performed with a 1:500 dilution of FITC-coupled goat anti-rabbit antibody and a 1:500 dilution of Cy3-coupled goat anti-mouse antibody (Molecular Probes, Eugene, OR). Confocal stacks of brains were obtained and merged with a Leica confocal microscope (Leica Microsystems Inc., Bannockburn, IL).

Statistics

Unless otherwise specified in figure legends, statistical analyses were typically one-way analysis of variance (ANOVA) tests of experimental means, with Bonferroni's multiple comparison *post hoc* tests to compare individual columns within data sets, or two-way ANOVA tests of experimental means with Bonferroni's multiple comparison *post hoc* tests when a second factor was involved (+/– antennae, 23/30°C). ANOVA was performed independently on oviposition preference data and position preference data. Error bars in figures represent means ± standard error of the mean (S.E.M.). All statistical analyses were

performed using GraphPad Prism, Version 4.0 (GraphPad Software, Inc., San Diego CA).

ACKNOWLEDGEMENTS

We thank W. Boll and M. Noll for *pox-neuro* flies and extremely useful insights, L. Luo and E. Marin for $GAL4^{5\text{-}120}$, $GAL4^{4\text{-}67}$, and $GAL4^{2\text{-}72}$ brain images, F. Wolf for single-fly traces, A. Rothenfluh for advice and use of *whir* alleles, and members of the Heberlein lab for exiting and heated discussions. Funding provided by an NSF predoctoral fellowship (A.D.), an NRSA/NIDA (I.K.) and grants from NIH/NIAAA (U. H.).

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FIGURE LEGENDS

Figure 1: Egg–laying and positional responses to acetic acid (AA).

(A) Apparatus for assaying oviposition and positional preference. Mated females were presented a dish in which one half contains food mixed with a compound of choice, and the other contains food mixed with an equivalent volume of water. An oviposition preference index (OI) and a positional preference index (PI) were calculated during the 3–h sampling period (see Methods for OI and PI formulas). **(B)** Comparison of OI and PI values of mated females, virgins, and males in response to 5% AA. As the egg–laying rate decreased for each consecutive group (left-to-right, shaded triangle), the positional aversion response increased (*$p<0.05$, **$p<0.01$, one–way ANOVA, Bonferroni's post–test; n=17). No significant differences in OI values were observed between female groups (Student's unpaired t-test). **(C)** Females showed no preference for consuming food containing AA. Mated females were presented the following two–choice food combinations: a) -AA/-AA: Blue #1/Green #3 (black bar); b) +AA/-AA: Blue #1 + 5% AA/ Green #3 (green bar); c) -AA/+AA: 5% AA + Blue #1/Green #3 (blue bar). After feeding, the amount of dye in fly gut contents was quantified by TLC. **(D)** Females deposited the majority of eggs on 5% AA substrate (+AA). **(E)** A single time point showing females spending time on media lacking AA. Photos were taken after 3 h **(D)** and at the 1–h time point **(E)**. To facilitate photography in **D** and **E**, 0.5% agarose was utilized instead of regular food.

Figure 2: Egg–laying and positional responses for different acids and pH values.

(A) Mated female egg–laying and positional responses were assayed using food titrated to

pH=3.5 with AA, HCl, or H_2S0_4. Control H_2O–supplemented food has pH=4.5. Significant responses at pH=3.5 were only observed for AA-containing substrate (**p<0.01, one–way ANOVA, Dunnett's multiple comparison post–test; n≥9). **(B)** Total number of eggs laid were comparable on food supplemented with AA, HCl, or H_2S0_4 (n≥9). **(C)** Egg–laying preferences and **(D)** positional preferences of mated females for foods titrated to different pH values using AA, HCl, and H_2S0_4 (see Table S1 for acid concentrations). There were significant differences between the dose–response curves for AA when compared to HCl and H_2S0_4 (linear regression; ***p<0.0001, n=4-8).

Figure 3: Role of olfaction in oviposition and positional choices.

(A) *whir*^{1} exhibited reduced OI to 5% AA when compared to wild–type (*wt*) females. Restoration of normal OI values was seen in *whir*^{1} lacking antenna (–ant) when compared to unoperated (+ant) *whir*^{1} females. **(B)** *whir*^{1} exhibited excessive repulsion to 5% AA when compared with *wt* females. Removal of antenna in *wt* and *whir*^{1} females caused a loss of positional aversion. (***p<0.001; one–way ANOVA, Bonferroni's post–test for comparisons between genotypes or between +ant and –ant columns of individual genotypes. n=8-12).

Figure 4: Role of gustatory system in oviposition and positional choices.

(A) OI values for *poxn* mutants and transgenic rescue lines at 5% AA (red bars); two–way ANOVA revealed genotype as the primary source of variation (80.42%, F=153.3, p<0.0001), with negligible interaction with antennal condition (0.37%, F=0.71, p=0.61; n≥7). OIs were significantly lower in *poxn*$^{\Delta M22\text{-}B5}$ homozygotes and in *poxn*$^{\Delta M22\text{-}B5\text{-}/\text{-}}$ flies carrying the *full-1-* and *full-152* transgenes, when compared to wild type *(Ctl)*, *poxn*$^{\Delta M22\text{-}B5+/\text{-}}$ heterozygotes, and

poxn carrying the complete rescue *SuperA* transgene. (*a* = ***p<0.001 when compared to *Ctl, poxn*$^{\Delta M22\text{-}B5+/-}$, and *SuperA* lines; one–way ANOVA, Bonferroni's post–test for comparisons between genotypes of the same antennal condition; n≥12). Antennaectomy had no effect on OI values (yellow bars). **(B)** PIs of the flies shown in **A**. Most genotypes (unoperated, purple bars) maintained positional repulsion to 5% AA, although *poxn*$^{\Delta M22\text{-}B5-/-}$ flies showed significantly reduced aversion when compared to *Ctl* flies. *poxn*$^{\Delta M22\text{-}B5-/-}$ carrying the *full-1* and *full-152* transgenes showed enhanced aversion, when compared to *poxn*$^{\Delta M22\text{-}B5-/-}$ carrying the complete–rescue *SuperA* transgene (*b* = *p<0.05, when compared to *SuperA*; *c* = *p<0.05 when compared to *Ctl*; one–way ANOVA, Bonferroni's post–test; n≥12). All genotypes demonstrated significant reductions in positional aversion upon removal of antennae (magenta bars), with the exception of *poxn*$^{\Delta M22\text{-}B5-/-}$ (two–way ANOVA, Bonferroni's post–test within genotypes for +ant vs. –ant ; n≥12).

Figure 5: Effect of silencing specific neuronal subsets with *Shibire*^{ts}.

OI and PI values for *UAS-Shi*^{ts}/+ and *GAL4/*+ controls, and *GAL4/UAS-Shi*^{ts} experimental females at permissive (23°C) and non–permissive (30°C) temperatures. **(A)** *GAL4*$^{5\text{-}120}$*/UAS-Shi*^{ts} exhibited reduced egg–laying preference for 5% AA at 30°C, while maintaining normal positional aversion. **(B)** Brain GAL4 expression of *GAL4*$^{5\text{-}120}$, visualized by crossing to *UAS-CD8.GFP*, revealed strong expression in the mushroom body (MB) and a few lateral neurons (LNs). **(C)** *GAL4*$^{4\text{-}67}$*/UAS-Shi*^{ts} exhibited strong reduction in positional aversion at 23°C and 30°C, while maintaining normal egg–laying attraction. **(D)** *GAL4*$^{4\text{-}67}$ drives strong expression in neurons that project to the ellipsoid body ring (EB). The locations of cell bodies (cb), dendrites (d), and axonal terminals (t) are indicated. (**A, C**: *p<0.05, **p<0.01, ***p<0.001

by one–way ANOVA with Bonferroni's post–test for comparisons between columns within the 23ºC or 30ºC groups; n≥8). (**B, D**: Green=*UAS-CD8.GFP*, red=neuropil marker nc82).

Figure 6: Models for the interaction between egg–laying attraction and positional repulsion to AA.

The gustatory (GS) and olfactory (OS) systems simultaneously detect input from a single compound, AA. Both sensory systems relay the signals to higher order centers of their respective circuits for processing and subsequent execution of motor programs (MS=motor systems) leading to oviposition preference (OP) or positional avoidance (PA). Competition between behavioral drives could occur: 1) in the female brain where neurons of the two pathways interact to simultaneously evaluate competing signals, such that either oviposition preference (OP) or positional avoidance (PA) is selected before motor program execution; 2) at the behavioral output level after motor–program selection where only the mutually exclusive nature of the behavioral outputs results in competitive shifts of behavioral responses; 3) a combination of both central integration and behavioral output competition; 4) and 5) as directional inhibitory interactions between either the gustatory (4) or olfactory (5) processing circuits and corresponding behavioral outputs. Red intersection lines represent negative interactions.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1: Average number of eggs laid per fly and percent total of flies sampling the substrate at 5% AA.

(A) Comparison of the average number of eggs laid per fly for the mated females, virgin females, and males of the *w Berlin* background tested in Figure 1B. As expected, on average mated females laid more eggs than their virgin counterparts (***$p<0.001$, Student's unpaired t-test, n=17). The number eggs/fly was calculated by adding the total eggs laid on both –AA and +AA halves of the dish, and dividing by the number of flies tested in that particular assay. (**B)** Mated females, virgin females, and males spent the same amount of time sampling the two-choice –AA/+AA dish (approximately 60% of their time), suggesting that differences in positional aversion in Figure 1B are not due to one group of flies spending less time actively sampling the food substrates ($p>0.05$, one-way ANOVA). The time spent on food was calculated by averaging the amount of flies on the dish at each time point, then dividing by the total number of flies being tested, yielding an average percent of flies on the food at any given time.

Figure S2: Male positional responses to different acids.

To test the effect of pH on positional preferences, male responses were characterized using food titrated to pH=3.5 with AA, HCl, or H_2S0_4. Control food titrated with equivalent volumes of H_2O had a pH=4.5. Significant repulsive responses at pH=3.5 were only observed for AA-containing substrate (**$p<0.01$, one-way ANOVA, Dunnett's multiple comparison post-test; $n\geq9$).

Figure S3: Dosage curves of egg-laying preferences, positional preferences, and average number of eggs laid per fly for various concentrations of AA.

(A) Egg–laying preferences for different concentrations of AA in individual two–choice assays. Females showed attraction for AA as an egg–laying substrate even at very low concentrations (0.01%AA), with a maximal preference for 3-5% AA. Preference diminished as the concentration increased. (B) Positional preferences for the flies assayed in panel S3A. Females demonstrate increasing aversion to AA at concentrations >3% AA; negligible positional responses are observed at doses lower than 1% AA. Of particular note, positional preference was slightly attractive at 1% AA, possibly indicating that egg-laying preference is still strong enough at this concentration to competitively exert a positive positional preference. (C) Comparison of the average number of eggs laid per fly for the mated females assayed in the dose-response curve depicted in panel S3A and S3B. Although there was variability between the number of eggs laid per fly at different concentrations of AA, statistical analysis revealed no significant differences between the number of eggs laid for each concentration of AA when compared to the 0.00% AA control (one-way ANOVA, Dunnett's post test).

Figure S4: Behavioral responses to AA in different experimental contexts.

(A) Multiple-choice stripe assay in which flies must traverse increasing concentrations of AA (***$p<0.001$; one–way ANOVA, Bonferroni's post–test revealed only 5% AA yielded significantly different preference compared to 0.25% AA, 10% AA, and all H_20 controls; $n\geq6$). See Suppl. Methods for detailed description of the assay. **(B)** Single-fly tracking

experiments. Representative traces of single females sampling behavior for 1 h in a modified two–choice assay. Positional responses (lower right corner) quantified by scoring the position of the fly at 10–min intervals for 1-h range from mildly (PI=-0.25), moderately (PI=-0.43), and highly repulsive (PI=-0.71).

Figure S5: Further antennactomy experiments investigating role of olfaction in responses to AA.

(A) OI and **(B)** PI values for *wt* females +/- antennae at 5% and 10% AA. Removal of antenna reduced the highly negative PI seen at 10% AA, and increased attractive OI. Although reductions in positional aversion were observed in –ant females at 10% AA **(B),** females still demonstrated some aversion to 10% AA. (**A-B:** ***p<0.001; one–way ANOVA, Bonferroni's post–test for comparisons between genotypes or AA% tests within either the +ant or -ant group. ***p<0.001; two–way ANOVA, Bonferroni's post–test for comparisons between +ant and –ant columns of individual genotypes or AA% tests; n=8-12). **(C)** Positional responses of antennaectomized and *whir*^{1} males to 5% AA. Similar to results obtained with females (Figure 3B), *whir*^{1} males exhibited increased repulsion to 5% AA when compared to wild-type (*wt*) males (**p<0.01, ***p<0.001; Student's unpaired t-test; n=10-12). In addition, significant PI reductions occurred between unoperated (+ant) and antennaectomized (–ant) males within each genotype (***p<0.001; two-way ANOVA, Bonferroni's post-test; n=10-12), demonstrating that olfactory input is also a factor in determining the aversive positional response to 5% AA in males. Of particular note, males lacking antenna still demonstrate some repulsion, suggesting that additional inputs are involved in sensing and responding to 5% AA. **(D)** Positional responses of antennaectomized

females to 0.25% AA. *wt* flies exhibited moderate egg–laying attraction to 0.25% AA with negligible positional aversion. Removal of the third antennal segment caused an increase in OI and a small yet significant positive shift in PI value (*p<0.05, **p<0.01; Student's unpaired t-test; n≥6).

Figure S6: Further *Shibire^{ts}* experiments implicating the mushroom body in egg-laying attraction and the ellipsoid body in positional aversion to 5% AA.

(A+C) OI and PI values for *UAS-Shi^{ts}*/+ and *GAL4/+* controls, and *GAL4/UAS- Shi^{ts}* experimental females at permissive (23°C) and non-permissive (30°C) temperatures. **(A)** *GAL4$^{5-98}$*/UAS- *Shi^{ts}* exhibited reduced egg-laying preference for 5% AA at the non-permissive temperature, while maintaining positional aversion comparable to *GAL4$^{5-98}$*/+ controls. *GAL4$^{5-98}$/UAS-Shi^{ts}* also displayed slightly diminished egg-laying preference for 5% AA at the permissive temperature (***p<0.001). This particular issue appears to be a result of mild developmental defects observed with this line. However, despite the statistical significance of the decrease, egg-laying attraction to AA remained robust at 23°C (PI=+0.61). Of additional note, *UAS-Shi^{ts}*/+ in these experiments were slightly less repulsed than *GAL4$^{5-98}$/UAS-Shi^{ts}* and *GAL4$^{5-98}$*/+ (**p<0.01). This trend towards less repulsion had been observed for this particular control, and the apparent increase in repulsion *GAL4$^{5-98}$/UAS-Shi^{ts}* and *GAL4$^{5-98}$*/+ is therefore not likely due to specific *GAL4$^{5-98}$* effects. **(B)** Neuronal *GAL4* expression patterns of *GAL4$^{5-98}$* was visualized by crossing to *UAS-CD8.GFP*, revealing strong expression in the mushroom body (MB) and a few lateral neurons (LNs). **(C)** *GAL4$^{2-72}$/UAS-Shi^{ts}* females exhibited reduction of positional aversion to 5% AA, while maintaining attractive egg-laying preference for the same substrate. **(D)** *GAL4$^{2-72}$* is strongly expressed in neurons that project

to the ellipsoid body (EB). The locations of cell bodies (cb), dendrites (d), and axonal terminals (t) are indicated. Similar to *GAL4$^{4-67}$/UAS-Shi^{ts}* flies (Figure 6C), loss of positional aversion was also seen in *GAL4$^{2-72}$/UAS-Shi^{ts}* at 23°C. Furthermore, the *GAL4$^{2-72}$/+* control showed a very small reduction in OI at 23ºC, when compared to some other lines at the same temperature. However, this effect is not likely biologically relevant as variances become compressed when OI values approach a ceiling for preference. (**A+C**, *p<0.05, **p<0.01, ***p<0.001; one-way ANOVA, Bonferroni's post-test for comparisons between columns within the 23ºC or 30ºC groups; ***p<0.001; two-way ANOVA, Bonferroni's post-test for comparisons between 23ºC and 30ºC columns, n=15-18). As described in Figure 6A and C, flies demonstrated increased repulsion at 30°C. (**B+D** Green=*UAS-CD8.GFP*, red=neuropil marker nc82.)

Figure S7: *Shibire^{ts}* silencing experiments testing the role of PDF^{+} lateral neurons in egg-laying attraction positional aversion to 5% AA.

GAL4$^{5-98}$, GAL4$^{5-120}$, and *GAL4$^{4-67}$* lines possessed some minimal *GAL4* expression in the lateral neurons (LNs), in addition to strong expression in the MB or EB. Since the *GAL4^{pdf}* line expresses strongly in the LNs (Taghert, et al., 2001), we assayed *GAL4^{pdf}/UAS-Shi^{ts}* flies at permissive (23°C) and non-permissive (30°C) temperatures to exclude the possibility that the LNs were responsible for the egg-laying or positional phenotypes seen in *Shibire^{ts}* experiments with *GAL4$^{5-98}$, GAL4$^{5-120}$,* and *GAL4$^{4-67}$* lines. Although *GAL4^{pdf}/UAS-Shi^{ts}* females demonstrated slightly diminished egg-laying preference at the non-permissive temperature (OI=+0.80) when compared to *GAL4^{pdf}/+* and *UAS-Shi^{ts}/+* controls (OI=+0.95) (*p<0.05; one-way ANOVA, Bonferroni's post-test; n>10), egg-laying attraction remained

robust, and did not decrease to the extent observed in *GAL4$^{5\text{-}120}$/UAS-Shi^{ts}* and *GAL4$^{5\text{-}98}$/UAS-Shi^{ts}* flies (OI=+0.08 and +0.12, Figure 6A and Figure S5A, respectively). Furthermore, there was no significant difference when compared to *GAL4^{pdf}/UAS-Shi^{ts}* at the permissive temperature. These data suggest that the slight decrease in OI is likely due to general genotypic variation between the different lines, rather than a specific *UAS-Shi^{ts}* silencing effect in the LNs. Unlike *GAL4$^{4\text{-}67}$/UAS-Shi^{ts}* (Figure 6C), *GAL4^{pdf}/ UAS-Shi^{ts}* did not demonstrate reduction in positional aversion to 5% AA at either the permissive or non-permissive temperature. Thus, the PDF^{+} LNs are not essential for generating either normal egg-laying or positional responses to 5% AA.

Figure S8: Effect of simultaneously silencing *GAL4$^{5\text{-}120}$* and *GAL4$^{4\text{-}67}$* neurons with *Shibire^{ts}*.

The egg–laying and positional responses of *GAL4$^{5\text{-}120}$/+; GAL4$^{4\text{-}67}$/UAS-Shi^{ts}* females were assayed at permissive (23°C) and non–permissive (30°C) temperatures. Additive behavioral defects were observed when compared to individual *GAL4$^{5\text{-}120}$/ UAS-Shi^{ts}* and *GAL4$^{4\text{-}67}$/UAS-Shi^{ts}* lines. OI and PI values for the experimental *GAL4$^{5\text{-}120}$/+; GAL4$^{4\text{-}67}$/UAS-Shi^{ts}* females were compared with OI and PI values from the following concurrently run controls: 1) *GAL4$^{5\text{-}120}$/+; GAL4$^{4\text{-}67}$/+*, 2) *GAL4$^{5\text{-}120}$/UAS-Shi^{ts}*, 3) *GAL4$^{5\text{-}120}$/+*, 4) *GAL4$^{4\text{-}67}$/UAS-Shi^{ts}*, 5) *GAL4$^{4\text{-}67}$/+*, and 6) *UAS-Shi^{ts}/+*; certain controls were omitted from the figure for simplicity (*a* = ***p<0.001 when comparing PIs of experimental and all control genotypes at 30°C; *b* = ***p<0.001 when comparing OIs of experimental and all control genotypes at 30°C; two–way ANOVA for comparisons between individual genotypes at 23ºC and 30ºC, one–way ANOVA for comparisons among 23ºC or 30ºC groups, Bonferroni's post–test; n≥8). The

GAL4$^{5\text{-}120}$/+; *GAL4*$^{4\text{-}67}$/+ control exhibited a small but significant increase in PI when compared to *UAS-Shi*^{ts}/+ (*$p<0.05$). Despite this minor caveat, our data strongly suggests that the MB and EB function in largely separate pathways to affect egg–laying attraction and positional repulsion to 5% AA, respectively.

Figure 1

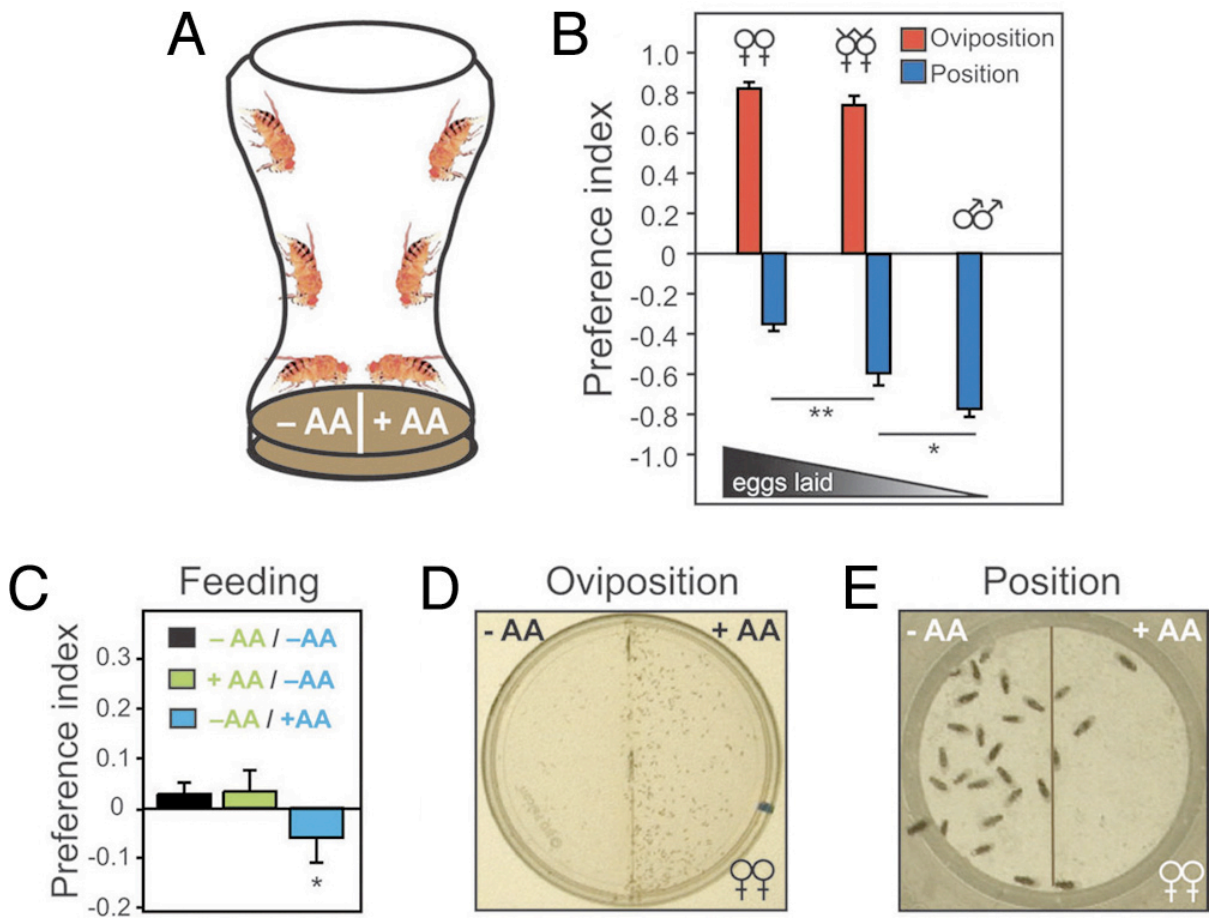
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Figure 2

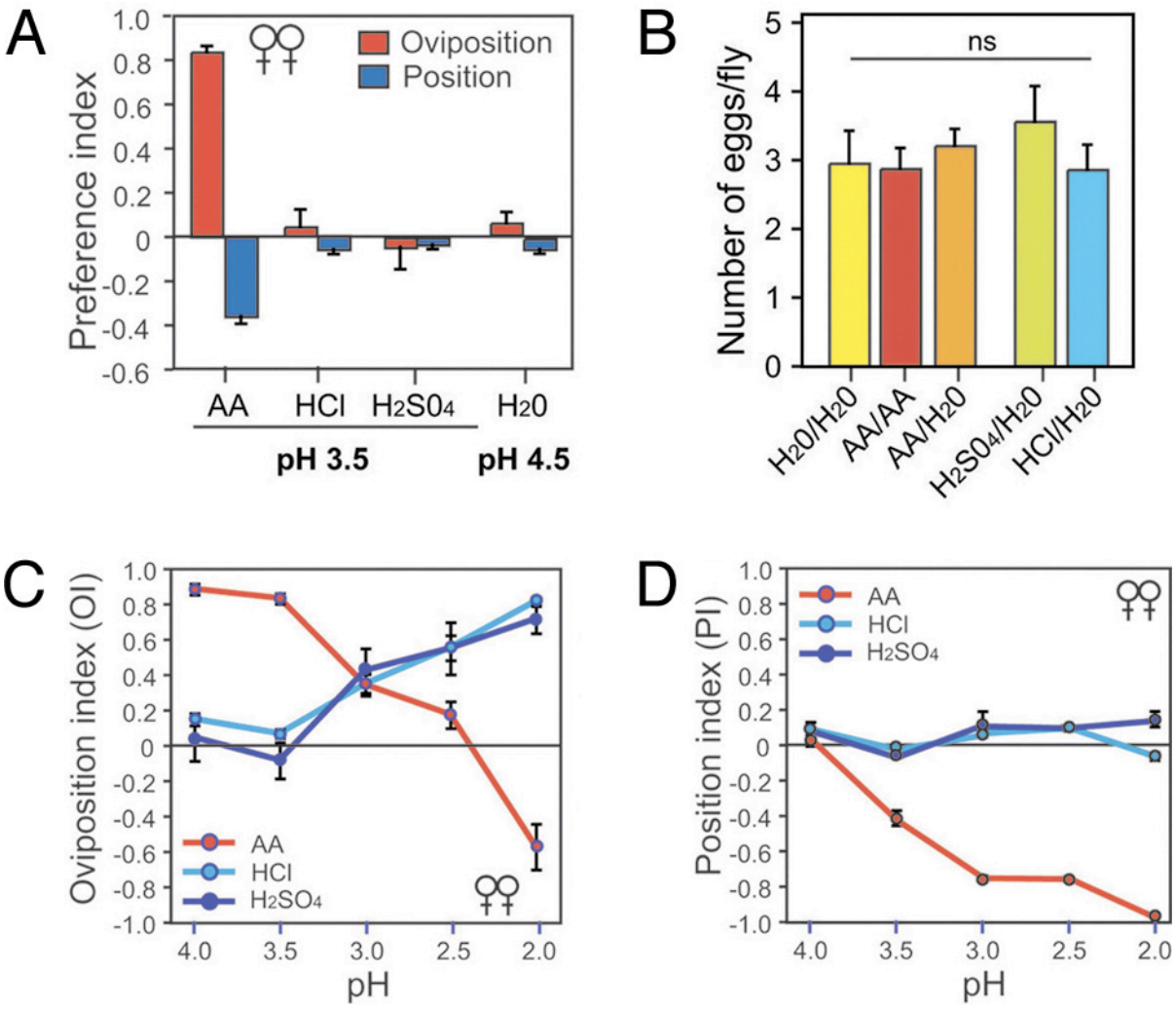
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Figure 3

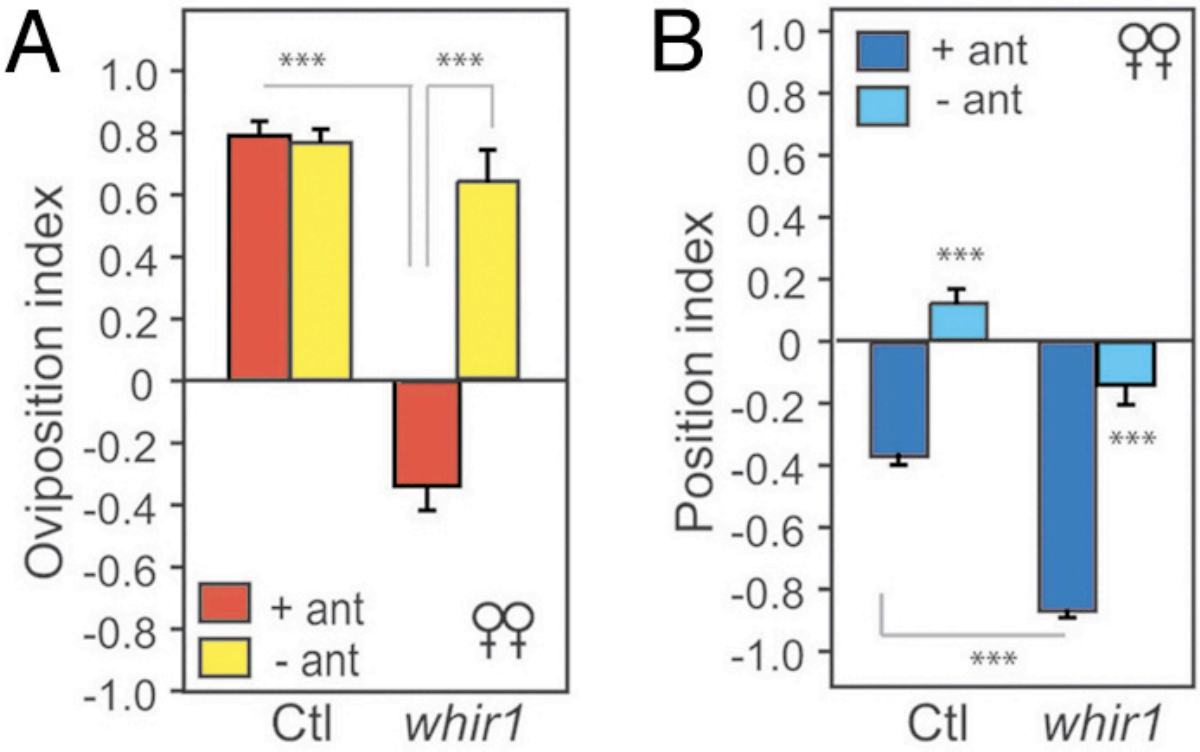
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Figure 4

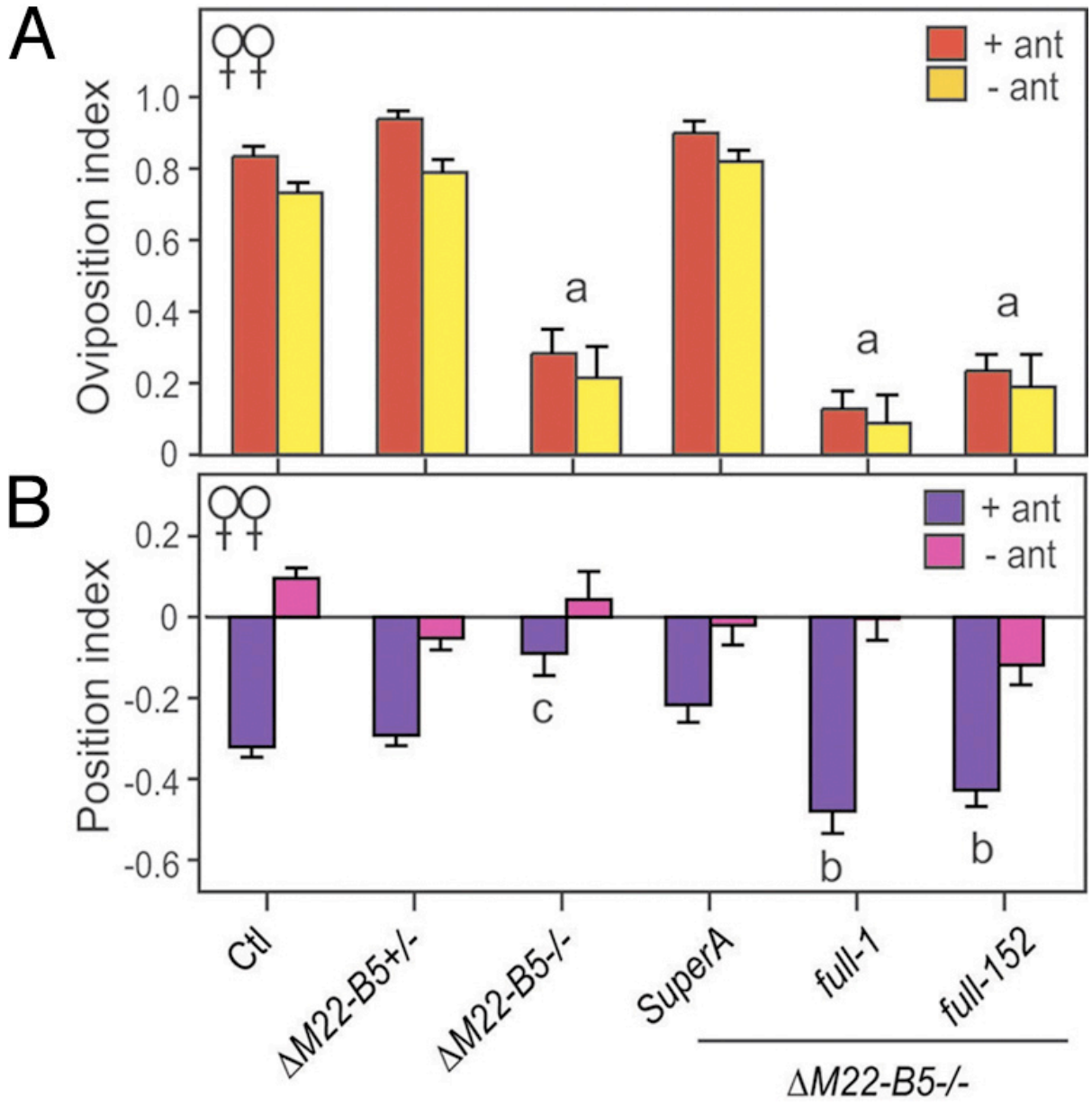
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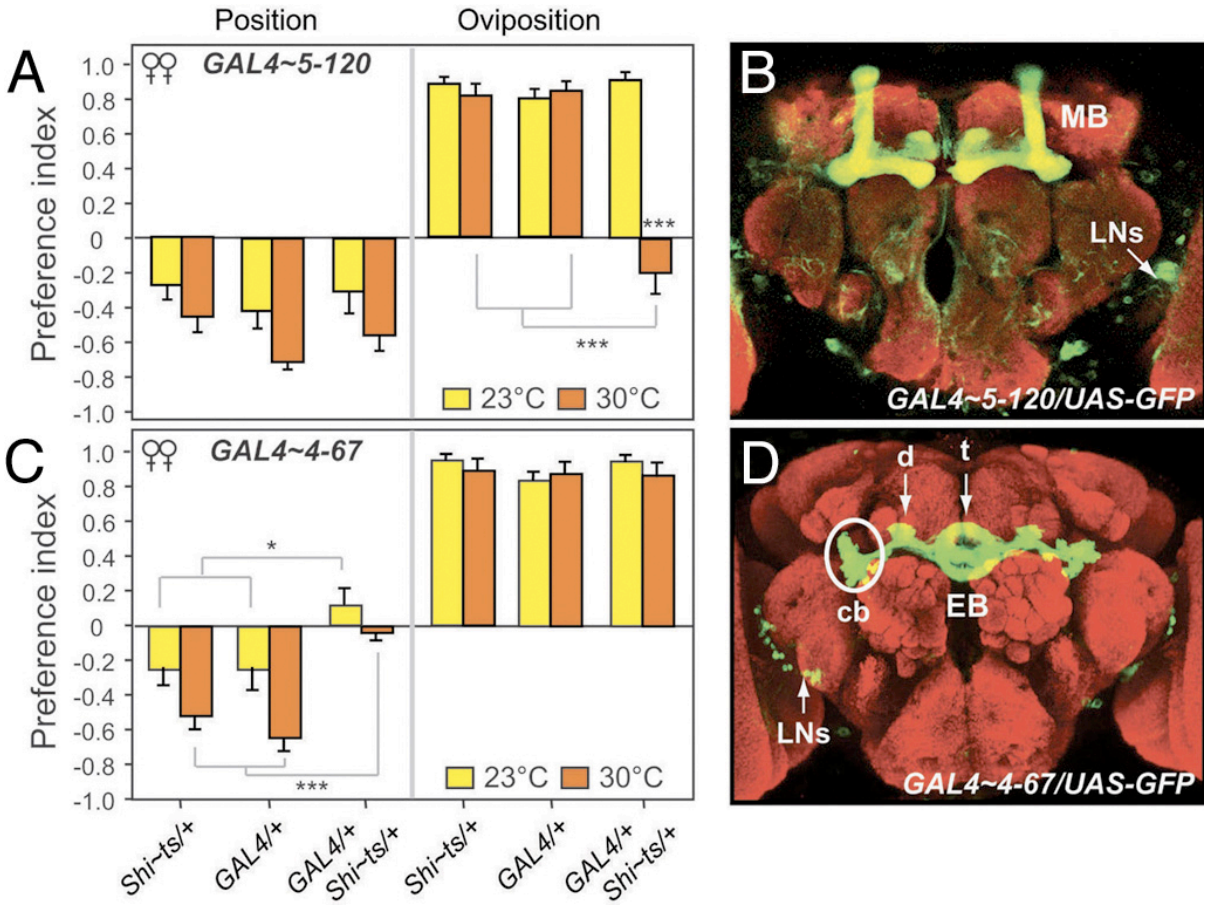
Figure 5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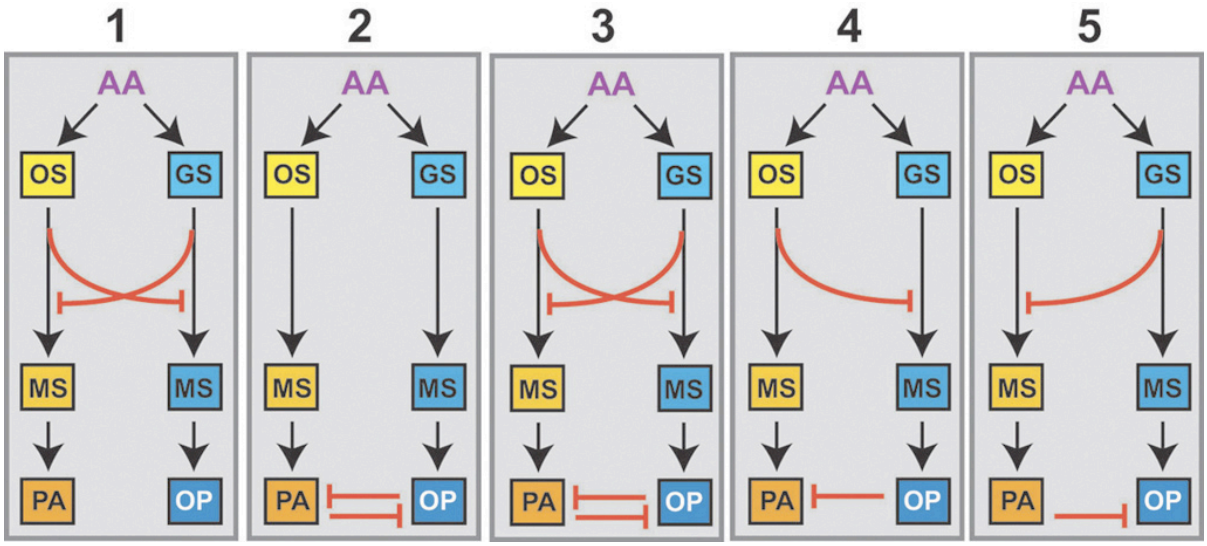
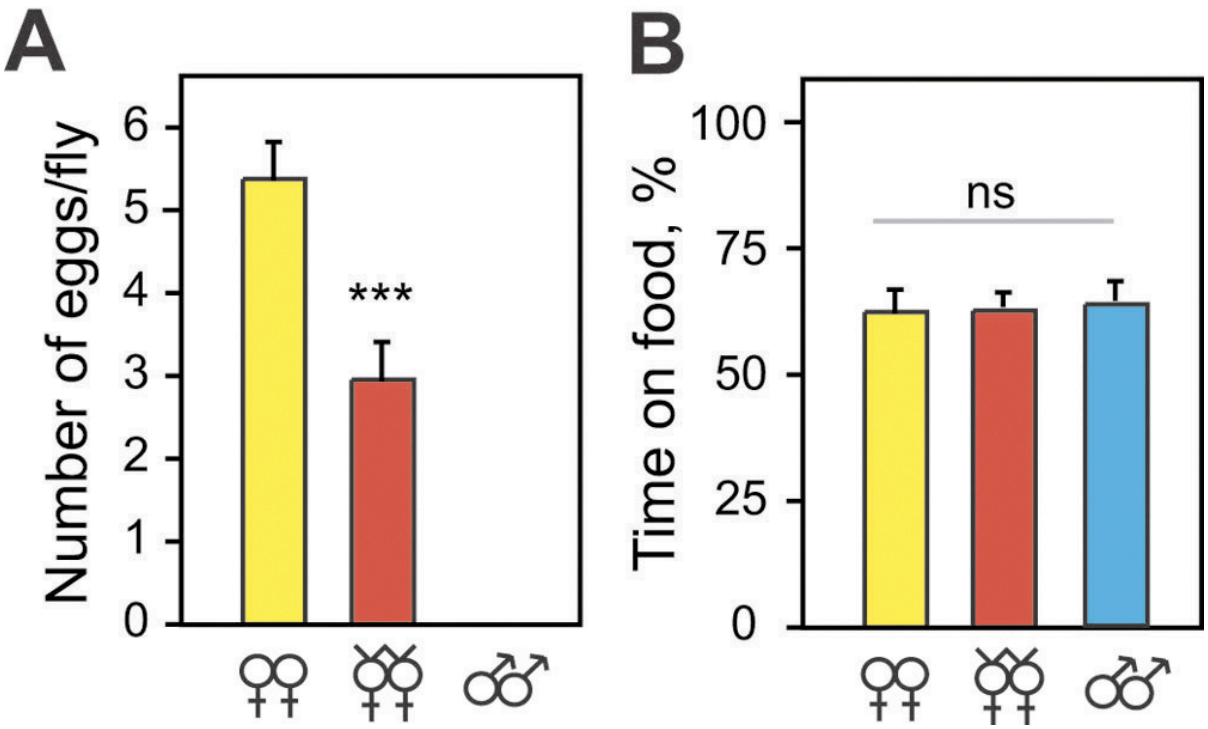
***
23°C
30°C
B
MB
LNs
GAL4~5-120/UAS-GFP
C
GAL4~4-67
*
***
23°C
30°C
D
d
t
cb
EB
LNs
GAL4~4-67/UAS-GFP
Shi~ts/+
GAL4/+
GAL4/+
Shi~ts/+
Shi~ts/+
GAL4/+
GAL4/+
Shi~ts/+

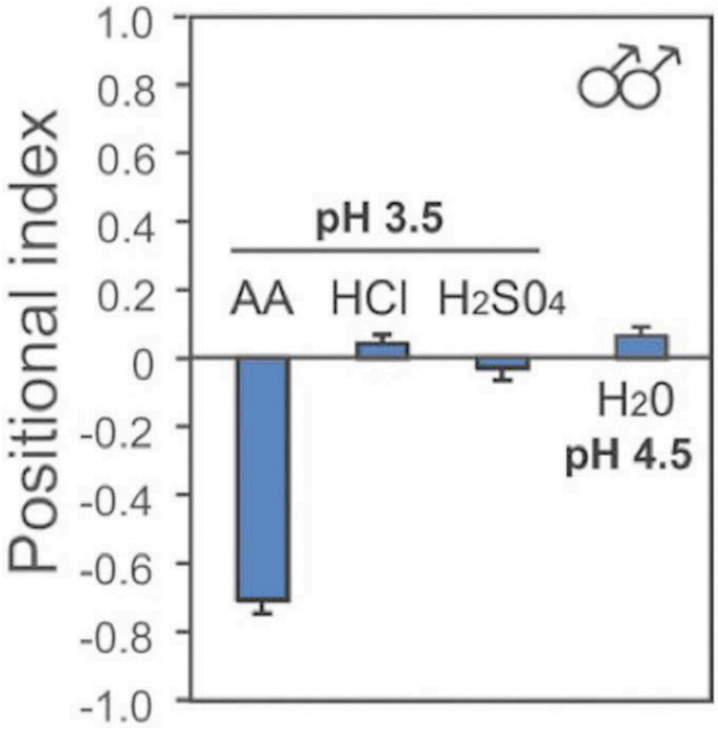
Figure 6



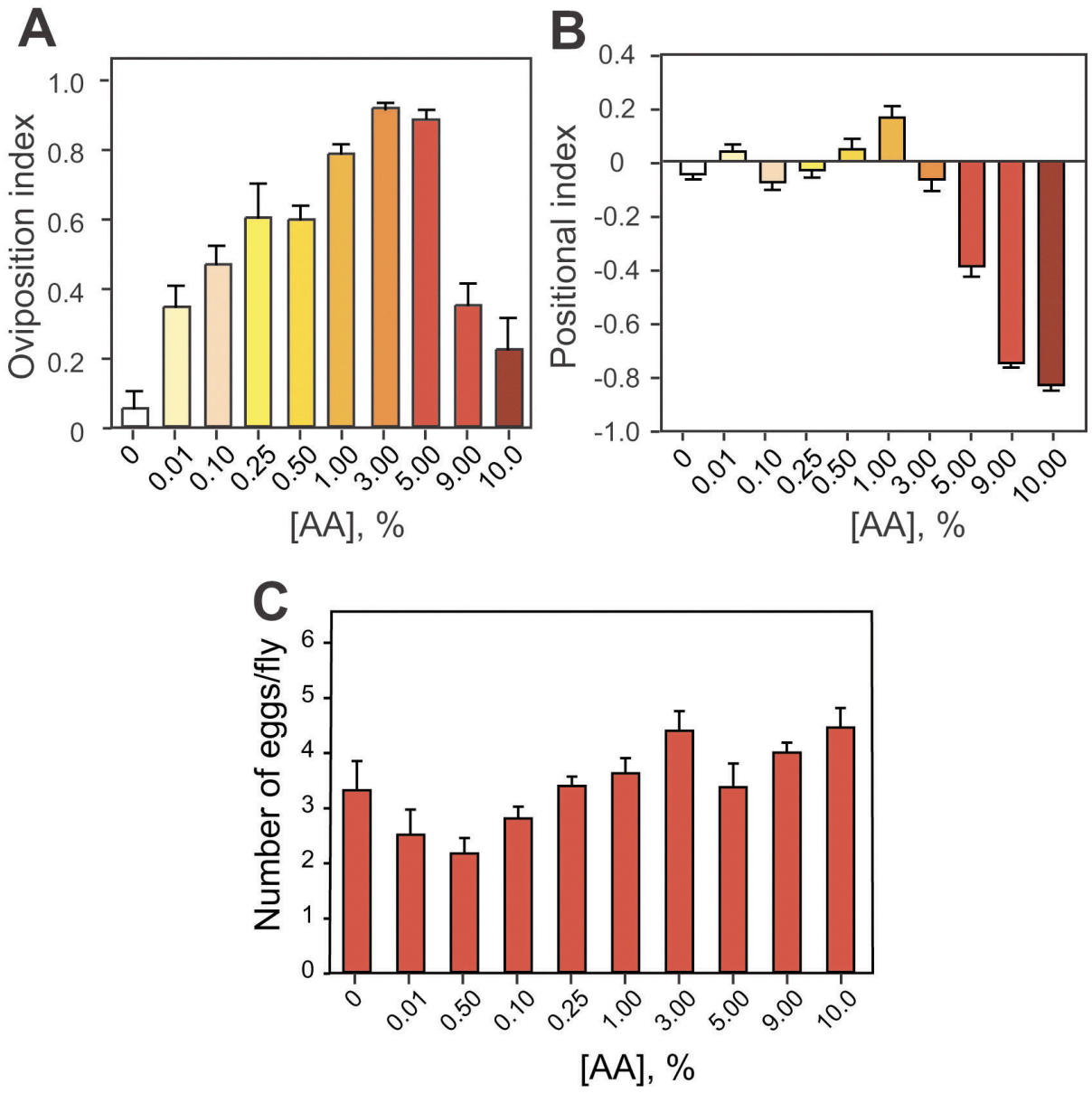
Supplementary Figure S1



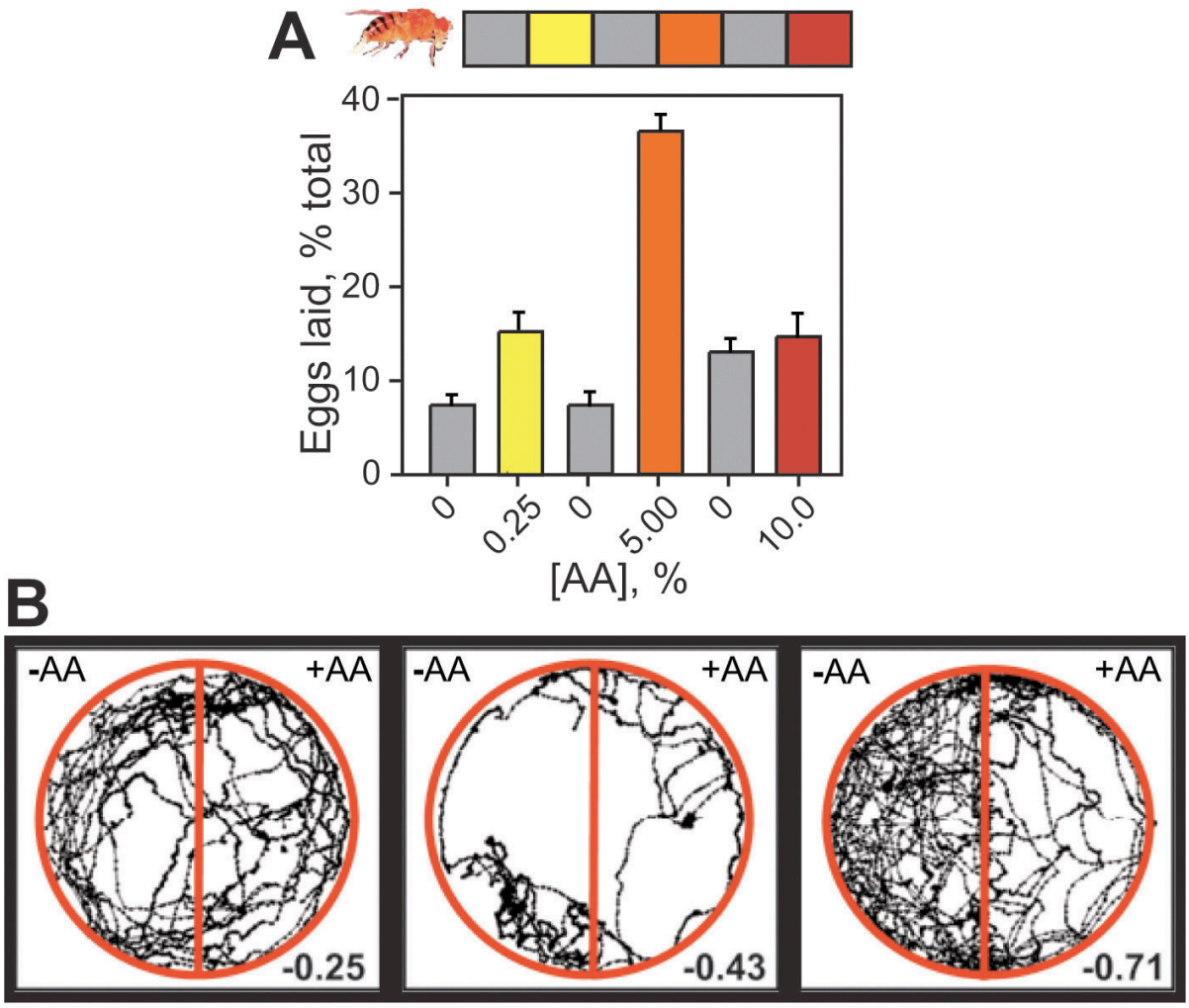
Supplementary Figure S2



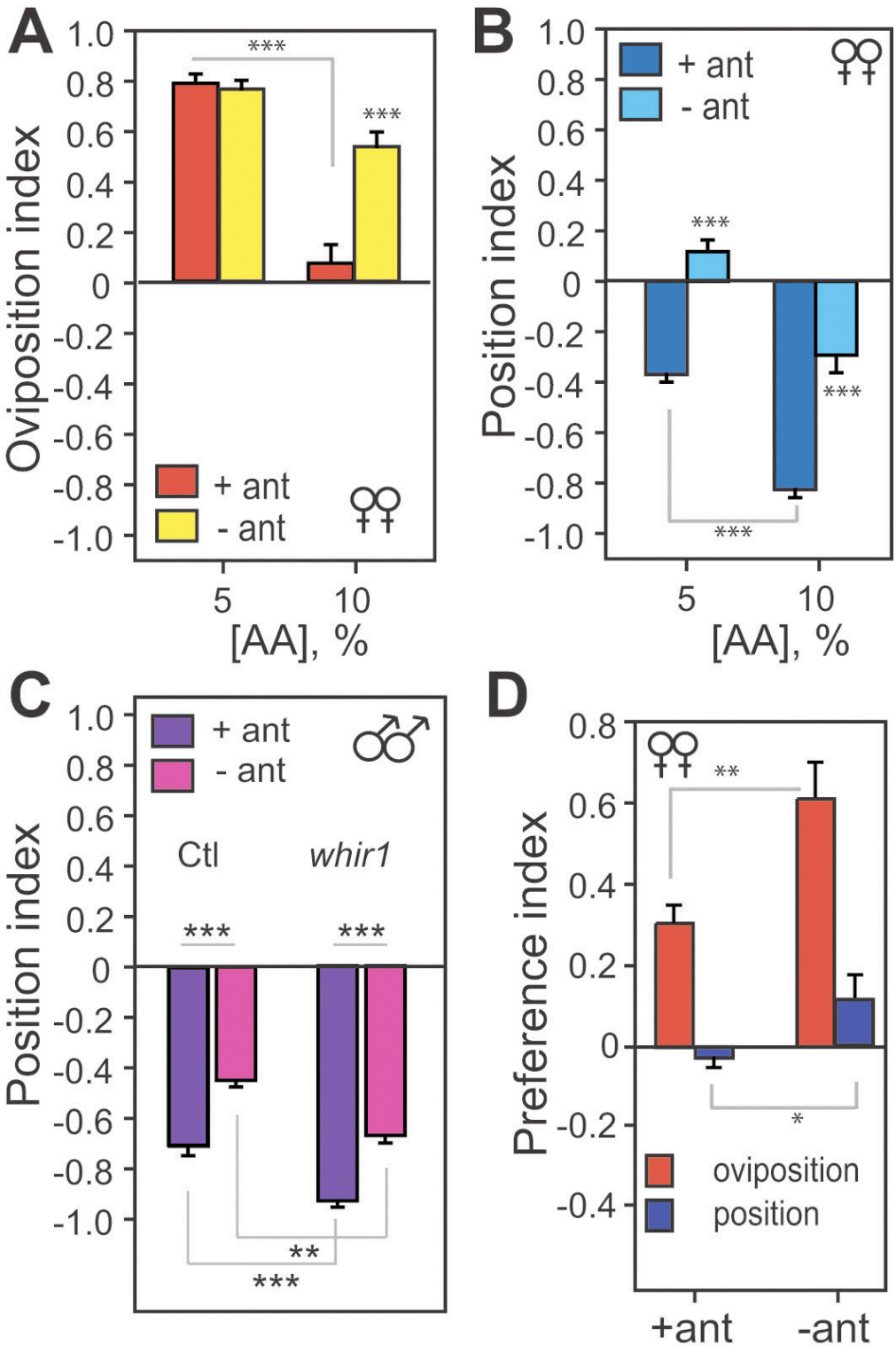
Supplementary Figure S3



Supplementary Figure S4



Supplementary Figure S5



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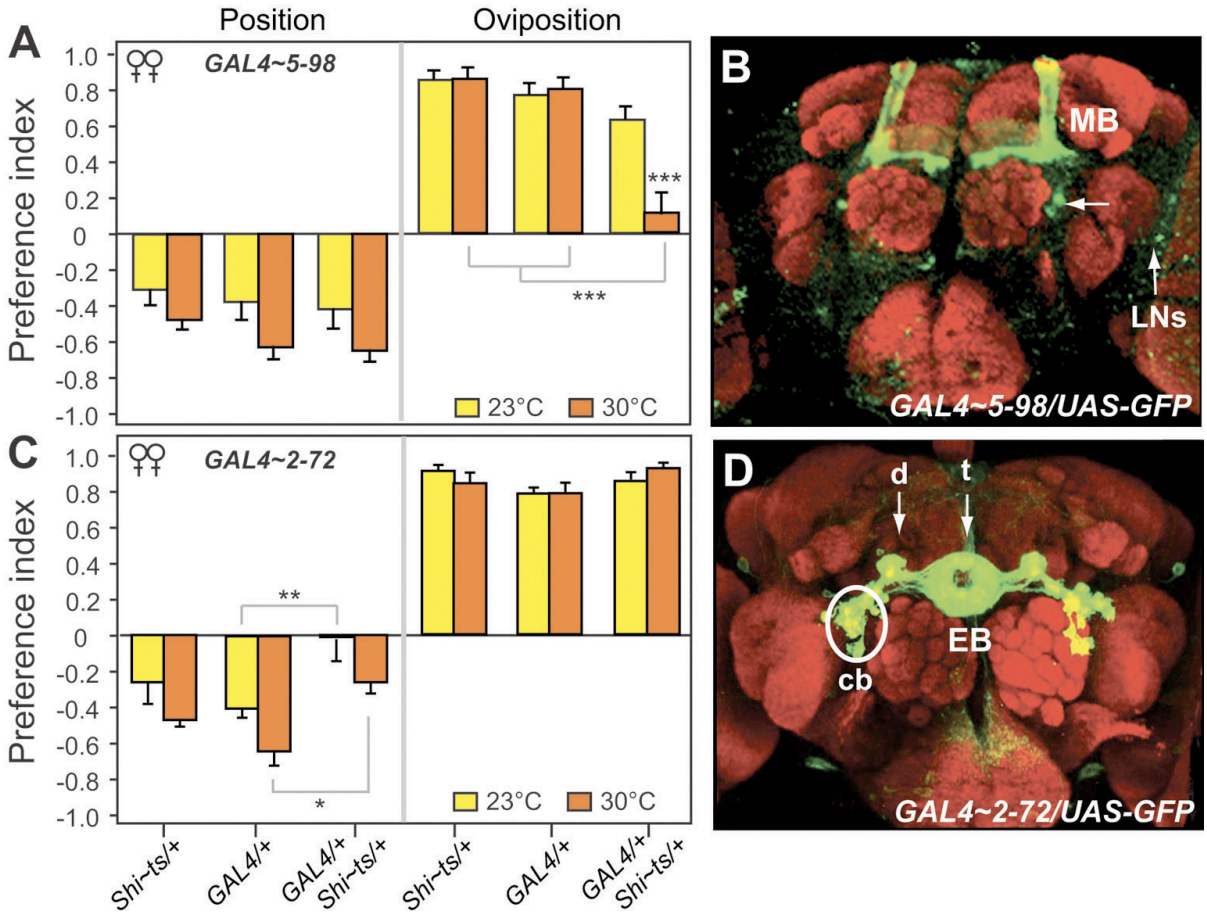

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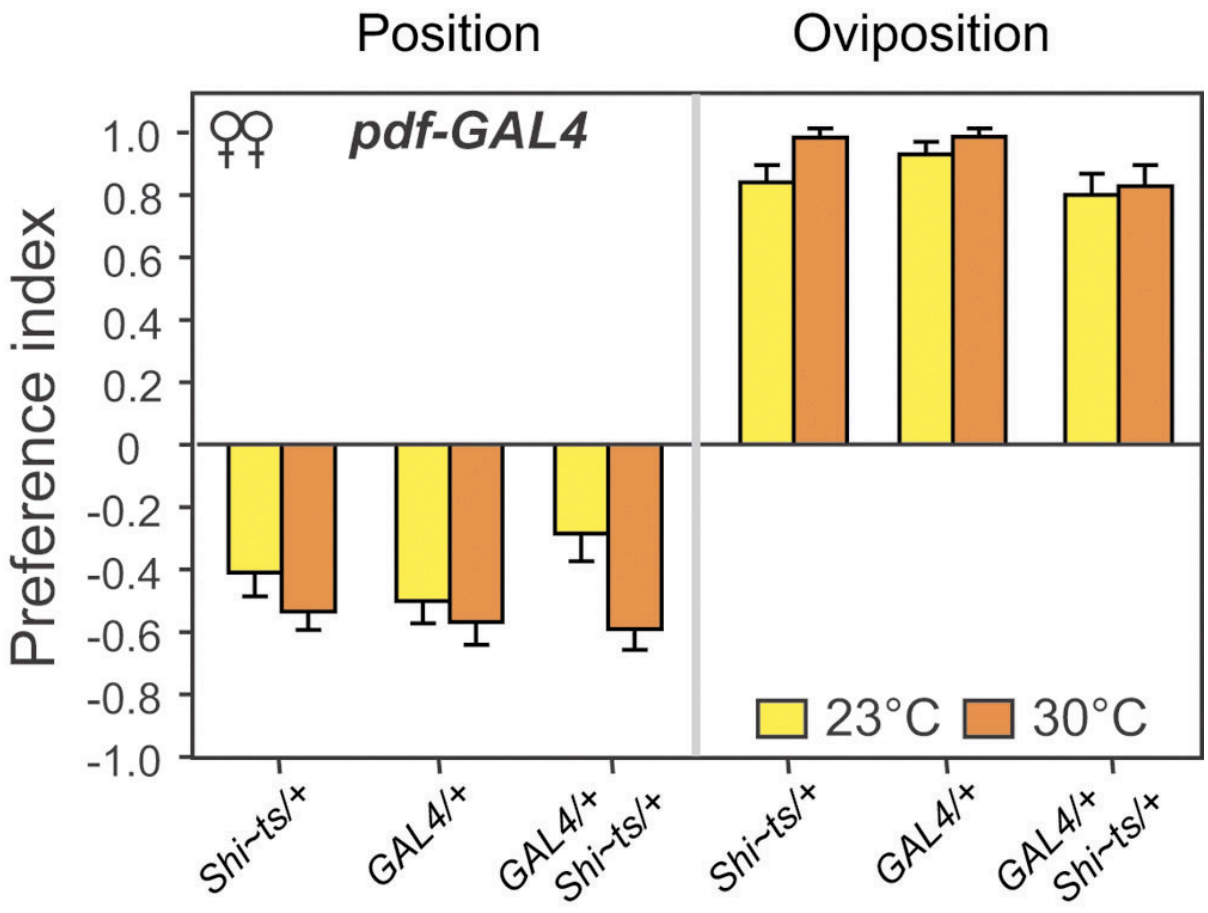
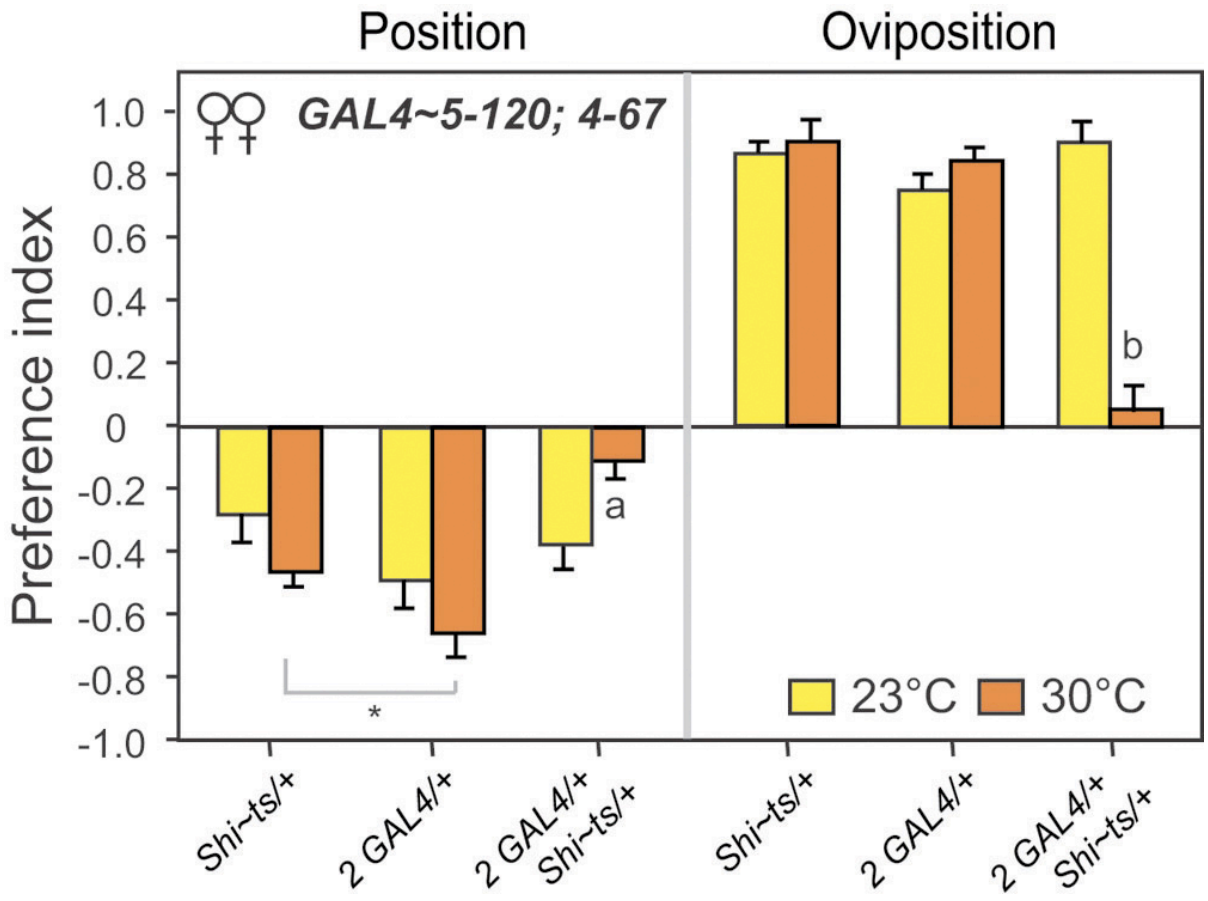


Supplementary Figure S8



Supplementary Table S1

Table S1. Concentrations of acetic acid (AA), hydrochloric acid (HCl) and sulfuric acid (H_2S0_4) that yield equivalent pH values when mixed into fly food

	pH				
Acid	4.0	3.5	3.0	2.5	2.0
AA	3.00%	5.00%	9.00%	10.0%	17.0%
HCl	0.05%	0.07%	0.10%	0.17%	0.25%
H_2S0_4	0.04%	0.06%	0.08%	0.11%	0.15%

Percentages are based on volume of acid before distilled water was added to control for dilution of the food mixture.

Supplementary Table S2

Table S2. Behavioral phenotypes observed upon *Shibire^{ts}* synaptic silencing in *GAL4* lines with expression in specific brain regions

GAL4-expressing brain structures/neurons tested with *UAS-Shi^{ts}*	Egg-laying preference phenotype	Position preference phenotype	Disruption in overall egg-laying	No observed *GAL4/UAS-Shi^{ts} effects*
Mushroom body	*5-98* *5-120* *10-229*‡	*8–239*†	*201Y* *OK107*	*17D* *mb247*
Ellipsoid body		*2-72* *4-67* *c232* *c561*		
Posterior protocerebrum		*5-138*		*104Y*
Subesophagael ganglion	*2-6*			
Antennal projection neurons		*GH146*		
Lateral neurons				*pdf*

These 19 lines possessed GAL4 expression primarily confined to a single brain structure/region; the remaining 39 lines had overlapping expression patterns in multiple structures, limiting conclusions with regard to which neurons were important to either egg-laying attraction or positional repulsion to 5% AA. Expression of GAL4 in the MB GAL4 lines that specifically disrupted egg-laying preference was found to overlap in the α and β MB lobes. Expression of GAL4 in the lines that specifically disrupt positional aversion: *GAL4^{c232} and GAL4^{c561}, drive expression in R3 and R4, and R1 cells, respectively. Since GAL4$^{4-67}$ drives expression in the R2, R4, and possibly small field EB neuronal subclasses, and GAL4$^{2-72}$ in the R2, R4, and possibly R3 EB neurons, we deduce that function of the R1 and R4 subclasses of EB neurons is necessary for positional repulsion to AA.*

†Peripheral nervous system expression.

‡10-229 appears to be cold-sensitive in the *UAS-Shibire^{ts} tests.*

Chapter 3

Description of Forward and Reverse Screens for Genes and Neurons Responsible for Competing Positional Aversion and Egg-Laying Attraction Responses

Ryan M. Joseph, Anita V. Devineni, Peter Sohn

INTRODUCTION

Drosophila are particularly amenable to genetic screening (St. Johnston, 2002), and countless mutants can be generated in a relatively short time-frame using either ethyl methane sulfonate (EMS) to produce nucleotide substitutions during DNA replication or mobilizing transposable sequences called P-elements to disrupt the reading frames of genes (Engels, 1983). P-element transposition is particularly useful, because P-element constructs can be engineered to include useful experimental sequences, including the *GAL4* transgene (Brand and Perrimon, 1993; Brand et al., 1994). Thus, the generation of a catalogue of *Drosophila* lines through P-element transposition, in essence, generates two separate libraries of fly strains: (1) a library of mutants with genes disrupted by random insertion of P-elements into open reading frames, and (2) an enhancer trap library of flies in which *GAL4*-containing P-elements integrated in non-detrimental genomic locations that are close enough to promoter and/or enhancer regions to co-opt transcriptional machinery. As a result, when a particular cell-type activates expression of the gene adjacent to the P-element insertion, the transcriptional machinery also induces the expression *GAL4*. Given that genes are differentially activated in different cell-types, *GAL4* is therefore only expressed in restricted areas of the fly, including specific neuronal circuits and structures.

These *GAL4* expression patterns are normally imaged by using a *UAS*-fusion of Green Fluorescent Protein (*UAS-GFP*) to detect tissues have active *GAL4/UAS* expression (Gao et al., 1999), providing anatomical targets for reverse-genetic screens. When used with the *UAS*-fusion constructs that silence or activate synaptic signaling (described in Chapter 1), libraries of P-element *GAL4* strains can thus be used to screen for neurons that are important for different behaviors. Our laboratory possesses one such library of $P\{GAL4^{GawB}\}$ flies that

can be used in both forward-genetic and reverse-silencing screens, as demonstrated by previous work from our laboratory (Wolf et al., 2002; Kaun et al., 2011). In order to characterize the genetic elements and neuronal structures involved in *Drosophila* oviposition behavior, we utilized this $P\{GAL4^{GawB}\}$ library in forward-genetic screens for lines that exhibited mutant phenotypes with regard to positional aversion and egg-laying attraction to acetic acid (AA), and reverse-genetic screens for neuroanatomical structures that are important to positional and egg-laying responses to both AA and lobeline. The results of these screens are described below.

RESULTS

Note: With regard to the experiments described in the following four sections: (*i*) the primary forward-genetic mutant screen for *Drosophila* lines defective in egg-laying behavior was conducted by Ryan M. Joseph; (*ii*) the secondary screen verifying egg-laying phenotypes and testing positional aversion in the initial candidates from the first screen were performed by Peter Sohn; *(iii)* the reverse-genetic screen for brain regions important to AA-based responses was initially performed by Anita V. Devineni, as accredited in Chapter 2; additional characterization of *GAL4* lines and behavioral phenotypes were performed by Ryan M. Joseph; (*iv)* the reverse-genetic screen for brain regions important to lobeline-based responses was performed in its entirety by Ryan M. Joseph.

i.) Forward-Genetic Screen for Mutants in Acetic Acid Egg-Laying Preference

In combination with studies conducted in Chapter 2, a forward-genetic screen was initiated to identify genes involved in egg-laying attraction to AA. Ideally, we hoped to

isolate genes that affected both positional aversion and egg-laying attraction to AA, using the rationale that genes important to the *selection* step in the *Drosophila* oviposition decision-making process would be important to the appropriate execution of both behaviors. However, because little was known about genes involved in egg-laying behavior, we felt that an amenable starting point would be to first screen for mutants with aberrant egg-laying preferences, and then perform secondary screen experiments on these isolated mutants to see if they also exhibited aberrant positional preferences. Thus, we would be able to additionally identify genes involved in the relatively uncharacterized *Drosophila* oviposition program, while searching more specifically for encoded proteins that directly affect decision-making and choice-like selection in our behavioral paradigm.

550 mutant *GAL4* lines were assayed using the two-choice egg-laying assay described in the Materials and Methods of Chapter 2, except that 3% AA was used for the screen. The lower concentration of 3% AA was used to stimulate a slightly lower egg-laying attraction than 5% AA, such that we could possibly isolate mutants in our forward-genetic screen that exhibited an increase in egg-laying preference to AA. *white-Berlin* females were also tested for their responses to 3% AA, to ensure that there were no substantial difference in egg-laying preference was caused by genetic background. Egg-laying preference was scored and tabulated as an oviposition index (OI) for each line (Figure 1). A screen average for oviposition index was subsequently calculated, yielding an OI=+0.896±0.0031; *white-Berlin* females had an average OI=+0.901±0.0102. Females that exhibited an OI value three standard deviations from the mean of the screen average were classified as mutant (OI_{cutoff}=+0.678), given this standard in numerous outlier tests. Using this criterion, we obtained 6 behavioral mutants, and 1 borderline strain that possessed an OI value right at the

cutoff point, for a total of 7 lines that potentially had mutant genes important to AA-based egg-laying behavior (Figure 2). A complete list of all lines tested, and their corresponding OI values are offered below as well (Figure 3).

5-131 and 10-184: white rabbit (RhoGAP18B)

The two strongest mutants were *5-131* and *10-184*, which are both insertions in the X-chromosome locus of *white rabbit* (*whir*), or *RhoGAP18B*, a RhoGTPase activating protein that has been implicated in behavioral pathways relating to ethanol sensitivity (Rothenfluh et al., 2006). Although there could be overlap ethanol-related and AA-related behavioral pathways, including those involving egg-laying (Eisses, 1997), we demonstrated in Chapter 2 of this dissertation that *whir* possesses increased olfactory sensitivity, which causes an excessive positional aversion response to 3% AA that negatively affects egg-laying attraction. As such, the effect of the *whir* mutation in our two-choice assay is a sensory defect rather than a perturbation in a gene that specifically affect the *valuation* and/or *selection* stages of decision-making process.

3-41b: flea and/or jing

The next strongest mutant phenotype line was *3-41b*. Unfortunately, BLAST results after obtaining inverse-PCR sequences revealed DNA homology to naturally occurring transposon elements (Quesneville et al., 2005), which are present throughout the *Drosophila* genome in the reading frames of multiple CGs. As a result, exact identification of which gene perturbed by the P-element insertion will not be possible without more in depth sequence analysis. However, of the top BLAST hits, one candidate insertion site is in the *jing* locus on

the second-chromosome. *jing* encodes a protein with a zinc-finger binding domain with transcriptional repressor activity, and may play an essential role in the development of neuroanatomical structures that could be important to egg-laying behavioral responses (Sun et al., 2006; McClure and Schubiger, 2008). Numerous testable alleles of *jing* exist, so it should be relatively easy to ascertain if *jing* is indeed the gene responsible for the mutant behavioral phenotypes seen in *3-41b*. However, if *3-41b* is not an insertion in the *jing* locus, future study will be comparatively more difficult.

4-76b: Homologue for Death-associated protein kinase related (DAPK), Drak

Unfortunately, the *4-76b* insertion was difficult to amplify using inverse-PCR; four trials were necessary to obtain a relatively weak BLAST sequence alignment with the *Drosophila* homologue of *Death-associated protein kinase related*, or *Drak*, located on the X-chromosome. Possible biological functions include regulation of tissue morphogenesis and cellular adhesion, via potential ATP-binding and serine/threonine-protein kinase activity (Neubueser and Hipfner, 2010). Interestingly, *Drak* appears to operate in pathways that perform functions associated with RhoGTPase activity; one can speculate that maybe *Drak* would also exhibit an olfactory-sensitivity phenotype similar to *whir/RhoGAP18B*, which might cause the decrease in egg-laying attraction for 3% AA. However, given the weak sequence alignment, further conclusions cannot be drawn without more specific characterization of *4-76b*.

4-20: Rab-protein 6 (Rab6)

The *4-20* insertion mapped to the second-chromosome locus containing *Rab-protein 6* (*Rab6*), which encodes a Rab-type GTPase (Pereira-Leal and Seabra 2001), leaving the possibility that the mutant phenotype in *Rab6* is also due to a sensory defect, as in *whir/RhoGAP18B*. However, *Rab6* has also been implicated in mechanosensory bristle formation in adult *Drosophila* (Purcell and Artavanis-Tsakonas, 1999); since gustatory bristles contain a mechanosensory neuron as well (Miyazaki and Ito, 2010), the specific sensory defect may result from aberrant development of chemosensory neurons, instead of changes in olfactory-sensitivity. Furthermore, the related *Rab5* protein has been shown to be involved in proper dendrite morphogenesis (Satoh et al., 2008). Thus, there are several possible ways *4-20* may be disrupting egg-laying attraction to AA.

10-142: CG31522

The *10-142* insertion mapped to a third-chromosome locus belonging to *CG31522*, which has unknown biological function and has not been extensively characterized. However, studies suggest that it would share sequence homology with fatty acid biosynthesis or long-chain fatty acid metabolism (Szafer-Glusman et al., 2008). Involvement in these processes would make some sense, considering that acetic acid and its derivatives, like acetyl-coA, are byproducts and/or chemical components of these pathways. Thus, a mutation affecting the detoxification or processing of acetic acid from the fly's tissues could alter sensitivities to this compound, thereby affecting behavioral preferences for acetic acid.

3-21a: amnesiac

The borderline mutant was *3-21a*, which after sequencing using inverse-PCR was revealed to be an X-chromosome insertion in the locus of *amnesiac* (*amn*), which has protein sequence similarity to neuropeptide hormones that bind G-coupled protein receptors (Feany and Quinn, 1995; Korzus, 2003). As such, it has been implicated in a multitude of different behavioral responses, including: learning and memory (Keene et al., 2006), salience and attention-related processes (van Swinderen, 2007), ethanol-sensitivity and responses (Wolf et al., 2002). Unsurprisingly, different alleles of *amn* are isolated in nearly all behavioral screens performed in our laboratory. However, given the expression of *amn* in the mushroom body and functional importance to behaviors that are mediated by this structure (Yu et al., 2006), it is possible that *amn* could play a role in decision-making processes relating to the *Drosophila* oviposition program. Of particular note, the mushroom body may in fact be an integration center for the selection between positional aversion and egg-laying attraction responses to bitter-tasting compounds (Chapter 4), and therefore future study reveal functional importance of *amn* in this decision between competing behavioral outputs.

Of note, the *3-21a* insertion is contained within predicted *CG32529*, which is in the reading frame opposite of *amn* on the X-chromosome. Annotation suggests the encoded protein has DNA binding properties, but molecular and/or biological functions are unknown. However, the possibility still remains that *CG32529* is responsible for the egg-laying phenotypes seen in the *3-21a* line. Thus, before future investigations are pursued with *3-21a* and egg-laying preferences, additional alleles of *amnesiac* will need to be assayed.

Summary

The forward-genetic screen provided some initial candidate genes that could be important to egg-laying behavior in *Drosophila*. Some mutated genes, such as those encoding RhoGTPases like *whir,* may be involved in sensory perception and thus disrupt egg-laying preference to AA at the input level of processing. In contrast, other mutated genes like *jing* may be involved in the appropriate morphogenesis of the nervous system during development. Although interesting for the characterization of *Drosophila* oviposition behavior, these mutations do not necessarily affect decision-making in an acute manner. In other words, the disruption caused by the gene mutation do not directly affect the selection process between positional aversion or egg-laying attraction, but rather alter how input signals are detected and relayed upstream in the decision-making process (either neuoroanatomically or developmentally). Given the known expression pattern and likely molecular function of *amnesiac*, the *3-21a* insertion provides the best candidate for an encoded protein that could directly affect either the *valuation* or *selection* steps of the decision-making process in the mushroom body, and may provide interesting investigations in the future.

***ii.*) Secondary Screen for Mutants in Acetic Acid Egg-Laying and Positional Preferences**

After initial egg-laying mutants had been identified in the forward-genetic screen described above, candidates were then assayed in a secondary screen that tested both positional and egg-laying responses to 5% AA. Additional candidates from the $P\{GAL4^{GawD}\}$ library that were not assayed in the original egg-laying screen were also selected for testing, based on their known *GAL4/UAS-GFP* expression patterns in discrete higher-order brain

regions that could be potentially involved in decision-making and/or selection of behavioral output. These regions included: the subesophageal ganglion (SOG), central complex (CC), ellipsoid body (EB), and the mushroom body (MB). We hypothesized that mutating genes important to the *valuation* or *selection* steps in the decision-making process that chooses either positional aversion or egg-laying attraction would cause a disruption in both behaviors, and support the *central integration model* described in Chapter 1. Meanwhile, if mutated genes induced a loss of only one behavioral response, it would suggest that the neurons expressing those genes were important for sensory perception before a decision is made, or motor neuron output after a decision made. In either case, both results would be interesting from an entomological standpoint, since characterization of the genes and circuits involved in *Drosophila* oviposition site-selection had only recently be initiated at the neurogenetic level (Yang et al., 2008). Additional assays accounting for defects in overall locomotion and/or health of mutant females would also have to be performed to ensure that observed defects were not due to non-specific effects that were not directly related to oviposition behavior or decision-making.

42 mutant *GAL4* lines were assayed for both positional aversion and egg-laying attraction to 5% AA. Of note, the *GAL4* lines tested were backcrossed for five generations into the *white-Berlin* background, to ensure that mutant effects were specific to the P-element insertion and not a result of additional modifiers. 5% AA was employed in order to generate a positional aversion response (Chapter 1). For this secondary screen, position indexes (PI) and oviposition indexes (OI) were compared to the corresponding values for *white-Berlin* using unpaired t-tests (two-tailed), since: (1) using a screen average would be an unreliable measure of wild-type behavior, since of the 42 lines at least half were selected for their

decreased OI values observed in the first egg-laying screen, and (2) sufficient sample sizes were obtained to perform direct statistical comparison of each line.

With regard to positional aversion to 5% AA, 4 mutant lines exhibited PI-values statistically different from the *white-Berlin* control (Figure 4). However, these lines exhibited *increased* positional aversion, which was explained when sequencing revealed most of these lines to be alleles of *whir*. With regard to egg-laying attraction to 5% AA, 6 mutant lines had OI-values statistically different from the *white-Berlin* control (Figure 5). Notably, some lines isolated in the original screen exhibited more wild-type egg-laying preferences after backcrossing (i.e. they "lost" their mutant phenotypes and did not demonstrate statistical significance when compared to *white-Berlin*). However, for the most part these lines still exhibited trends towards decreased egg-laying preference to 5% AA, which would likely become significant when higher sample sizes are obtained. Thus, both statistically significant mutants and lines that trended towards aberrant positional or egg-laying responses were selected for sequencing with inverse PCR. A complete list of lines tested and their phenotypes is also provide below (Figure 6):

11-96: white rabbit (RhoGAP18B)

The insertion in *11-96* was revealed to be another allele of *whir*, which was described above in Section (*i*). As such, it exhibited mutant egg-laying phenotypes similar to *5-131* and an independent *whir* allele tested in this secondary screen (Figure 5). Of note, *5-131*, *11-96*, and *whir* demonstrated increases in positional aversion to 5% AA (Figure 4), as described by the characterization of *whir* in Chapter 2. Interestingly, the *10-184* allele of *whir* that was isolated in the original forward-genetic screen for egg-laying preferences to 3% AA did not

exhibit statistical differences in behavioral responses to 5% AA. Possible explanations for this different include: (1) backcrossing the *10-184* insertion reveled that the mutant effects in this particular *GAL4* line are due to other genomic modifiers; (2) the insertion *10-184* may have generated a weaker allele of *whir*, which does not exhibit as dramatic phenotypes. If the latter explanation is correct, then *10-184* could be useful in future studies where the behavioral phenotypes associated with other *whir* alleles are too strong to facilitate characterization of responses.

4-12: CG15626

The *4-12* line possessed a strong decrease in egg-laying attraction to 5% AA, but a relatively normal positional aversion to 5% AA. The *4-12* insertion mapped to a second-chromosome locus, inside the open reading frame containing *CG15626*, an uncharacterized gene with unknown biological functions. This gene was also isolated by Anita V. Devineni in a separate screen for ethanol-related behaviors, and will be a subject of her dissertation research. As such, further description of *4-12* will be left for Anita's thesis.

3-21a and 5-15: amnesiac

3-21a and *5-15* both map to loci within the *amn* gene. As described earlier, the *3-21a* insertion exhibited significant decrease in egg-laying attraction to 3% AA in the original mutant screen. In contrast, in this secondary screen the decrease in egg-laying preference for *3-21a* and *5-15* were not significant in t-test comparisons with *white-Berlin.* However, these lines still exhibited a trend towards diminished egg-laying preference to 5% AA, suggesting that these effects seen in the first screen are in fact real. Interestingly, *3-21a* and *5-15* had

normal positional aversion responses to 5% AA, suggesting that they may not necessarily be involved directly in decision-making centers in the brain, as described by the *central integration model* for selection between competitive behavioral responses (Chapter 1). These results are not entirely unexpected, given our findings with acetic acid in Chapter 2. Notably, when dealing with AA-related behaviors, the mushroom body regulates egg-laying attraction but not positional aversion (Chapter 2, Figure 5). Thus, the expression of *amn* in the mushroom body would be expected to correlate with decreased egg-laying attraction to AA, but not necessarily positional aversion to AA. In future studies, it will be interesting to test the responses of different *amn* alleles to lobeline, since the regulation of both egg-laying attraction and positional aversion to lobeline does appear to proceed through the mushroom body (Chapter 4), and thus *amn* may be directly involved in decision-making in this particular context.

4-67: F4/AMP-activated protein kinase gamma subunit (SNFAγ)

4-67 was the only line to exhibit statistically significant changes in both egg-laying attraction and positional aversion to 5% AA. Specifically, *4-67* simultaneously demonstrated an increase in positional aversion and a loss of egg-laying attraction when compared to *white-Berlin* controls. (Figure 6). The *4-67* insertion mapped to the third-chromosome locus of *SNF4/AMP-activated protein kinase gamma subunit* (*SNFAγ*), a protein with serine/threonine kinase activity and AMP-activated kinase activity (Yoshida, 1998). Interestingly, *SNFAγ* has been shown to play a role in the development of peripheral sensory organs (Abdelilah-Seyfried et al., 2000) and the central nervous system during embryonic development (Wheeler et al., 2006). One can therefore speculate about possible role of *SNFA*

in the appropriate evaluation of sensory input and/or the direct selection processes between egg-laying attraction and positional aversion responses in the *Drosophila* oviposition.

Of note, *4-67*^{GAL4} expresses strongly in the ellipsoid body, as described in Chapter 2, where this line was utilized in silencing experiments with *UAS-Shibire*^{ts} to demonstrate that the ellipsoid body is required for appropriate execution of the positional aversion response to 5% AA. One could argue that the positional aversion defect observed in *4-67* homozygotic mutants could be related to the role of the ellipsoid body in this response. Although it is tempting to claim the ellipsoid body could represent an integration center or decision-making region of the brain, our additional ellipsoid body-*GAL4* silencing experiments argue against this conclusion. Notably, these experiments demonstrate that silencing ellipsoid body neurons has negligible effect on AA-based (Chapter 2, Figure 5) and lobeline-based (Chapter 3, Section (*iv*)) egg-laying responses. Thus, the simultaneous disruption of positional aversion and egg-laying attraction seen in in *4-67* homozygotic mutants is more likely due to developmental defects that the *4-67*^{GAL4}*/UAS-Shibire*^{ts} flies do not possess since they likely *SNFAγ/+* heterozygotes.

10-142: CG31522

As described in Section (*i*), the *10-142* maintained the loss of egg-laying attraction phenotype. However, this line did not exhibit any mutant phenotypes with regard to positional aversion to 5% AA.

4-20: Rab-protein 6 (Rab6)

Although the egg-laying decrease that *4-20* displayed in the original genetic screen was not maintained at a statistically significant level, this *GAL4* lines still exhibited a trend towards decreased egg-laying attraction to 5% AA. Interestingly, *4-20* also demonstrated a trend towards increased positional aversion to 5% AA. However, further experiments are necessary to see if this change in positional behavior is a real effect, or just variability.

3-48: CG8034 Adjacent

The *3-48* line possessed a strong trend towards a loss in egg-laying attraction. The insertion mapped to a region of the X-chromosome adjacent to *CG8034*, but was not mobilized into the open reading frame of this gene. The biological function of the encoded protein is unknown, and future investigation would be required to identify if *CG8034* is indeed responsible for the decrease in egg-laying attraction, or if the P-element is exerting effects elsewhere on other genomic sequences.

4-76b: Homologue for Death-associated protein kinase related (DAPK), Drak

The decrease in egg-laying attraction observed in *4-76b* in the initial screen described above was recapitulated as a trend towards diminished egg-laying attraction in the secondary screen. Interestingly, *4-76b* did also demonstrate a trend towards increased positional aversion towards 5% AA, suggesting this gene could play a role in both behaviors. However, the positional aversion trend is relatively weak, and would need to be validated before further conclusions can be drawn.

17-31: Inositol 1,4,5-triphosphate kinase 1 (IP3K1)

The *17-31* line exhibited a small trend towards both a loss of egg-laying attraction and positional aversion to 5% AA, suggesting it could play a role in the selection process between these two behaviors. The *17-31* P-element mapped to the second-chromosome locus containing *Inositol 1,4,5-triphosphate kinase 1* (*IP3K1*), which encodes a kinase involved in oxidative stress responses in *Drosophila* (Seeds et al., 2004; Terhzaz et al., 2010). It is difficult to speculate how *IP3K1* could affect neural circuitry regulating the *detection-valuation-selection* steps involved in decision-making behavior pertaining to the *Drosophila* oviposition program. However, acetic acid can be chemically associated with oxidative stress pathways, and thus any alterations to how AA is handled with regard to oxidative stress responses could have modest effects on AA-related behaviors, as with the phenotypes observed in *17-31*.

8-174: raspberry (ras)

The *8-174* line exhibited a small trend towards a loss of egg-laying attraction, while exhibiting normal positional aversion to 5% AA. *8-174* mapped to the X-chromosome locus of *raspberry* (*ras*), which encodes an IMP-dehydrogenase/GMP-reductase that has been showed to regulate remodeling of the nervous system that is needed for appropriate axon guidance during *Drosophila* neural development (Long et al., 2006), and possibly plays a role in bristles development (Norga et al. 2003). As such, the phenotype of *8-174* could result from developmental defects associated with *ras* mutation.

Summary

With regard to positional aversion and egg-laying attraction to 5% AA, only *4-67* exhibited statistically significant modification in both behaviors when compared to controls(Figure 6), highlighting this line as a possible candidate gene that plays a direct role in decision-making processes related to *Drosophila* oviposition behavior. However, *3-21a* and *5-15* insertions also exhibited trends towards loss of egg-laying attraction and are known to express in the mushroom body; future experiments testing lobeline responses in these lines should thus prove interesting in identifying if *amn* plays a role in decision-making in the context of responses to bitter-tasting substances (Chapter 4). Of additional note, several lines that were isolated in the original genetic screen described in Section (*i*) above did not maintain significant decreases in egg-laying attraction to acetic acid, nor did they exhibit decreases in positional aversion. Although their behavioral trends are still promising, these results highlight the difficulty in using forward-genetics to identify specific genes directly involved in decision-making processes. Rather, it seems likely that several of the isolated mutations exert their effects by detrimentally affecting nervous system development, rather than acutely disrupting a gene directly involved in the decision-making process at the precise moment of selection between two competing behavioral preference responses.

Thus, to continue our search for neural circuits and integration centers that directly effect the *detection-valuation-selection* steps of decision-making between positional aversion and egg-laying attraction in *Drosophila*, we subsequently employed reverse-genetic *GAL4/UAS* screens that conditionally silenced neuronal structures, thereby eliminating developmental issues associated with homozygotic mutants.

***iii.*) Reverse-Genetic Screen for Brain Regions Important to Acetic Acid Responses**

The elimination of a single protein through genetic mutation does not necessarily eliminate overall signaling in a neuron that may be important to a particular behavioral response. Thus, using genetic mutants to identify neural circuitry involved in *Drosophila* decision-making behaviors can sometimes be an inefficient and thus risk endeavor. Fortunately, the when *Drosophila* enhancer trap libraries are employed in conjunction with *GAL4/UAS*-fusion silencing techniques described in Chapter 1, reverse-genetic screens can be performed in which entire neuronal subsets are silenced, and behavioral phenotypes are characterized. This method allows for more efficient identification of neural circuits involved in behavioral preference pathways. Furthermore, the expression of genes can be linked to these neuronal subsets through a variety of methods, such as RNA-interference. As such, we also conducted reverse-genetic screens for neuroanatomical structures important to either positional aversion or egg-laying attraction to AA. Ideally, we hoped to find structures that mediated both behaviors, which would therefore invoke the *central integration model* for competitive behavioral responses.

As described in Chapter 2, we conducted conditional silencing experiments using *UAS-Shibire*^{ts} to screen through 58 lines with *GAL4* expression in known neuronal subsets within the *Drosophila* brain. These candidate neurons included the mushroom body (MB), ellipsoid body (EB), subesophageal ganglion (SOG), and antennal lobe (AL), which have already been described in Chapter 1and Chapter 2. Furthermore, we also selected regions related to the central complex, including the fan-shaped body (FSB), protocerebral bridge (PCB), and neuronal noduli within the posterior brain. Briefly, the central complex is made up of the EB, PCB, FSB, noduli, and superior arch neurons that are interconnected at the

neural circuitry level (Hanesch et al., 1989). The EB sits anterior to the FSB and the PCB; dendrites from the EB project to the FSB, while nerve terminals from the PCB are received by the EB. Furthermore, the PCB also seems to send neurons to the noduli and the superior arch (Hanesch et al., 1989). Given the interconnected nature of these neurons, it is often difficult to separate behavioral responses among the different sub-structures of the central complex, despite the existence of very discrete *GAL4*-expressing lines (Renn et al., 1999).

From the 58 candidate *GAL4* lines, reliable comparisons with controls could be obtained for 27 strains; the remaining lines had either overlapping expression patterns that made neuronal structure identification difficult, or had problems with controls and/or viability with *UAS-Shibire^{ts}* that prevented the collection of useable behavioral data. A summary of the expression patterns and behavioral phenotypes of the 27 *GAL4/UAS-Shibire^{ts}* lines is provided below (Figure 7). Rather than discussing specific *GAL4* lines, we describe results and conclusions pertaining to the primary brain structures being silenced, since the was a reverse-genetic screen for neuroanatomical structures;

Mushroom Body (MB)

With regard to AA-based behavioral responses, the importance of the mushroom body (MB) in mediating egg-laying preference and its dispensability in relaying positional avoidance has already been discussed in Chapter 2. However, a few additional notes are worth mentioning. In particular, *mb247*, *17D*, *4-67*, and *8-239* did not show disruption in egg-laying attraction to 5% AA; however, closer analysis reveals that *GAL4* only expresses in partial subsets of the neuronal lobes that comprise the mushroom body in this line (Aso et al., 2009). Of the MB expressing lines that did lose egg-laying preference, these strains had

more complete coverage within the neuronal subsets that make up the MB as a whole (further discussion of the different lobes that could be affecting decision-making and/or egg-laying behaviors will be addressed in Chapter 4). Similarly, there were also lines like *201Y* and *OK107* that did not lay *any* eggs (Figure 7); the *GAL4*-expression in these lines is noticeably stronger than other lines. We hypothesize that, in addition to egg-laying site preferences, the MB may also be involved in more general oviposition mechanisms, and that excessive silencing of the neurons in the *201Y* and *OK107* expression pattern might cause a global shutdown of oviposition.

Alternatively, these expression patters are also very broad, and additional MB-related neurons that the other strains do not possess could be causing the defective behaviors. For example, it has recently been shown that octopamine is important to proper *Drosophila* oviposition (Lee et al., 2009). If *201Y* and *OK107* contained octopaminergic neurons that synapse on the MB, then silencing neurons within these *GAL4*-expression patterns could stop ovulation. Indeed, this is why we also tested ddc^{GAL4} (Yarali and Gerber, 2010), which expresses in dopaminergic and serotonin neurons in the *Drosophila* brain. When we originally began this study, before octopamine had been shown to be the neuropeptide critical to ovulation, it was thought that dopamine or serotonin could be necessary for egg-laying behaviors. However, our experiments revealed no effects in either egg-laying attraction or positional aversion when these neurons were silenced with $UAS\text{-}Shibire^{ts}$, suggesting that these neuropeptides play a minor role in egg-laying preference for AA. Likewise, we also noticed that there might be *GAL4*-expression in *pdf* neurons of the *5-120* and *5-98* lines; as such we tested pdf^{GAL4} (Taghert et al., 2001; Kaneko et al., 2005) with $UAS\text{-}Shibire^{ts}$ and also

saw no changes from wild-type behavioral responses, further substantiating the MB as being specifically required for normal egg-laying preferences for AA.

Surprisingly, some MB-*GAL4* lines demonstrated losses in positional aversion to AA, namely *8-239* and *4-64*. However, further imaging with *UAS-GFP* revealed that *8-239* may have some expression in the legs, where silencing could have locomotion-related effects that would affect positional aversion. Additionally, further imaging with *UAS-GFP* revealed that *4-64* also expresses strongly in the FSB, which may also be involved in positional aversion responses to both AA (line *11-33*; Figure 7) and lobeline (Figure 8). Thus, these positional phenotypes are likely not due to specific silencing of the mushroom body.

Ellipsoid Body (EB) and Fan-Shaped Body (FSB)

As described in Chapter 2, the ellipsoid body is required for the generation of appropriate positional aversion responses to AA. Indeed, all *EB-GAL4* lines (*4-67, 2-72, c232, C561,* and *11-27*) demonstrate disruptions in positional aversion to AA. This finding fits with previous researching detailing that the EB is important to spatial orientation and locomotor-related behaviors (Neuser et al. 2008; Kong et al., 2010). Additionally, *FSB-GAL4* lines also demonstrate losses in positional aversion, such as in the *11-33* and *4-64* lines, although further study will be necessary to see if these effects are specific to the FSB, or are derived from other neurons within the *GAL4* expression patterns (Figure 7). However, given the interconnectedness of the EB and the FSB in the central complex (Hanesch et al., 1989), and that both structures have been implicated in related memory-based visual behaviors (Pan et al., 2009), it appears possible that the FSB could also play a role in AA-based positional aversion responses.

Protocerebral Bridge (PCB)

Although the protocerebral bridge has been implicated in distance-related visual targeting of motor neurons, specifically with the assessment and decision to "jump" a gap that exceeds the fly's body length (Triphan et al., 2010). However, a potential role in decision-making behaviors associated with *Drosophila* oviposition is less clear, especially since visual sensory input is not considered a primary sensory modality required for both egg-laying and positional responses to AA, as demonstrated by infrared video recordings under dark conditions (Ryan M. Joseph, unpublished data). The PCB has also been shown to be important to walking speed and locomotion, so it could affect positional aversion in this manner (Poeck et al, 2008). However, of the 3 reliable *PCB-GAL4* lines tested, only 2 exhibited aberrant positional responses: *11-27* and *5-138*. Furthermore, of these 2 *PCB-GAL4* lines, *11-27* has *GAL4* expression in the EB and *5-138* has incredibly strong *GAL4* expression in the peripheral nervous system, thereby preventing any strong conclusions being drawn about the PCB.

Antennal Lobe (AL)

As expected, silencing lines with *GAL4* expression in the antennal lobe caused a disruption in the olfactory-based positional aversion response. Notably, *GH146* expresses in the secondary neurons that link the AL to the MB (Sen et al., 2005); silencing these cells with *UAS-Shibire*^{ts} caused females to lose their positional aversion to 5% AA (Figure 7). Interestingly, *5-40/UAS-Shibire*^{ts} flies did not lose positional aversion to 5% AA at the restrictive temperature (Figure 7). However, not all glomeruli express *GAL4* in the *5-40*

lines; thus the lack of behavioral phenotypes could be due to the GAL4 expression pattern in the 5-*40* line not covering the AA-detecting glomeruli. Alternatively, it has been recently shown that AA is also detected by sensory neurons that contain ionotropic receptors instead of olfactory receptors (Ai et al., 2010), suggesting the AA may be detected by more than one sensory neuron type. Thus, silencing a large portion of the AL still may not necessarily disrupt AA-based positional aversion.

Subesophageal Ganglion (SOG)

With regard to the subesophageal ganglion, it makes sense that silencing neurons in this brain region would disrupt taste-based egg-laying attraction to AA, given the role of the SOG in processing gustatory input (Miyazaki and Ito, 2010). Expectedly, the *2-6*, *4-15*, and *5-40* lines possessing *GAL4* expression in the SOG exhibited losses in egg-laying attraction to 5% AA (Figure 7).

Summary

Although the reverse-genetic screen for neural circuitry important to egg-laying attraction and positional aversion responses to 5% AA revealed numerous brain regions important to each response, we still did not identify a discrete brain region responsible for *both* behaviors, and subsequently could not draw conclusions regarding possible integration centers that perform the *valuation* or *selection* steps of a decision-making process based on the *central integration model* described in Chapter 1. Although we had identified two behavioral pathways that directly competed, the possibility remained that a common integration center might be difficult to find because the two competing pathways proceeded

through neural circuits governed by different sensory modalities: olfaction and gustation. As such, there may be too much non-overlapping neural circuitry to cause a complete disruption in both the smell-based positional aversion and taste-based egg-laying attraction responses, even if a central integration center was in fact knocked out by our silencing techniques.

iv.) Reverse-Genetic Screen for Brain Regions Important to Lobeline Responses

Given that competing AA-based responses in our two-choice paradigm utilize independent olfactory and gustatory systems to detect competing environmental cues, there may be too much independent neural circuitry to cause a complete disruption in both the smell-based positional aversion and taste-based egg-laying attraction responses, even if a central integration center was in fact silenced in our *GAL4/UAS-Shibire*^{ts} experiments. We hypothesized that, if we could identify competing behavioral responses that were induced by sensory circuits and relay neurons within a single sensory modality—such as gustation—then there would be a higher likelihood that the neural circuits of the competing preference pathways would converge in common centers within the brain. As a result, there would be a higher possibility that silencing these common centers could simultaneously disrupt both the positional aversion and egg-laying attraction responses, thereby implicating these brain regions as possible decision-making centers.

Fortunately, we had noticed that bitter compounds like lobeline and quinine simultaneously induced competing positional aversion and egg-laying attraction responses in our two-choice assay (Chapter 4). Since lobeline and quinine are known for their qualities as non-appetitive tastants (Detzel and Wink, 1993; Sellier et al., 2010), rather than volatile odorants, we reasoned that both behavioral responses proceeded through the gustatory system

and therefore had a higher likelihood of intersecting in the *Drosophila* brain for concurrent evaluation. In addition to expanding our behavioral paradigm to work with bitter-tasting compounds (Chapter 4), we conducted a reverse-genetic neuroanatomical screen for brain regions important to positional aversion and egg-laying attraction to 0.50mM lobeline.

We assayed *GAL4/UAS-Shibire^{ts}* females as described in Materials and Methods of Chapter 4, screening 29 lines for the same brain regions discussed above in Section (*iii*): the MB, EB, FSB, PCB, and SOG (the AL was excluded after initial trials revealed olfaction was not required for either the positional aversion and egg-laying attraction response to lobeline). A summary of the expression patterns and behavioral phenotypes of the 29 *GAL4/UAS-Shibire^{ts}* lines is provided below (Figure 8). Rather than discussing specific *GAL4* lines, we describe results and conclusions pertaining to the primary brain structures being silenced, since the was a reverse-genetic screen for neuroanatomical structures;

Mushroom Body (MB)

Results from *UAS-Shibire^{ts}* experiments involving the mushroom body will be a primary source of study in Chapter 4, and detailed discussion will be reserved for that section of the thesis. In summary, the MB is involved in both positional aversion and egg-laying attraction to 0.50mM lobeline (Figure 8), including two lines that show a simultaneous disruption in both behaviors: *5-120* and *30Y*. These two lines cover most neuronal subsets within the MB (Kaun et al., 2011; Aso et al., 2009), and provide evidence that the MB is an intersection point in the gustatory related neural circuits that mediate the competition positional aversion and egg-laying attraction responses. Furthermore, these findings suggest that the MB may act as an integration center that performs decision-making processes related

to the *Drosophila* oviposition program. Furthermore, tentative conclusions can be drawn about which neuronal lobes are responsible for receiving input from either the repulsive or attractive preference pathway (Chapter 4: Figure S6), based on the expression patterns of the different *MB-GAL4* lines tested.

Subesophageal Ganglion (SOG)

Interestingly, not all *GAL4* lines with expression in the SOG exhibited a disruption the taste-based positional aversion and egg-laying attraction behaviors, as one would expect given the role of the SOG as a gustatory processing center that receives bitter-sensing input from *Gr66a* neurons (Miyazaki and Ito, 2010). However, closer examination of expression patterns of *SOG-GAL4* lines revealed that flies with *GAL4* expression in the upper/dorsal region of the SOG (*71Y*, *c687*, and *11-81*) tended to lose behavioral responses to lobeline, while flies with *GAL4* expression in the lower/ventral region of the SOG (*5-43* and *2-6*) tended to exhibit normal behavioral responses to lobeline (Ryan M. Joseph, unpublished observations). These results suggest that neurons in the upper/dorsal SOG may be the relay point in neural circuitry governing both responses; this conclusion is supported by studies showing the lobeline-detecting *Gr66a* neurons do send axons to these regions of the SOG (Thorne et al., 2004; Wang et al., 2004; Sellier et al. 2010; Miyazaki and Ito, 2010).

Fan-Shaped Body (FSB)

Compared to the AA-based screen performed in Section (*iii*) above, we conducted more *FSB-GAL4* experiments in this lobeline-based screen, in order to better ascertain the role of the FSB in executing avoidance-related positional behaviors. Indeed, we found that

most *FSB-GAL4* lines exhibited disrupted positional aversion to lobeline when the FSB was silenced with *UAS-Shibire*^{ts} (Figure 8). Surprisingly, most lines also exhibited some degree of aberrant egg-laying attraction to lobeline (Figure 8), including *OK348,* which actually demonstrated *increased* egg-laying preference for the bitter compound. However, a caveat with most of these lines was that they also possessed *GAL4* expression in the upper regions of the SOG, which connect to the pars-intercerebralis. Our findings with *SOG-GAL4* lines suggest that this region operates in the relay of sensory information through the egg-laying pathway; as such, conclusions regarding the FSB as a possible integration center for both the positional aversion and egg-laying attraction pathways are not as strong as those associated with the MB. Still, *OK348* and *3-15* lacked this upper-SOG *GAL4* expression, therefore suggesting that the FSB may be an interesting structure for future study in the *Drosophila* oviposition decision-making paradigm.

Ellipsoid Body (EB)

Interestingly, the same *EB-GAL4* lines that caused a loss of AA-induced positional aversion when silenced by also caused a loss of lobeline-induced positional aversion when inactivated by *UAS-Shibire*^{ts} (Figure 8). This finding supports the notion that the EB is required for general execution of multiple locomotor-related behavioral responses (Neuser et al. 2008; Kong et al., 2010).

Protocerebral Bridge (PCB)

Initially, during the screen we identified *5-138* as having a dramatic loss in both positional aversion and egg-laying attraction to lobeline, and hypothesized that the PCB

could also be a candidate integration and/or decision-making center. However, subsequent analysis of the peripheral nervous system in *5-138* females revealed incredibly strong *GAL4* expression in the legs, including in sensory neurons that appeared to overlap with *Gr66a* neurons. Thus, the loss in behavioral responses was likely due to silencing of sensory neurons, as in $Gr66a^{GAL4}/UAS\text{-}Shibire^{ts}$ flies (Chapter 4: Figure 2).

Summary

The reverse-genetic screen for brain regions important to lobeline-based responses achieved what we had intended it to achieve: the identification of higher order brain structures important to both positional aversion and egg-laying attraction to lobeline. As such, the mushroom body was identified as an intersection point between the competing behavioral responses, and thus could potentially act as a decision-making center according to the *central integration model* described in Chapter 1. In addition, the FSB may also be an intersection point between the two preference pathways; further investigations will be required to determine if this is the case.

DISCUSSION

Most discussion regarding the genes and neurons identified in the aforementioned screens have already been addressed in the Results and Summary section. One issue worth noting is the fact that relatively few mutants were obtained from both of the forward-genetic screens. In addition to the difficulties associated with identifying gene sequences directly involved in decision making processes (described in the Summary of Section (*ii*) above), the possibility also remains that our experimental conditions and statistical requirements were

too stringent, as we only had a mutant identification rate of 7/550 lines, roughly equal to 1.3%, which is surprisingly low. Thus, future work may entail repeating the screen with modified parameters.

MATERIALS AND METHODS

Fly Stocks

Flies for the forward-genetic mutant screens and reverse-genetic neuroanatomy screens were flies were reared in constant light, 25°C, 70% humidity on cornmeal/molasses/yeast food. For all screens described in Section (*i*) and Section (*ii*), lines from our laboratory's P-element insertion library were used (collection was generated using $P\{GAL4^{GawB}\}$ transposon element in the *white-Berlin* background). Lines tested in Section (*ii*) were backcrossed for five generations to the *white-Berlin* background employed as a control in these experiments. In addition to the $P\{GAL4^{GawB}\}$ strains from our library, additional *GAL4* lines were also tested in the neuroanatomy screens detailed in Section (*iii*) and Section (*iv*): *71Y* and *104Y* are from lines used in Young and Armstrong, 2010; *c687* is the from the line studied in Foltenyi et al., 2007; *OK348* is the line studied in Connolly et al., 1996; $GH146^{GAL4}$ is the line studied in Sen et al., 2005; pdf^{GAL4} is the line studied in Taghert et al., 2001; *007Y* is studied in Poeck et al., 2008; fru^{GAL4} was provided by the authors of Manoli et al., 2005; *201Y, 30Y, OK107, mb247* are described in detail in Aso et al., 2009; *c232* and *C561* are studied in Renn et al., 1999). These lines were typically backcrossed to the *white-Berlin* background used by our laboratory before being assayed.

Forwards-Genetic Mutant Screens

In the initial forward-genetic screen for homozygotic mutant females with defective egg-laying behavior, the assays in Section (*i*) were performed according to the protocol detailed in Chapter 2, with the following modifications: (1) 3% acetic acid was used, (2) positional indexes were not calculated. A screen average of oviposition indexes was calculated, and a cutoff of three standard deviations from this mean was assigned for determining if a particular line was mutant. In the secondary-screen for homozygotic mutant females with defective egg-laying behavior, the assays in Section (*ii*) were performed as described in Chapter 2, using 5% acetic acid. Individual unpaired t-tests (two-tailed) were performed between average oviposition indexes and position indexes of *white-Berlin* controls and each mutant line.

Reverse-Genetic Neuroanatomy Screen

With regard to the reverse-genetic neuroanatomy screens, experiments were performed as described by Materials and Methods in Chapter 2 for the AA-based screen and Chapter 4 for the lobeline-based screen. In the AA-based screen, differences were dramatic enough that statistical analysis were not run unless necessary, in which case 2-Way ANOVA's with Bonferonni post-test were run to compare OI-values and PI-values of different temperature columns within the same genotype, while 1-Way ANOVA's with Bonferonni post-test were run to compare OI-values and PI-values between different genotypes of the sample temperature group. In the lobeline-based screen, these statistical analysis were typically performed for all lines tested.

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FIGURE LEGENDS

Figure 1: Forward-Genetic Screen for Egg-laying Preferences Towards 3% Acetic Acid

550 mutant lines from a *GAL4*-expressing P-element library were tested for egg-laying attraction towards 3% acetic acid (AA). Two-choice assays were conducted and an oviposition index (OI) for each line was calculated as described in Chapter 2, with the exception that 3% AA was used instead of 5% AA for the genetic screen. Experiments were repeated on independent days to ensure reproducibility of results; $n \geq 2$ for most lines in which $OI > +0.82$. If a particular line had an $OI < +0.82$, additional experiments were performed to obtain at least $n \geq 4$, in order to confirm that the decrease in egg-laying preference was a real effect. OI values for each line tested are plotted in descending order, ending in the 7 mutant lines with the largest decrease in egg-laying preference: *5-131, 10-184*, *3-41b*, *4-76b*, *4-20*, *10-142*, and *3-21a*. The light blue line labels the screen average for egg-laying attraction (OI=+0.896). The dark blue line labels the *white-Berlin* egg-laying attraction (OI=+0.901). The red line labels the cutoff value for classifying a line as mutant (OI=+0.678), which was assigned as three standard deviations from the screen average (standard deviation=0.0726).

Figure 2: Egg-laying Preferences of Seven Candidate Mutants Towards 3% Acetic Acid

A magnified representation of the oviposition indexes of the 7 candidate mutants pulled from the screen shown in Figure 1. The screen average and *white-Berlin* OI values are shown for comparison. *3-21a* is labeled red to distinguish that this candidate exhibited an OI value equal to the calculated cutoff that determines if a line has a mutant phenotype.

Figure 3: List of Oviposition Indexes for 550 Tested Mutants in Egg-Laying Screen

A complete list of all the lines tested and their corresponding oviposition indexes (OI) in the forward-genetic screen for egg-laying responses to 3% AA. Mutant lines are grouped numerically for ease of identification; the 6 clear mutants are colored yellow, the 1 borderline mutant is colored red, and *white-Berlin* is colored blue. n≥2 for all lines, unless otherwise specified by n=1 label in the column containing the standard error of the mean (S.E.M.).

Figure 4: Secondary-Screen for Mutants in Positional Aversion to Acetic Acid

42 mutants from a *GAL4*-expressing P-element library were tested for positional aversion to 5% acetic acid (AA). Two-choice assays were conducted and a positional index (PI) for each line was calculated as described in Chapter 2. Experiments were repeated on independent days to ensure reproducibility of results; n≥3 for most lines (See Figure 6 for specific sample sizes). Average PI-values were compared to the mean PI-value obtained for *white-Berlin* (light-blue). Significant increases in positional aversion were observed in 4 mutants (orange), although trends were observed in others as well (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; unpaired t-test (two-tailed); n≥2). Error bars are omitted for clarity.

Figure 5: Secondary-Screen for Mutants in Egg-laying Attraction to Acetic Acid

42 mutants from a *GAL4*-expressing P-element library were tested for egg-laying aversion to 5% acetic acid (AA). Two-choice assays were conducted and a oviposition index (OI) for each line was calculated as described in Chapter 2. Experiments were repeated on independent days to ensure reproducibility of results; n≥3 for most lines (See Figure 6 for specific sample sizes). Average OI-values were compared to the mean PI-value obtained for

white-Berlin (blue). Significant increases in positional aversion were observed in 6 mutants (orange), although trends were observed in others as well (*, P<0.05; **, P<0.01; ***, P<0.001; unpaired t-test (two-tailed); n≥2). Error bars are omitted for clarity.

Figure 6: List of Positional Indexes and Oviposition Indexes for 42 Mutants Tested in Secondary-Screen for Responses to Acetic Acid

A complete list of all the lines tested and their corresponding position indexes (PI) and oviposition indexes (OI) in the secondary screen for behavioral responses to 5% AA. Mutant lines are grouped numerically for ease of identification. Yellow labels P<0.05 significance when compared to *white-Berlin* for either PI or OI. Orange labels P<0.01 significance when compared to *white-Berlin* for either PI or OI. Red labels P<0.01 significance when compared to *white-Berlin* for either PI or OI. Blue labels *white-Berlin* index values. S.E.M = standard error of the mean.

Figure 7: Neuroanatomical Screen for Brain Structures Responsible for Acetic Acid Induced Positional Aversion and Egg-laying Attraction Responses.

Neuronal subsets of 25 *GAL4* lines with known expression patterns in discrete *Drosophila* brain regions were silenced using *UAS-Shibire*^{ts}, and were assayed for disrupted behavioral responses to 5% acetic acid (AA). Black boxes indicate strong *GAL4* expression in a particular brain structure. Gray boxes indicate moderate *GAL4* expression in a particular brain structure. SOG = subesophageal ganglion, AL = antennal lobe, MB = mushroom body, EB = ellipsoid body, FSB = fan-shaped body, PCB = protocerebral bridge, VNC/Legs = the ventral nerve cord and/or expression in leg-neurons, ser/dopa = expression in serotonin and

dopaminergic neurons, PDF = expression in *pdf* neurons. OI = oviposition index; PI = position index. LOSS indicates that silencing caused a disruption in either egg-laying (OI) or positional (PI) responses to AA. wt = wild-type behavioral response. na = data was not able to be obtained, since flies did not lay eggs or had developmental defects. Of particular note, *11-33* homozygote flies exhibited mutant egg-laying responses to AA, but were normal in the *11-33/UAS-Shibire^{ts}* females.

Figure 8: Neuroanatomical Screen for Brain Structures Responsible for Lobeline Induced Positional Aversion and Egg-laying Attraction Responses.

Neuronal subsets of 29 *GAL4* lines with known expression patterns in discrete *Drosophila* brain regions were silenced using *UAS-Shibire^{ts}*, and were assayed for disrupted behavioral responses to 0.50mM lobeline. Black boxes indicate strong *GAL4* expression in a particular brain structure. Gray boxes indicate moderate *GAL4* expression in a particular brain structure. SOG = subesophageal ganglion, AL = antennal lobe, MB = mushroom body, EB = ellipsoid body, FSB = fan-shaped body, PCB = protocerebral bridge, VNC = ventral nerve cord, Legs = expression in leg-neurons. OI = oviposition index; PI = position index. LOSS indicates that silencing caused a disruption in either egg-laying (OI) or positional (PI) responses to lobeline. wt = wild-type behavioral response. na = data was not able to be obtained, since flies did not lay eggs or had developmental defects. Of note, *OK348* exhibited an increase (indicated by GAIN) in egg-laying preference. 2-Way ANOVA's with Bonferonni post-test were used to compare indexes of experimental females versus control females of different temperature columns within the same genotype; 1-Way ANOVA's with Bonferonni post-test were used to compare indexes of experimental females versus control

females between different genotypes of the same temperature group; typically n≥4 for each trial.

Figure 1

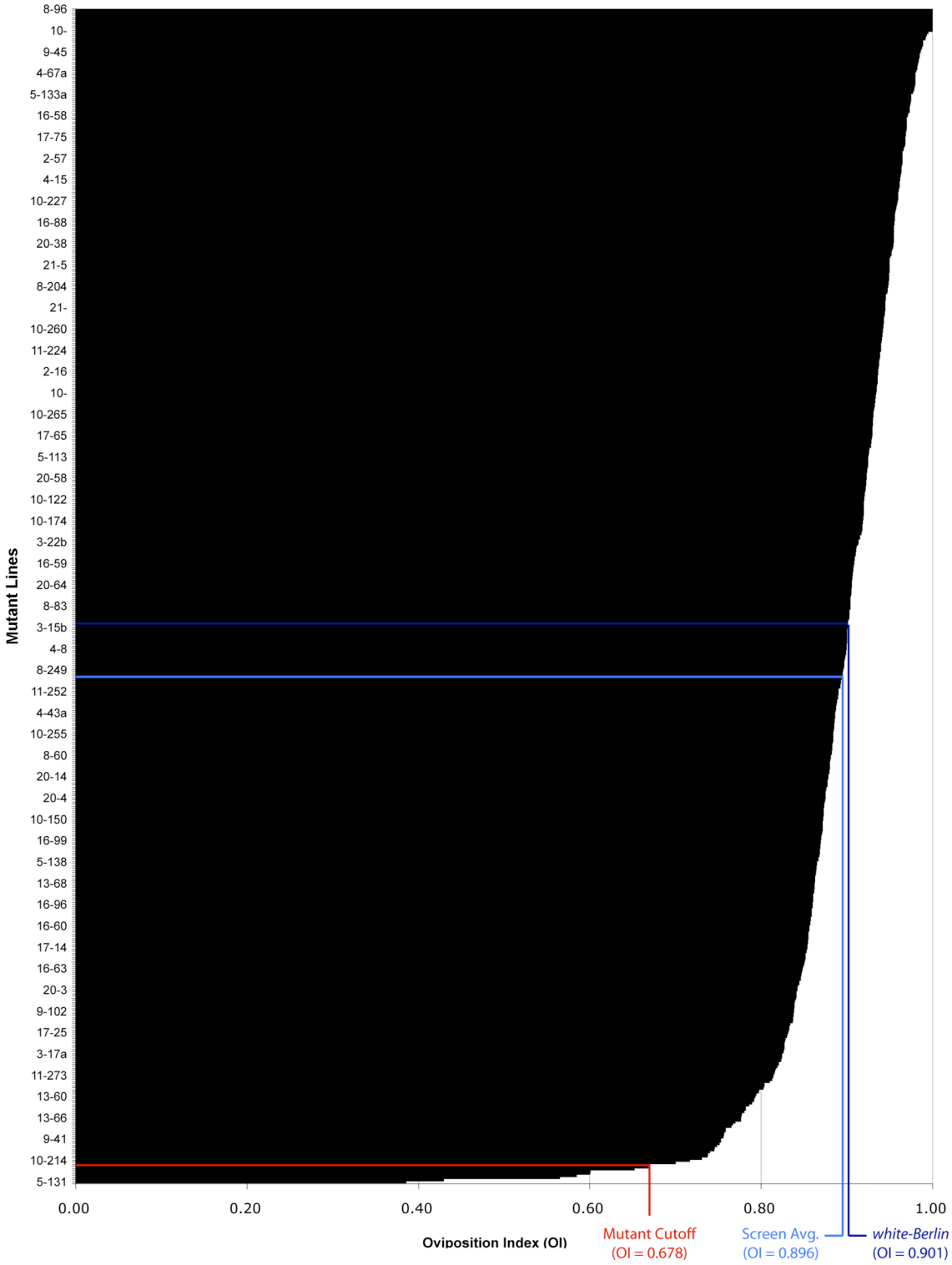

Mutant Lines
8-96
10-
9-45
4-67a
5-133a
16-58
17-75
2-57
4-15
10-227
16-88
20-38
21-5
8-204
21-
10-260
11-224
2-16
10-
10-265
17-65
5-113
20-58
10-122
10-174
3-22b
16-59
20-64
8-83
3-15b
4-8
8-249
11-252
4-43a
10-255
8-60
20-14
20-4
10-150
16-99
5-138
13-68
16-96
16-60
17-14
16-63
20-3
9-102
17-25
3-17a
11-273
13-60
13-66
9-41
10-214
5-131
0.00
0.20
0.40
0.60
0.80
1.00
Oviposition Index (OI)
Mutant Cutoff
(OI = 0.678)
Screen Avg.
(OI = 0.896)
white-Berlin
(OI = 0.901)

Figure 2

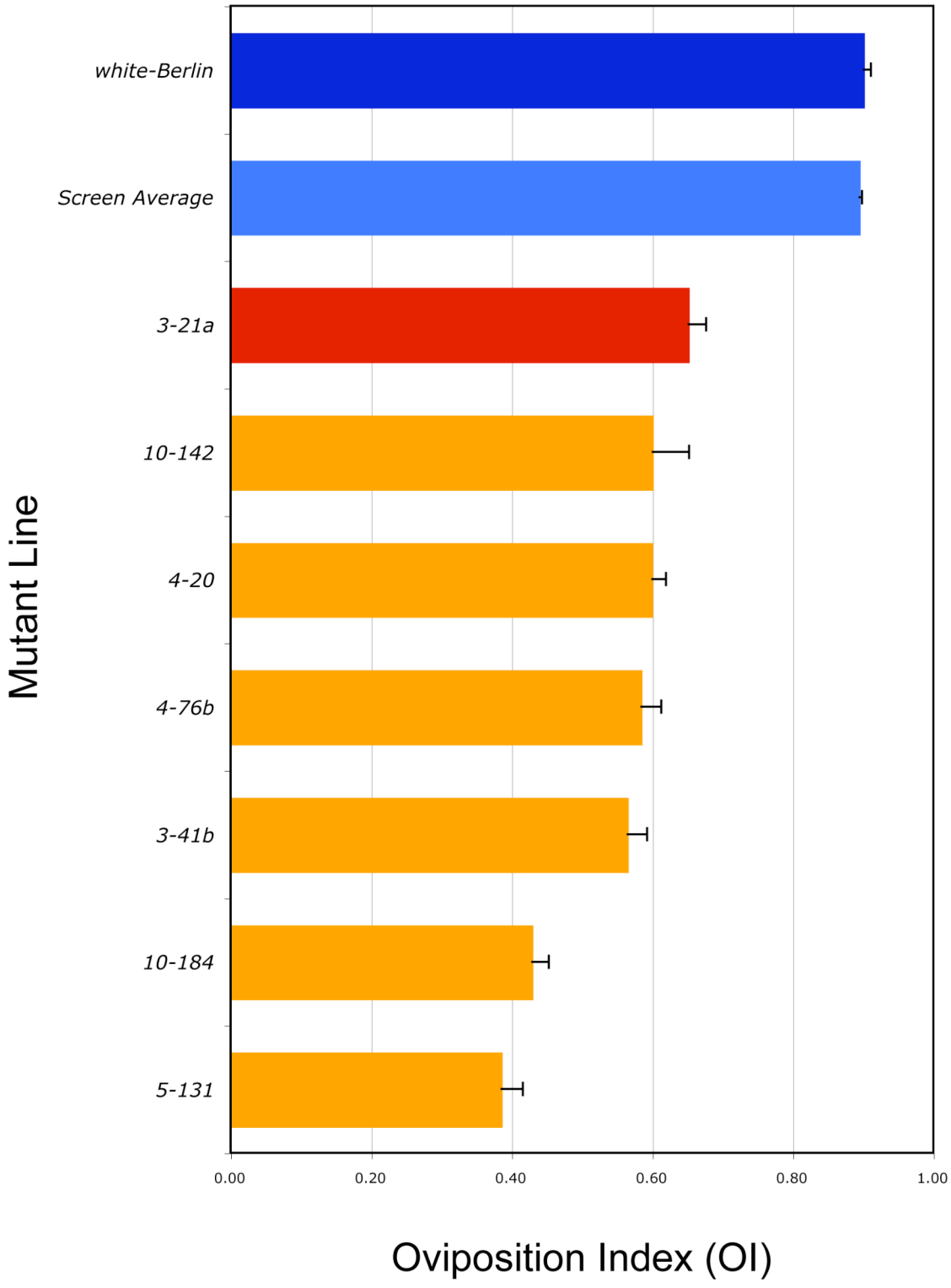

white-Berlin
Screen Average
3-21a
10-142
4-20
4-76b
3-41b
10-184
5-131
Mutant Line
0.00
0.20
0.40
0.60
0.80
1.00
Oviposition Index (OI)

Figure 3

Line	*OI*	*S.E.M*
2-10*	0.92	0.0330
2-11	0.92	0.0442
2-12	0.93	0.0346
2-13	0.96	0.0287
2-14	0.89	0.0468
2-15	0.96	0.0075
2-16	0.94	0.0055
2-2	0.84	0.0127
2-21	0.98	0.0179
2-27	0.96	0.0045
2-28	0.97	0.0194
2-29	0.97	0.0106
2-3	0.85	0.1158
2-30	0.95	0.0129
2-31	0.81	0.0285
2-32	0.90	0.0171
2-33	0.92	0.0139
2-34	0.98	0.0156
2-35	0.88	0.0251
2-4	0.86	0.0503
2-57	0.97	0.0096
2-6	0.76	0.0305
2-69	0.90	0.0320
2-79	0.97	0.0096
2-8	0.85	0.0978
2-9	0.96	0.0361
3-1	0.95	0.0282
3-11b	0.88	0.0581
3-15b	0.90	0.0259
3-16	0.83	0.0408
3-17a	0.82	0.0388
3-18b	0.86	0.0750
3-2	0.96	0.0310
3-20	0.99	0.0132
3-20b	0.99	0.0111
3-21a	0.65	0.0494
3-22b	0.91	0.0038
3-23	0.84	0.0319
3-26	0.93	0.0009
3-27	0.83	0.0653
3-29b	0.87	0.0844
3-30	0.76	0.0493
3-41b	0.57	0.0554
3-46a	0.92	0.0216
3-48a	0.96	0.0082
3-5	0.98	0.0080
3-62a	0.86	0.0183
3-77	0.77	0.0541

Line	*OI*	*S.E.M*
3-8	0.88	0.0066
3-81	0.90	0.0141
3-81	0.90	0.0063
3-89b*	0.90	0.0216
3-9	0.98	0.0071
4-10	0.91	0.0374
4-11	0.88	0.0174
4-12a	0.67	0.0609
4-13b	0.94	0.0265
4-14	0.96	0.0258
4-15	0.96	0.0268
4-17	0.88	0.0581
4-1a	0.94	0.0278
4-20	0.60	0.0387
4-22	0.87	0.0135
4-24	0.86	0.0350
4-25b	0.82	0.0257
4-26	0.86	0.0225
4-26	0.97	0.0050
4-27	0.87	0.0172
4-28	0.98	0.0200
4-29	0.81	0.0939
4-2b	0.92	0.0226
4-3	0.87	0.0292
4-30	0.82	0.0267
4-32	0.78	0.2222
4-33	0.78	0.0599
4-35b	0.83	0.0644
4-36	0.92	0.0417
4-4	0.75	0.0645
4-42	0.89	0.0071
4-43a	0.89	0.0315
4-43a	0.94	0.0245
4-44b	0.83	0.0658
4-47	0.93	0.0016
4-48	0.95	0.0182
4-49	0.90	0.0258
4-5	1.00	0.0050
4-50	0.82	0.0503
4-52a	0.90	0.0584
4-54a	0.87	0.0549
4-56	0.94	0.0410
4-58	0.88	0.0848
4-59	0.84	0.0467
4-6	0.83	0.0283
4-65a	0.95	0.0126
4-67a	0.98	0.0141
4-7	0.95	0.0196

Figure 3 (Continued)

Line	OI	S.E.M
4-76b	0.59	0.0561
4-8	0.90	0.0151
5-1	0.86	0.0435
5-10	0.91	0.0293
5-11	0.89	8.9815
5-113	0.93	0.0189
5-128	0.97	0.0222
5-131	0.39	0.0601
5-133a	0.98	0.0126
5-134	0.84	0.0928
5-134	0.95	0.0126
5-135	0.83	0.0115
5-136	0.83	0.0450
5-136	0.96	0.0160
5-138	0.87	0.0054
5-14	0.86	0.0241
5-140a	0.85	0.0192
5-142	0.75	0.1329
5-15	0.78	0.0219
5-16a	0.88	n=1
5-17b	0.90	0.0293
5-21	0.86	0.0931
5-3b	0.95	0.0208
5-5	0.96	0.0206
5-61	0.98	0.0126
5-72	0.88	0.0650
5-84	0.99	0.0050
8-1	0.89	0.0576
8-12	0.90	0.0112
8-16	0.90	0.0370
8-17	0.79	0.0067
8-2	0.80	0.0240
8-204	0.95	0.0162
8-206	0.86	0.0279
8-212	0.93	0.0185
8-219	0.88	0.0376
8-23	0.93	0.0029
8-233	0.97	0.0120
8-249	0.90	0.0343
8-27	0.94	0.0390
8-29	0.90	0.0341
8-3	0.96	0.0370
8-32	0.85	0.0400
8-34	0.92	0.0561
8-36	0.87	0.1125
8-37	0.84	0.0006
8-38	0.70	n=1
8-4	0.84	0.0207

Line	OI	S.E.M
8-42	0.94	0.0105
8-44	0.89	0.0798
8-46	0.98	0.0027
8-47	0.79	0.0212
8-49	0.87	0.0269
8-5	0.93	0.0482
8-50	0.77	0.0346
8-51	0.95	0.0036
8-52	0.90	0.0221
8-55	0.95	0.0209
8-59	0.99	0.0085
8-6	0.91	0.0581
8-60	0.88	0.0136
8-61	0.96	0.0183
8-62	0.88	0.0496
8-65	0.86	0.0340
8-68	0.96	0.0390
8-70	0.90	0.0467
8-72	0.90	0.0532
8-74	0.77	0.0375
8-76	0.92	0.0486
8-77	0.86	0.0094
8-79	0.93	0.0033
8-80	0.93	0.0694
8-81	0.95	0.0270
8-82	0.87	0.0408
8-83	0.90	0.0467
8-85	0.90	0.0200
8-86	0.86	0.0433
8-87	0.87	0.0220
8-88	0.95	0.0138
8-89	1.00	0.0000
8-91	0.91	0.0009
8-92	0.90	0.0326
8-96	1.00	0.0000
9-1	0.88	0.0390
9-10	0.94	0.0043
9-101	0.89	0.0594
9-102	0.84	0.0394
9-105	0.84	0.0209
9-106	0.95	0.0476
9-118	0.97	0.0049
9-12	0.91	0.0086
9-13	0.89	0.0259
9-14	0.85	0.0435
9-140	0.91	0.0258
9-148*	0.92	0.0219
9-15	0.87	0.0505

Figure 3 (Continued)

Line	OI	S.E.M
9-150*	0.88	0.0358
9-16	0.87	0.0169
9-17	0.86	0.0366
9-18	0.88	0.0014
9-19	0.90	0.0286
9-2	0.96	0.0120
9-20	0.97	0.0300
9-21	0.93	0.0438
9-23	0.78	0.0375
9-24	0.82	0.0819
9-26	0.91	0.0041
9-27	0.92	0.0203
9-28	0.83	0.0549
9-29	0.90	0.0276
9-3	0.85	0.0569
9-30	0.93	0.0459
9-31	0.86	0.0058
9-33	0.90	0.0172
9-34	0.85	0.0466
9-35	0.95	0.0362
9-36	0.89	0.0348
9-37	0.92	0.0800
9-38	0.79	0.0323
9-39	0.94	0.0566
9-40	0.85	0.0026
9-41	0.75	0.0377
9-42	0.91	0.0062
9-43	0.82	0.0312
9-44	0.96	0.0075
9-45	0.99	0.0139
9-46	0.92	0.0325
9-48	0.97	0.0057
9-49	0.94	0.0141
9-50	0.90	0.0215
9-52	0.99	0.0120
9-53	0.94	0.0214
9-54	0.97	0.0129
9-55	0.87	0.0324
9-58	0.95	0.0100
9-59	0.91	0.0267
9-60	0.84	0.0484
9-61	0.94	0.0093
9-62	0.92	0.0392
9-63	0.95	0.0500
9-64	0.92	0.0075
9-65	0.91	0.0409
9-7	0.75	0.0374
9-71	0.98	0.0075

Line	OI	S.E.M
9-8	0.86	0.0592
9-9	0.83	0.0876
10-102	0.89	0.0482
10-103	0.75	0.0462
10-107	0.87	0.0334
10-110*	0.78	0.0520
10-111	0.94	0.0180
10-112	0.82	0.0255
10-113	0.92	0.0273
10-114	0.92	0.0354
10-115	0.91	0.0338
10-119	0.91	0.0206
10-122	0.92	0.0071
10-123	0.90	0.0769
10-124*	1.00	0.0000
10-125*	0.92	0.0406
10-126*	0.90	0.0697
10-127	0.97	0.0111
10-129	0.87	0.0464
10-131	0.88	0.0453
10-132	0.92	0.0350
10-133	0.90	0.0581
10-135*	0.93	0.0299
10-136	0.88	0.0441
10-138	1.00	0.0000
10-140	1.00	0.0000
10-141	0.86	0.0579
10-142	0.60	0.1036
10-143	0.93	0.0456
10-144	0.98	0.0170
10-148	0.89	0.0399
10-149	0.95	0.0462
10-150	0.87	0.0675
10-151	0.98	0.0086
10-152*	0.92	0.0451
10-153	0.94	0.0556
10-155*	0.97	0.0270
10-156	1.00	0.0000
10-157	0.88	0.1005
10-159	0.96	0.0078
10-161	0.85	0.0383
10-164	0.86	0.0624
10-168	0.92	0.0407
10-169	0.84	0.0532
10-172	0.92	0.0417
10-174	0.92	0.0325
10-175	0.86	0.0416
10-179*	0.94	0.0388

Figure 3 (Continued)

Line	OI	S.E.M
10-182*	0.93	0.0437
10-183	0.88	0.0607
10-184	0.43	0.0473
10-185	0.93	0.0563
10-186	0.89	0.0093
10-187	0.93	0.0215
10-188	0.80	0.0301
10-189	0.94	0.0061
10-190	0.86	0.0396
10-191	0.96	0.0449
10-192	0.91	0.0376
10-193	0.85	0.0194
10-194	0.90	0.0360
10-195	0.91	0.0659
10-196	0.94	0.0131
10-197	0.98	0.0098
10-198	0.87	0.0233
10-199	0.94	0.0354
10-200	0.94	0.0044
10-201	0.95	0.0228
10-206	0.82	0.0656
10-209	0.87	0.0982
10-211	0.88	0.0151
10-213	0.73	0.0430
10-214	0.72	0.0780
10-215	0.93	0.0264
10-217	1.00	0.0000
10-218	0.92	0.0446
10-219	0.95	0.0185
10-223	0.89	0.0471
10-227	0.96	0.0278
10-228	0.89	0.0522
10-232	0.84	0.0298
10-234	0.95	0.0011
10-235	0.94	0.0256
10-237	0.90	0.0698
10-238	0.92	0.0450
10-247	0.94	0.0597
10-249	0.87	0.0427
10-251	0.97	0.0259
10-255	0.88	n=1
10-256	0.96	0.0403
10-257	0.93	0.0389
10-258	0.94	0.0588
10-259	0.94	0.0631
10-260	0.94	0.0577
10-263	1.00	0.0000
10-265	0.93	0.0691

Line	OI	S.E.M
10-267	1.00	0.0000
10-268	0.94	0.0116
10-270	0.90	0.0537
10-271	0.92	0.0181
10-290	0.98	0.0126
11-206	0.95	0.0208
11-211	0.93	0.0206
11-216	0.96	0.0000
11-224	0.94	0.0316
11-241	0.89	1.4142
11-244*	0.96	0.0263
11-247*	0.96	0.0096
11-250*	0.95	0.0173
11-252	0.89	2.4495
11-273	0.81	0.0497
11-288	0.92	0.0330
13-55	0.86	0.0700
13-56	0.86	0.0016
13-57	0.91	0.0636
13-58	0.86	0.0098
13-59	0.90	0.0431
13-60	0.79	0.0550
13-61	0.84	0.0128
13-62	0.91	0.0267
13-64	0.91	0.0454
13-66	0.78	0.0430
13-67	0.88	0.0485
13-68	0.86	0.0011
14-111	0.97	0.0100
14-113	0.98	0.0126
14-19	0.91	0.0289
14-46	0.97	0.0171
14-85	0.88	0.0408
14-87	0.97	0.0191
16-100	0.95	0.0124
16-52	0.92	0.0564
16-55	0.86	0.0006
16-57	0.67	0.0301
16-58	0.97	0.0010
16-59	0.91	0.0323
16-60	0.86	0.0552
16-63	0.85	0.0152
16-65	0.90	0.0479
16-66	0.85	0.0095
16-67	0.88	0.0815
16-68	0.93	0.0484
16-69	0.84	0.0554
16-71	0.74	0.0366

Figure 3 (Continued)

Line	*OI*	*S.E.M*
16-72	0.97	0.0008
16-73	0.76	0.0242
16-75	0.91	0.0067
16-78	0.99	0.0065
16-79	0.96	0.0187
16-80	0.93	0.0200
16-81orig	0.88	0.0003
16-81reb	0.96	0.0026
16-82	0.90	0.0149
16-83	0.92	0.0385
16-84	0.97	0.0143
16-85	0.86	0.0504
16-86	0.94	0.0097
16-87	0.94	0.0641
16-88	0.96	0.0300
16-90	0.82	0.0713
16-91	0.99	0.0080
16-92	0.90	0.0120
16-94	0.95	0.0113
16-95	0.95	0.0500
16-96	0.86	0.0210
16-97	0.98	0.0200
16-98	0.92	0.0292
16-99	0.87	0.0098
17-1	0.88	0.0352
17-101	0.87	0.0300
17-102	0.89	0.0207
17-103	0.87	0.0824
17-104	0.74	0.0319
17-107	0.93	0.0037
17-108	0.90	0.0341
17-11	0.92	0.0106
17-110	0.87	0.0258
17-111	0.96	0.0375
17-113	0.84	0.0452
17-114	0.94	0.0634
17-117	0.76	0.0256
17-14	0.85	0.0766
17-15	0.87	0.0846
17-16	1.00	0.0000
17-177	0.80	0.0433
17-18	0.94	0.0274
17-19	0.94	0.0301
17-21	0.86	0.0169
17-23	0.93	0.0222
17-24	0.96	0.0385
17-25	0.83	0.0408
17-26	0.97	0.0014

Line	*OI*	*S.E.M*
17-27	0.96	0.0030
17-29	0.98	0.0167
17-3	0.84	0.0570
17-31	0.85	0.0422
17-32	0.80	0.0195
17-33	0.83	n=1
17-34	0.83	0.0352
17-37	0.95	0.0328
17-38	0.99	0.0110
17-39	0.95	0.0131
17-4	0.93	0.0351
17-42	0.94	0.0169
17-43	0.95	0.0180
17-43	0.99	0.0050
17-44	0.89	0.0416
17-45	0.97	0.0057
17-46	0.92	0.0408
17-49	0.90	0.0152
17-5	0.81	0.0176
17-51	0.87	0.0313
17-54	0.98	0.0179
17-55	0.93	0.0433
17-56	0.96	0.0210
17-57	0.99	0.0111
17-58	0.96	0.0440
17-6	0.79	0.0405
17-62	0.93	0.0652
17-65	0.93	0.0141
17-66	0.96	0.0423
17-66X	0.89	0.0033
17-69	1.00	0.0000
17-72	0.96	0.0107
17-73	0.96	0.0135
17-74	0.86	0.0498
17-75	0.97	0.0121
17-76	0.93	0.0062
17-77	0.92	0.0071
17-78	0.93	0.0722
17-8	0.94	0.0097
18-52	0.97	0.0100
18-53	0.97	0.0080
18-61	0.98	0.0115
18-65	0.82	0.0696
18-76	0.94	0.0279
18-89	0.92	0.0098
20-1	0.86	0.0359
20-10	0.88	0.0219
20-11	0.88	0.0049

Figure 3 (Continued)

Line	*OI*	*S.E.M*
20-12	0.80	0.1053
20-13	0.93	0.0667
20-14	0.88	0.0525
20-17	0.91	0.0708
20-19	0.85	0.0180
20-2	0.75	0.0883
20-20	0.88	0.0090
20-205	0.87	0.0516
20-21	0.91	0.0130
20-22	0.88	0.0019
20-23	0.90	0.0296
20-25	0.91	0.0250
20-26	0.86	0.0360
20-27	0.84	0.0320
20-3	0.84	0.0715
20-31	0.92	0.0566
20-32	0.84	0.0219
20-33	0.86	0.0366
20-36	0.91	0.0238
20-37	0.78	0.0368
20-37	0.86	0.0737
20-38	0.95	0.0455
20-39	0.83	0.0384
20-4	0.88	0.0695
20-43	0.83	0.0835
20-47	0.85	0.0414
20-5	0.76	0.0415
20-51	0.87	0.0374
20-52	0.88	0.0515
20-53	0.85	0.0321
20-56	0.94	0.0192
20-58	0.92	0.0264
20-59	0.89	0.0361
20-6	0.84	0.0456
20-60	0.85	0.0349
20-63	0.86	0.0063
20-64	0.91	0.0430
20-66	0.93	0.0051
20-68	0.79	0.0334
20-69	0.93	0.0702
20-7	0.95	0.0545
20-71	0.96	0.0217
20-72	0.74	0.0304
20-73	0.84	0.0139
20-74	0.89	0.0266
20-77	0.89	0.0419
20-78	0.92	0.0115
20-79	0.97	0.0171

Line	*OI*	*S.E.M*
20-8	0.87	0.0053
20-80	0.85	0.0567
20-81	0.87	0.0450
20-82	0.94	0.0202
20-83	0.97	0.0055
20-84	0.96	0.0183
20-86	0.86	0.0230
20-88	0.86	0.0392
20-89	0.98	0.0219
20-9	0.97	0.0191
20-93	0.90	0.0294
21-2	0.87	0.0370
21-5	0.95	0.0208
21-7	0.97	0.0096
21-12**	0.95	0.0287
21-18	0.94	0.0082
21-28	0.95	0.0096
21-31	0.89	0.0206
21-35	0.88	0.0222
21-38	0.93	0.0208
21-39	0.98	0.0115
21-45	0.90	0.0275
white-Berlin	0.90	0.0102

Figure 4

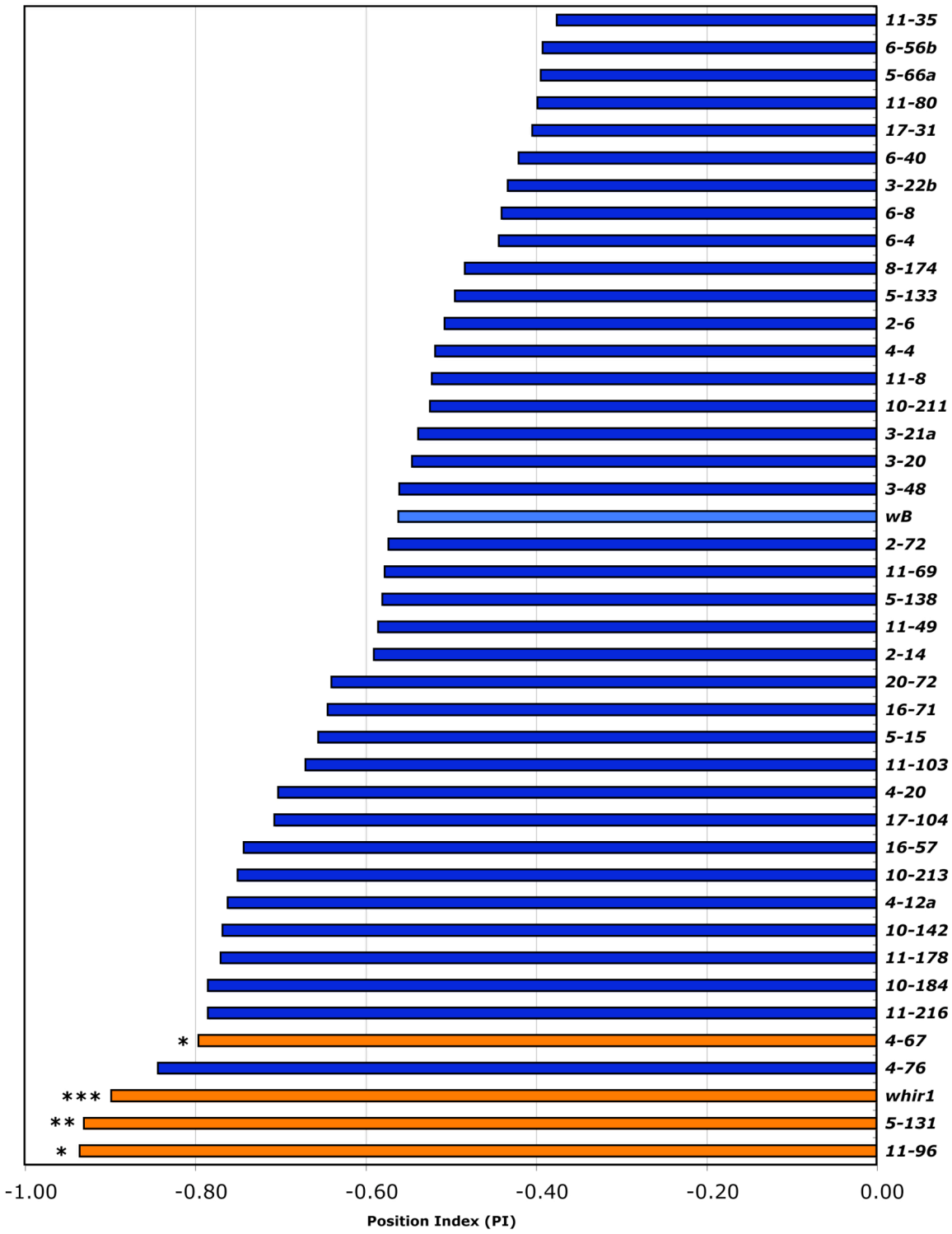

11-35
6-56b
5-66a
11-80
17-31
6-40
3-22b
6-8
6-4
8-174
5-133
2-6
4-4
11-8
10-211
3-21a
3-20
3-48
wB
2-72
11-69
5-138
11-49
2-14
20-72
16-71
5-15
11-103
4-20
17-104
16-57
10-213
4-12a
10-142
11-178
10-184
11-216
*
4-67
4-76
***
whir1
**
5-131
*
11-96
-1.00
-0.80
-0.60
-0.40
-0.20
0.00
Position Index (PI)

Figure 5

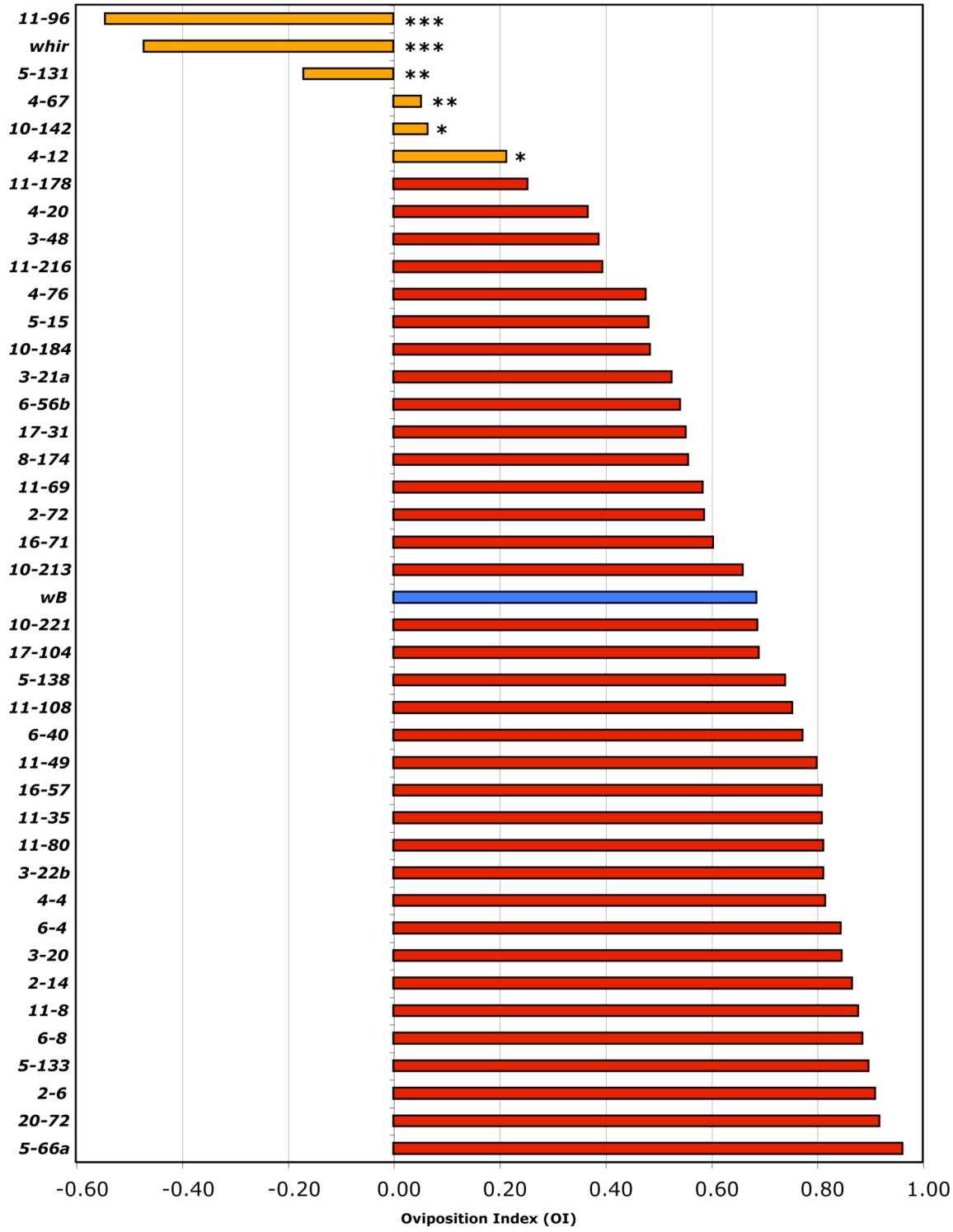
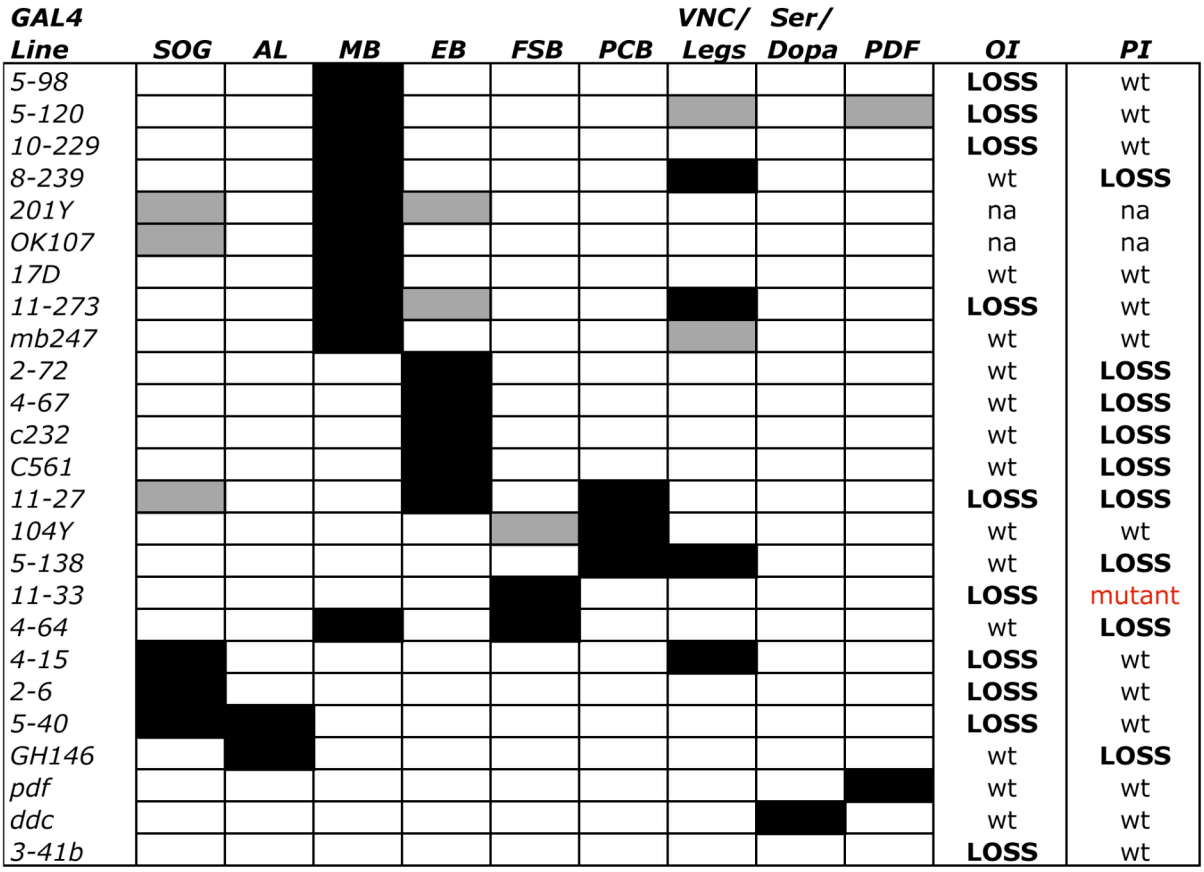

11-96
whir
5-131
4-67
10-142
4-12
11-178
4-20
3-48
11-216
4-76
5-15
10-184
3-21a
6-56b
17-31
8-174
11-69
2-72
16-71
10-213
wB
10-221
17-104
5-138
11-108
6-40
11-49
16-57
11-35
11-80
3-22b
4-4
6-4
3-20
2-14
11-8
6-8
5-133
2-6
20-72
5-66a
***
***
**
**
*
*
-0.60
-0.40
-0.20
0.00
0.20
0.40
0.60
0.80
1.00
Oviposition Index (OI)

Figure 6

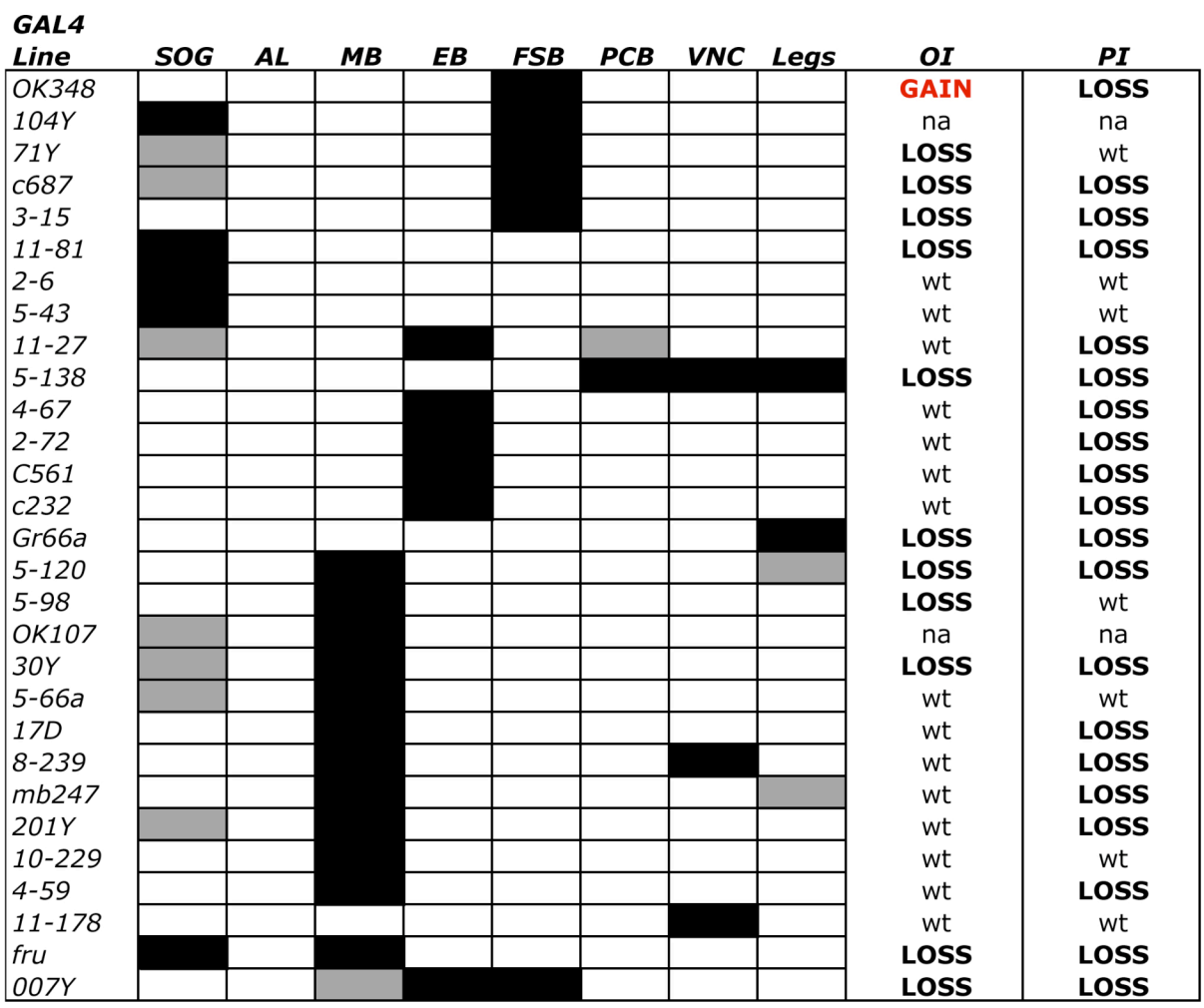
Line	*PI*	*S.E.M*	*OI*	*S.E.M.*	*N*
2-14	-0.590	0.0544	0.867	0.1065	4
2-6	-0.507	0.0725	0.910	0.0397	4
2-72	-0.573	0.1222	0.588	0.1773	4
3-20	-0.545	0.1026	0.847	0.0361	5
3-21a	-0.538	0.0648	0.526	0.0647	5
3-22b	-0.433	0.0739	0.813	0.0125	4
3-48	-0.560	0.0469	0.388	0.2008	4
4-12a	-0.762	0.0807	0.213	0.3071	5
4-20	-0.703	0.0975	0.368	0.3830	3
4-4	-0.518	0.1185	0.816	0.0574	3
4-67	-0.796	0.0172	0.052	0.2083	5
4-76	-0.843	0.0176	0.477	0.1790	3
5-131	-0.930	0.0300	-0.170	0.0000	2
5-133	-0.495	0.2183	0.898	0.0310	3
5-138	-0.580	0.0589	0.740	0.0671	16
5-15	-0.655	0.0630	0.483	0.2589	5
5-66a	-0.394	0.1475	0.962	0.0233	5
6-4	-0.443	0.1368	0.845	0.0457	6
6-40	-0.420	0.1638	0.773	0.0211	5
6-56b	-0.392	0.3187	0.542	0.3261	2
6-8	-0.440	0.0812	0.886	0.0513	4
8-174	-0.483	0.0914	0.557	0.2312	5
10-142	-0.768	0.0654	0.065	0.1835	6
10-184	-0.785	0.0737	0.485	0.3079	3
10-211	-0.524	0.1340	0.660	0.0994	5
10-213	-0.750	0.0510	0.688	0.2427	4
11-103	-0.670	0.0853	0.753	0.0840	4
11-178	-0.770	0.0328	0.253	0.3671	4
11-216	-0.785	0.0318	0.395	0.2086	4
11-35	-0.375	0.0050	0.810	0.0900	2
11-49	-0.585	0.0557	0.800	0.0466	5
11-69	-0.577	0.0768	0.584	0.1165	7
11-8	-0.522	0.0428	0.878	0.0523	5
11-80	-0.398	0.1581	0.812	0.0368	5
11-96	-0.935	0.0330	-0.545	0.2149	4
16-57	-0.743	0.0595	0.810	0.0815	3
16-71	-0.644	0.0933	0.604	0.1147	5
17-104	-0.707	0.0340	0.690	0.0654	4
17-31	-0.404	0.1309	0.552	0.2117	5
20-72	-0.640	0.0274	0.918	0.0749	6
whir1	-0.898	0.0822	-0.472	0.1397	8
wB	-0.561	0.0310	0.686	0.2075	5

Figure 7



GAL4 Line	SOG	AL	MB	EB	FSB	PCB	VNC/ Legs	Ser/ Dopa	PDF	OI	PI
5-98			■							**LOSS**	wt
5-120			■				▒		▒	**LOSS**	wt
10-229			■							**LOSS**	wt
8-239			■				■			wt	**LOSS**
201Y	▒		■	▒						na	na
OK107	▒		■							na	na
17D			■							wt	wt
11-273			■	▒			■			**LOSS**	wt
mb247			■				▒			wt	wt
2-72				■						wt	**LOSS**
4-67				■						wt	**LOSS**
c232				■						wt	**LOSS**
C561				■						wt	**LOSS**
11-27	▒			■		■				**LOSS**	**LOSS**
104Y					▒	■				wt	wt
5-138						■	■			wt	**LOSS**
11-33					■					**LOSS**	mutant
4-64			■		■					wt	**LOSS**
4-15	■						■			**LOSS**	wt
2-6	■									**LOSS**	wt
5-40	■	■								**LOSS**	wt
GH146		■								wt	**LOSS**
pdf									■	wt	wt
ddc								■		wt	wt
3-41b										**LOSS**	wt

Figure 8



GAL4 Line	SOG	AL	MB	EB	FSB	PCB	VNC	Legs	OI	PI
OK348									**GAIN**	**LOSS**
104Y									na	na
71Y									**LOSS**	wt
c687									**LOSS**	**LOSS**
3-15									**LOSS**	**LOSS**
11-81									**LOSS**	**LOSS**
2-6									wt	wt
5-43									wt	wt
11-27									wt	**LOSS**
5-138									**LOSS**	**LOSS**
4-67									wt	**LOSS**
2-72									wt	**LOSS**
C561									wt	**LOSS**
c232									wt	**LOSS**
Gr66a									**LOSS**	**LOSS**
5-120									**LOSS**	**LOSS**
5-98									**LOSS**	wt
OK107									na	na
30Y									**LOSS**	**LOSS**
5-66a									wt	wt
17D									wt	**LOSS**
8-239									wt	**LOSS**
mb247									wt	**LOSS**
201Y									wt	**LOSS**
10-229									wt	wt
4-59									wt	**LOSS**
11-178									wt	wt
fru									**LOSS**	**LOSS**
007Y									**LOSS**	**LOSS**

Chapter 4

Tissue-Specific Activation of *Gr66a* Induces Opposing Responses During the Decision-Making Process in *Drosophila* Oviposition Behavior.

Ryan M. Joseph

ABSTRACT

Proper sensory perception is essential for organisms to modulate behavior and make choices necessary for survival. Recently, *Drosophila melanogaster* oviposition has been validated as one such important decision-making process, in which females evaluate their environment and choose to lay eggs on substrates they otherwise find aversive. Understanding the sensory systems that perceive environmental input signals and the higher-order neural circuits that select appropriate motor outputs is critical for studying how organisms make decisions, including the choice *Drosophila* make when selecting between positional preference versus egg-laying preference.

We employed neurogenetic techniques to characterize sensory and central neurons regulating these repulsive positional and attractive egg-laying responses towards a bitter-tasting compound, lobeline. Surprisingly, we found that neurons expressing *Gr66a*–a broadly expressed gustatory receptor normally involved in avoidance behaviors–receives input for both attractive and aversive behaviors. We hypothesized that activation of distinct *Gr66a*-expressing neurons may induce opposing behavioral responses. Indeed, using tissue-specific rescue experiments, we found that *Gr66a*-expressing neurons on the legs mediate the aversive positional response. In contrast, pharyngeal taste-cells mediate the attractive egg-laying response, as determined by the analysis of individual mosaic flies in which subsets of *Gr66a* neurons were silenced. Finally, inactivating neurons of the mushroom body disrupted both aversive and attractive responses, suggesting that this brain structure is as an integration center for decision-making.

We therefore define sensory and central neurons important to the decision-making process employed during *Drosophila* oviposition. Furthermore, our findings provide

additional insights into the complex nature of gustatory perception in fruit flies. Notably, *Drosophila* chemosensory neurons co-express multiple receptors, presenting challenges in understanding how activation of taste-neurons translates into different behavioral outputs. We show tissue-specific activation of bitter-sensing *Gr66a*-neurons provides one mechanism by which the gustatory system can functionally encode differential aversive and attractive responses.

INTRODUCTION

Proper perception of the environment is essential for an organism to modulate its behavior and make choices necessary to both survival of individual animals and propagation of the species. In *Drosophila melanogaster*, the selection of appropriate oviposition sites that will benefit hatching progeny is one such behavior (Richmond and Gerking, 1979l Ruiz-Dubreuil et al., 1994; van Delden and Kamping, 1990; Chess and Ringo, 1985; Mery and Kawecki, 2002; Jaenike, 1982; Amlou et al., 1998). Recent studies have demonstrated that during egg-laying site selection the female fruit fly undergoes a complex decision-making process (Kable and Glimcher, 2009; Kristan, 2008) in which females actively sense and assign value to the different oviposition options available, and then choose an optimal site to lay their eggs (Yang et al., 2008; Miller et al., 2011). Interestingly, females do not always remain on the same substrate where they have deposit their eggs (Joseph et al., 2009). Instead, *Drosophila* will often attractively lay eggs on substrates they normally find aversive for foraging, positional, and feeding responses (Moreteau et al., 1994; Matsuo et al., 2007; Fuyama, 1976; Eisses, 1997; Lee et al., 2009; Sellier et al., 2010; Weiss et al., 2011; Lee et al., 2010). Simply stated, a fly cannot be in two places at once, and a choice must be made

between these competing, contradictory preference pathways. Thus, with regard to *Drosophila* oviposition behavior, a decision is defined as the selection between one of two mutually exclusive responses: (1) avoid the substrate and hold eggs, or (2) not avoid the substrate in order to lay eggs. Taken together, these findings demonstrate that the *Drosophila* oviposition program employs an evaluation process that can be utilized as a model for decision-making.

Although previous studies have identified compounds that induce both avoidance-related responses and attractive egg-laying preferences in *Drosophila* (Yang et al., 2009; Miller et al., 2011; Joseph et al., 2009; Moreteau et al., 1994; Matsuo et al., 2007; Fuyama, 1976; Eisses, 1997; Lee et al., 2009; Sellier et al., 2010; Weiss et al., 2011), characterization has been performed independently on either the aversion or the attraction response to these compounds. In fact, only a few studies have directly investigated the actual decision between the two opposing preferences, in which positional aversion and egg-laying attraction to the same compound are simultaneously presented as available options, and their selection can be compared quantitatively in the same assay (Joseph et al., 2009). Given that the selection event itself is critical to the decision-making process, additional characterization of the sensory systems that receive relevant signal input and the neural circuitry that chooses between these competing positional and egg-laying motor outputs is necessary for understanding how decisions are made in *Drosophila* oviposition behavior.

Lobeline is one such compound shown to induce avoidance-related responses (Lee et al., 2009; Sellier et al., 2010; Marella et al., 2006; Weiss et al., 2011) and elicit egg-laying attraction (Yang et al., 2008) in independent behavioral assays. Interestingly, lobeline is an alkaloid naturally produced by the diverse genus of *Lobelia* plants (Krochmal et al., 1972),

and serves as a feeding repellent for several insect species (Detzel and Wink, 1993). Bitter-sensing *Gr66a* neurons in the *Drosophila* gustatory system have also been shown to detect lobeline (Lee et al., 2010), providing cellular and molecular targets for investigation. Thus, when employed with our previously established two-choice assay that concurrently measures opposing positional and egg-laying preferences (Joseph et al., 2009), lobeline is an ideal substrate to study the selection event that flies undergo when deciding between these two competing responses in the *Drosophila* oviposition program.

Given the bitter-tasting qualities of lobeline, it is likely that gustatory neurons play a role in the decision-making process associated with the oviposition program. However, the unique properties of the *Drosophila* gustatory system make characterization of taste-related behaviors relatively difficult. Unlike *Drosophila* olfactory neurons, which typically express a unique odorant receptor/co-receptor pair that defines its identity (Hallem et al., 2004; Larsson et al., 2004), *Drosophila* gustatory neurons co-express multiple gustatory receptors (Lee et al., 2009; Weiss et al., 2011l Thorne et al., 2004; Wang et al., 2004; Jiao et al., 2008; Isono and Morita, 2010). In particular, most sensory neurons that detect bitter compounds such as lobeline express *Gr66a* (Lee et al., 2009; Sellier et al., 2010; Weiss et al., 2011), presenting challenges in understanding how activation of bitter-taste neurons with overlapping gustatory receptor profiles can translate into diverse behavioral responses to specific compounds. In fact, there is still debate over whether flies identify different bitter compounds specifically, or if *Drosophila* simply utilizes the broad expression profiles of gustatory receptors to indiscriminately avoid any bitter compound (Weiss et al., 2011; Masek and Scott, 2010).

The *Drosophila* gustatory system also possesses complexities at the level of neural circuitry and anatomical organization. Gustatory sensory neurons are present in taste bristles

that are located in numerous tissues of the fly, including: the labellum, pharynx, legs, wings, and abdomen (Dunipace et al., 2001; Stocker and Schorderet, 1981; Stocker, 1994; Gendre et al., 2003; Mitri et al., 2009; Taylor, 1989; Thorne and Amrein, 2008; Shimono et al., 2009). Notably, the *Gr66a*-expressing neurons that detect bitter compounds are present in most of these tissues (Weiss et al., 2011; Dunipace et al., 2001; Mitri et al., 2009; Shimono et al., 2009), and axons from these gustatory sensory neurons subsequently project from taste bristles to the subesophageal ganglion (SOG) (Thorne et al., 2004; Wang et al., 2004; Miyazaki and Ito, 2010). Until recently, the construction of neural maps within the SOG has been limited, given the lack of obvious compartmentalization of axonal projections into discrete glomeruli. The SOG has been hypothesized to perform a role similar to the *Drosophila* antennal lobe, where signals undergo first-order processing and are sent to higher-order structures for further comparison and integration (Miyazaki and Ito, 2010). One candidate brain structure potentially downstream of the SOG is the mushroom body, which has been implicated in the integration of multiple sensory inputs (Xi et al., 2008), switches between motivational states (Krashes et al., 2009), and additional *Drosophila* decision-making behaviors (Zhang et al., 2007; Brembs et al., 2009; Wu et al., 2011). However, second-order neurons directly connecting the SOG to higher-order brain regions have yet to be identified, and investigation into potential decision-making centers involved in both positional and egg-laying preferences have only be recently initiated. Thus, important questions remain unanswered about the gustatory-related circuits involved in selecting motor outputs in decision-making processes like the *Drosophila* oviposition program.

In particular, what sensory systems detect environmental cues that the fly perceives as either repulsive or attractive, and what central neurons subsequently choose the positional or

egg-laying response most appropriate to the immediate situation? To address these questions, we used the *GAL4/UAS* binary expression system to inactivate signaling in specific neurons, and subsequently studied lobeline-induced responses using our previously established two-choice assay (Joseph et al., 2009). Surprisingly, we found that sensory cells expressing the same gustatory receptor, *Gr66a*, receive input for both the aversive positional and attractive egg-laying pathways. Our findings directly showing *Gr66a*-expressing neurons can mediate an attractive preference are distinct from previous studies, which have generally implicated bitter-sensing *Gr66a* cells in only aversive responses (Lee et al., 2009; Sellier et al., 2010; Weiss et al., 2011; Less et al., 2010; Marella et al., 2006). Furthermore, to the best of our knowledge, we describe a previously uncharacterized phenomenon in *Drosophila*, in which both attractive and repulsive responses are simultaneously induced by activation of neurons expressing a common taste receptor complex within the same gustatory circuitry.

We hypothesized that activation of *Gr66a*-expressing in distinct tissue regions of the fly could account for how a single gustatory input can generate opposing behavioral outputs. Inactivation of *GAL4* with a thorax-specific *GAL80* transgene revealed that *Gr66a*-neurons on the legs receive input for the aversive positional response. In contrast, performing single-fly behavioral and imaging assays on genetically mosaic females with restricted subsets of silenced *Gr66a*-neurons revealed that sensory cells in the pharynx mediate the attractive egg-laying response. Our findings therefore demonstrate that tissue-specific activation of the *Gr66a* receptor complex provides an additional level in which the *Drosophila* gustatory system can functionally encode bitter input as either attractive or repulsive, despite the compound being detected by neurons with overlapping gustatory receptor expression profiles.

In addition, we observed that silencing the mushroom body simultaneously disrupts both the positional aversion and egg-laying attraction responses to lobeline, providing neuroanatomical evidence that this structure is an intersection point in the neural circuitry of these competitive preference pathways. Furthermore, our data suggests that the mushroom body is a possible candidate higher-order brain region that compares and integrates signals from each sensory pathway, in order to selects one the opposing motor outputs. Taken together, we characterize sensory and central neurons involved in the *Drosophila* oviposition program, and demonstrate that when making a decision to lay an egg on a bitter compound like lobeline, flies activate a single *Gr66a* taste receptor complex in a tissue-specific manner to generate opposing attractive and repulsive behaviors.

RESULTS

Detection of Lobeline by *Gr66a*-expressing Neurons Induces Opposing Egg-Laying and Positional Responses

In a previous study, we characterized how simultaneous egg-laying attraction and positional aversion responses toward acetic acid can be effectively used as a model for choice behavior in female *Drosophila* (Joseph et al., 2009). Seeking to expand this paradigm, we asked whether other compounds could induce attractive oviposition preference and competitive positional aversion in our experimental setup. Lobeline, a bitter-tasting compound, has been previously implicated as an egg-laying attractant (Yang et al., 2008); however, the positional responses to lobeline in free-moving flies have not been extensively characterized. We confirmed that in our assay female flies preferentially lay eggs on media containing lobeline, as reflected by positive oviposition index (OI) values (Figure 1A-1B; red

labeling). Furthermore, we observed that female flies concurrently avoid the same lobeline-containing media as a resting substrate, as reflected by negative positional index (PI) values (Figure 1A-1B; blue labeling). We additionally confirmed that females perceive lobeline as less appetitive (i.e. repulsive) in feeding assays (Figure S1A). Both behavioral responses were dose-dependent, and our data suggests that each preference pathway competes with the other: at higher doses of lobeline (0.75-1.00mM), females exhibited extreme positional aversion (PI>-0.62), which substantially decreased egg-laying indices (Figure 1A). Both the positional repulsion and egg-laying attraction to 0.50mM lobeline remained constant over a 24-hour period (Figure 1C) and were similar in various genetic backgrounds (Figure S1D). We therefore selected the 0.50mM lobeline dosage for subsequent experiments in our study. Interestingly, 10mM quinine produces similar behavioral responses in female flies (Figure S1B), suggesting that the simultaneous induction of positional aversion and egg-laying attraction is not just specific to lobeline, but rather a more general behavioral paradigm *Drosophila* exhibit towards bitter-tasting chemicals. In summary, our data demonstrated that lobeline concurrently induces attractive egg-laying and repulsive positional responses in our experimental model for studying choice-like behavior in *Drosophila*.

Given past studies in which attractive and repulsive behavioral outputs were induced by simultaneous activation of the olfactory and gustatory circuits (Joseph et al., 2009; Suh et al., 2004; Fischler et al., 2007; Ai et al., 2010), we asked whether lobeline-induced behaviors diverged in a similar manner. To determine whether olfactory input was necessary for lobeline responses, we tested females in which the primary olfactory organs, the third antennal segments (Hallem et al., 2004), had been surgically removed. In addition, we assayed mutants lacking the critical *Or83b* co-receptor, which is required for most olfactory

signaling (Larsson et al., 2004). Flies with compromised olfactory systems exhibited normal responses to lobeline (Figure S2), indicating that both egg-laying attraction and positional aversion are not mediated by the olfactory system.

Our data therefore suggested that the gustatory system receives input for the positional aversion and egg-laying attraction responses to lobeline, especially since lobeline has been shown to induce aversive responses in other taste-based behavioral assays (Sellier et al., 2010, Weiss et al., 2011; Lee et al., 2010; Marella et al., 2006). Given the broad expression of *Gr66a* in most bitter sensing neurons (Weiss et al., 2011; Isono and Morita, 2010; Shimono et al., 2009; Miyazaki and Ito, 2010), we predicted that *Gr66a*-expressing sensory neurons were potential targets for at least the positional aversion response. To test this hypothesis, we used temperature sensitive *Shibire* transgene (*UAS-Shi^{ts}*) to deplete neurotransmitter endocytosis and subsequently silence signaling at the synaptic terminals of *Gr66a*-expressing neurons (Kitamoto, 2001). Indeed, silencing *Gr66a*-expressing neurons disrupted the positional aversion to lobeline in *Gr66a^{GAL4}/+; UAS-Shi^{ts}/+* females at the non-permissive temperature (Figure 2A, lower axis). Interestingly, silencing *Gr66a*-expressing neurons caused a loss of egg-laying attraction to lobeline as well (Figure 2A, upper axis). To independently verify these results, we used *UAS-tetanus toxin* (*UAS-TNT*) to abolish synaptic vesicle release (Sweeney et al., 1995). Similarly, *Gr66a^{GAL4}/UAS-TNT* females also exhibited disruptions in both the positional and egg-laying responses to lobeline (Figure 2B). Finally, to demonstrate the *Gr66a* bitter-receptor is necessary for the detection of lobeline and production of resulting behavioral responses, we assayed *ΔGr66a^{ex83}* mutant flies that lack the taste receptor but still have sensory neurons capable of synaptic signaling (Moon et al., 2006). Indeed, *ΔGr66a^{ex83}* females exhibited disrupted lobeline responses (Figure 2C),

demonstrating the molecular necessity of the *Gr66a* bitter-receptor in mediating the competing responses to lobeline. Taken together, our results show that active signaling through the *Gr66a* receptor in bitter-sensing gustatory neurons is required for the proper execution of positional aversion and egg-laying attraction preferences for lobeline.

Positional Aversion to Lobeline is Mediated by *Gr66a*-expressing Neurons on the Anterior Legs

How do females induce two opposing behavioral outputs from a single gustatory input? Gustatory sensory neurons that express *Gr66a* are present in diverse regions of the fly, including: bristles on the labellum (Weiss et al., 2011), internal mouthparts lining the pharynx (Lee et al., 2009; Dunipace et al., 2001; Mitri et al., 2009), tarsal leg segments of the legs (Isono and Morita, 2010; Dunipace et al., 2001; Mitri et al., 2009), and abdominal tissues (Shimono et al., 2009). We hypothesized that activation of *Gr66a* receptors in distinct sensory organs that have different axonal projection targets could induce discrete neural circuits, and thereby explain the divergence in attractive and repulsive responses to lobeline.

Gr66a^{GAL4} expresses in gustatory neurons that innervate sensory bristles on the anterior legs (Figure 3C; Dunipace et al., 2001; Mitri et al., 2009). To begin dissecting which neurons in the *Gr66a* expression pattern mediate the behavioral responses to lobeline, we assayed *Gr66a*^{GAL4}*/+; UAS-Shi*^{ts}/+ females that also possessed *teashirt-GAL80* (*TSH{GAL80}*), a thorax-specific *GAL4* repressor with expression in the *Drosophila* leg segments (Clyne and Miesenböck, 2008). If synaptic activation of *Gr66a* neurons on the anterior legs are necessary for the repulsive response to lobeline, *GAL80* inhibition of *Gr66a*^{GAL4}*/UAS-Shi*^{ts} expression in the thoracic leg segments should restore positional

aversion in these females. Indeed, *Gr66a*^{GAL4}*/TSH{GAL80}; UAS-Shi*^{ts}*/+* females exhibited normal positional aversion to lobeline (Figure 4A, lower axis). In addition, *TSH{GAL80}* did not rescue the loss of egg-laying attraction, confirming that the legs are not responsible for mediating the oviposition preference response (Figure 4A, upper axis).

Given that expression of *TSH{GAL80}* covers anterior, medial, and posterior thoracic leg segments, the possibility remained that *TSH{GAL80}* was rescuing the aversive lobeline response by restoring signaling in thorax neurons other than the characterized *Gr66a* cells on the forelegs. To confirm that sensory bristles on the anterior legs were responsible for the behavioral phenotypes, we performed bilateral removal of the first tarsal segments on the forelegs, midlegs, and hindlegs. Females were allowed to recover from microsurgeries for two days, and were subsequently assayed for positional aversion and egg-laying attraction to lobeline. Flies lacking the first tarsal segments of the forelegs lost positional aversion, while removal of tarsi on either the midlegs or hindlegs did not disrupt this response (Figure 4B). Furthermore, egg-laying attraction to lobeline was normal in all flies tested, confirming that taste bristles on the anterior legs play a minor role in the oviposition response. Taken together, our findings demonstrate that *Gr66a*-expressing gustatory neurons on the first tarsi of the forelegs receive input for the positional aversion response to lobeline.

Egg-Laying Attraction to Lobeline is Mediated by *Gr66a*-expressing Neurons in the Internal Mouthparts of the Pharynx

We previously used *pox-neuro* (*poxn*) mutant flies to demonstrate that sensory input for egg-laying attraction to acetic acid proceeds through taste-bristles on the labellum. The *poxn* defect causes gustatory bristles in most organs to undergo a transformation into

mechanosensory bristles that lack taste receptors (Awasaki and Kimura, 1997). Given our previous findings, we assayed lobeline responses in transgenic *poxn* strains in which the defects of the null mutant $poxn^{\Delta M22\text{-}B5}$ are selectively rescued in different tissues of the fly (Boll and Noll, 2002). In contrast to our previous findings with acetic acid, we surprisingly found *poxn* females lacking labellar taste receptors still exhibited normal egg-laying attraction to lobeline (Figure S3, upper axis). In addition, null $poxn^{\Delta M22\text{-}B5}$ females also exhibited normal egg-laying responses, suggesting that any abdominal bristles undergoing the mutant bristle transformation are not required for detecting lobeline, with regard to oviposition preferences. As expected, *poxn* females lacking taste receptors on the legs exhibited a loss of positional aversion to lobeline, indicating that the developmental transformation incurred by the *poxn* mutation is sufficient to render *Gr66a* neurons non-functional, at least in sensory bristles on the legs (Figure S3, lower axis).

Our results therefore indicate that *Gr66a*-expressing neurons mediating lobeline-induced egg-laying attraction are likely to be taste cells that are not transformed by the *poxn* mutation. *Gr66a*-expressing neurons are present in the pharyngeal tissues, and these gustatory cells have indeed been shown to still express taste-related proteins in the *poxn* mutant background (Galindo and Smith, 2001). Thus, we hypothesized that *Gr66a* expressing cells in the internal mouthpart organs, namely the ventral cibarial sensory organ (VCSO) or the labial sensory organ (LSO) (Figure 3A-B), are responsible for receiving input for the egg-laying attraction preference for lobeline. To test this possibility, we used mosaic analysis techniques to generate females expressing *UAS-TNT* in clones restricted to only a few cells within the *Gr66a* expression pattern, and subsequently conducted single-fly experiments to assay their behavioral responses to lobeline. We generated $Gr66a^{GAL4}$*; UAS-*

CD8-GFP flies and crossed them to *tubulin-FRT-GAL80-FRT; UAS-TNT; heat shock-FLP-recombinase* flies, in which the gene for *GAL80* repressor of *GAL4* is flanked by *FRT* recombination sites (Gordon and Scott, 2009)**.** Briefly, activation of *heat shock-FLP-recombinase* in resulting progeny causes recombination at the *FRT* sites in random sets of cells, which excises *GAL80* and allows for GAL4 expression in those particular cells. As a result, *UAS-TNT* and *UAS-CD8-GFP* expression is de-repressed, and the stochastically silenced subset of *Gr66a* neurons is therefore labeled with GFP. Progeny were heat shocked for 1-hour at 37°C during the pupal stage of development; females were collected after eclosion, and allowed to mate for two days before testing. Single females were then assayed for both their positional aversion and egg-laying attraction responses to lobeline. Immediately following behavioral tests, the heads, legs, and abdomens of individual experimental flies were dissected and imaged with a confocal microscope to determine which *Gr66a* cells possessed *UAS-TNT* silencing.

We obtained OI values, PI values, and GFP expression data for approximately 90 single clonal females, and approximately 20 individual control flies of the same genotype that did not undergo heat shock. To determine if silencing the *Gr66a*-expressing cells in a particular tissue region induced a loss of egg-laying attraction, we divided the assayed females into two groups: (1) flies possessing GFP-positive, and hence *UAS-TNT* silenced clones in a particular tissue region within the $Gr66a^{GAL4}$ expression pattern, and (2) flies that were GFP-negative, and thus lacked *UAS-TNT* activity in the tissue region. We then compared mean OI values of each group to see whether there was a significant decrease in egg-laying attraction in the GFP-positive females when compared to the GFP-negative

females. We compared GFP-positive and GFP-negative groups for the following tissue regions: labellum, legs, abdomen, LSO, and VCSO.

We found that flies containing silenced clones in the VCSO had significantly decreased egg-laying preference, when compared to flies lacking VCSO clones (Figure 5A). In contrast, egg-laying preference was not affected by silencing clones in the labellum, legs, abdomen, or LSO (Figure 5A). Interestingly, silencing a single cell within the VCSO was often sufficient to induce a decrease in egg-laying preference (Figure 5B). Furthermore, the egg-laying preference of flies lacking any silenced neurons in the VCSO was nearly identical to that of controls that were not heat shocked (*GFP* negative OI=+0.29, no heat shock OI=+0.32; Figure 5C). In addition, similar clonal analysis of PI values demonstrated a trend towards loss of positional aversion in females with clones in *Gr66a* neurons on the legs (Figure S4), supporting our previous findings implicating the legs in positional aversion and validating the experimental techniques of the clonal analysis. Thus, the *Gr66a*-expressing gustatory neurons present in the internal mouthparts lining the pharynx, specifically in the VCSO, receive input for the egg-laying attraction response to lobeline.

The Mushroom Body is an Intersection Point for Neural Circuits that Mediate the Positional Aversion and Egg-Laying Responses

Gr66a sensory neurons project axons into the subesophageal ganglion (SOG) Thorne et al., 2004; Wang et al., 2004; Miyazaki and Ito, 2010). The SOG has been postulated to act analogously to the antennal lobe, an olfactory relay center where signal input from peripheral sensory neurons undergoes primary processing before being sent to higher-order brain regions for further evaluation. Although the selection of positional aversion or egg-laying

attraction could theoretically occur within the SOG and be transmitted directly to neuromuscular junctions for execution of behavioral responses, previous work argues there are additional neuronal processing centers in the circuit maps that connect the SOG and motor output neurons (Gordon and Scott, 2009). Given its involvement in other decision-making behavioral paradigms (Krashes et al., 2009; Serway et al., 2009; Zhang et al., 2007; Brembs, 2009; Wu and Guo, 2011), we asked whether the mushroom body is one candidate brain structure that could be involved in choice-like processing of gustatory signals, after they have undergone primary processing in the SOG.

To assess whether the mushroom body could be involved in the selection of either positional aversion or egg-laying attraction to lobeline, we used *5-120^{GAL4}* flies that have broad expression in all lobes of the mushroom body (Figure 6B) (Joseph et al., 2009; Kaun et al., 2011). We assayed responses in *5-120^{GAL4}*/+; *UAS-Shi^{ts}*/+ females, and observed both a loss of positional aversion and a loss of egg-laying attraction to lobeline at the non-permissive temperature (Figure 6A). To confirm that phenotypes in *5-120^{GAL4}*/+; *UAS-Shi^{ts}*/+ flies resulted from specific silencing of the mushroom body, we utilized *mushroom body-GAL80* (*MB{GAL80}*) to specifically repress *GAL4* induction of *UAS-Shi^{ts}* in mushroom body neurons (Krashes et al., 2007). Indeed, *5-120^{GAL4}/MB{GAL80}; UAS-Shi^{ts}*/+ flies with restored neuronal signaling in the mushroom bodies exhibited normal responses to lobeline (Figure 6A). Imaging with *UAS-GFP* confirmed that *GAL4* expression was repressed in the mushroom bodies, but not in other neurons (Figure 6C). To verify that the loss of both lobeline responses was not specific to *5-120^{GAL4}*, we tested another *GAL4* line with broad expression in the mushroom body: *30Y^{GAL4}* (Aso et al., 2009). As expected, silencing synaptic signaling in *30Y^{GAL4}/UAS-Shi^{ts}* females also induced a loss of both the aversive and

attractive responses (Figure S5). In summary, these results suggest that the mushroom body is an intersection point in the neural circuitry underlying both lobeline responses, and possibly acts as a higher-order integration center that compares signals from both pathways.

Beyond *5-120^{GAL4}* and *30Y^{GAL4}*, using *UAS-Shi^{ts}* to silence the mushroom body in additional *GAL4* lines also yielded losses in either the positional aversion or egg-laying attraction at the non-permissive temperature (Figure S6). Notably, unlike *5-120^{GAL4}* and *30Y^{GAL4}*, these additional *GAL4* lines typically would only have one behavioral response disrupted. This difference is likely due to the other *GAL4* lines only expressing in restricted subsets of neurons within the mushroom body, when compared to the relatively broad expression pattern of *5-120^{GAL4}* (Aso et al., 2009; Kaun et al., 2011). Detailed analysis of which mushroom body neurons regulate which behavior will be the subject of a future study, but preliminary breakdown of *GAL4* expression in the different mushroom body lobes suggests that the γ/γ'-lobes mediate the egg-laying attraction response, while α/β and α'/β' are likely involved with processing the positional aversion response (Figure S6). Taken together, these results suggest that the mushroom body is an intersection point in the neural circuitry underlying both lobeline responses, and possibly acts as an integration center for signals from both pathways.

DISCUSSION

Understanding both the sensory systems that receive relevant signal input and the neuronal structures that select the appropriate motor output are critical to effectively use *Drosophila* oviposition behavior as a model for decision-making. Using our results from this study, we propose an initial model for the neural circuits that flies employ when deciding

whether to avoid bitter-tasting compounds, or to approach these non-appetitive substrates for egg-laying purposes (Figure 7).

Using lobeline as a representative bitter-tasting compound, we observed that sensory input for both the positional aversion and egg-laying attraction responses of the *Drosophila* oviposition program is received by gustatory neurons containing the *Gr66a* gustatory receptor (Figure 2). *TSH{GAL80}* and anatomical ablation experiments demonstrated that silencing signaling in *Gr66a*-expressing neurons in first tarsal gustatory bristles of the anterior legs only disrupts the avoidance of lobeline (Figure 4), thereby demonstrating that these *Gr66a* foreleg neurons primarily receive input for the positional aversion response (Figure 7, blue lines). These results are supported by previous work showing that contact of bitter compounds to the legs can induce repulsive behavioral outputs like the proboscis extension reflex (Wang et al., 2004), and by our genetic mosaic experiments, in which we observed a trend towards decreased positional aversion in single flies that possessed silenced *Gr66a* clones in both anterior legs (Figure S5). Surprisingly, clonal analysis experiments revealed that silencing gustatory neurons in the pharyngeal ventral cibarial sensory organ (VCSO) disrupted attraction to lobeline as an oviposition substrate (Figure 5), while silencing abdominal *Gr66a*-expressing cells did not significantly affect egg-laying preference. Thus, *Gr66a*-expressing neurons in the VCSO primarily receive input for the egg-laying attraction response (Figure 7, red lines).

Signal input for lobeline thus enters the positional and egg-laying preference pathways through *Gr66a*-expressing neurons in the anterior legs and the pharynx, respectively (Figure 7). Both leg and pharyngeal *Gr66a*-expressing neurons have been shown to project to the subesophageal ganglion (SOG) (Thorne et al., 2004; Wang et al., 2004).

Recent high-resolution imaging studies demonstrate that *Gr66a*-expressing neurons originating from the legs and pharynx project to non-overlapping regions within the SOG (Miyazaki and Ito, 2010), leaving open the possibility that positional aversion and egg-laying attraction relay signals through independent parallel pathways that compete only at the level of behavioral output (Figure 7, gray box). As such, motor output neurons from each pathway could theoretically project from the SOG directly to neuromuscular junctions for the execution of each corresponding response, without intersecting at the neuroanatomical level in higher-order decision-making centers.

However, we that silencing the mushroom body causes a disruption in both positional aversion and egg-laying attraction, suggesting that the neural circuits from each pathway converge in this higher-order brain structure (Figure 6; Figure S5). Therefore, our behavioral results strongly arguing against this alternative parallel pathway model for the competition between lobeline responses. In addition, although a direct neuroanatomical connection between the SOG and higher-order brain regions like the mushroom body has yet to be discovered in *Drosophila melanogaster* (Figure 7, dashed arrows), such a neural link is present in other insects (Schroter and Menzel, 2003). Thus, our behavioral data allows us to place the mushroom body as an intersection point between the positional aversion and egg-laying attraction responses in our model (Figure 7, purple box). Furthermore, sensory integration has been defined as the processing of two signal inputs into one behavioral output, notably in an environmental context in which two contradictory stimuli are simultaneously introduced and their signals converge on common neurons (Shinkai et al., 2011; Meredith, 2002). Our results, which demonstrate that input signals from the competing repulsive positional and attractive egg-laying pathways converge in the mushroom body

before a single behavioral output is selected, fit this model for sensory integration. Taken together with previous studies that implicate its role in other decision-making behaviors (Krashes et al., 2009; Serway et al., 2009; Zhang et al., 2007; Brembs, 2009; Wu and Guo, 2011), we tentatively propose the mushroom body is a candidate sensory integration center that receives and compares lobeline input from both the positional aversion and egg-laying attraction pathways, before selecting an contextually relevant behavioral output response (Figure 7, purple lines). In summary, we have defined sensory and central neurons that mediate positional aversion and egg-laying attraction responses to bitter compounds like lobeline, allowing us to construct an initial model of the pathways governing the decision-making selection process employed during *Drosophila* oviposition behavior.

Our clonal analysis results that abdominal *Gr66a* neurons do not mediate egg-laying preference (Figure 5) are interesting in that past studies have attributed bristles with chemosensory-like morphology on the *Drosophila* ovipositor and vagina as likely being necessary for egg-laying behaviors (Stocker, 1994; Taylor, 1989). However, electrophysiology and behavioral experiments directly testing the function of these bristles have not been performed. Furthermore, our observations and previous studies have noted that *Gr66a* abdominal neurons do not project to these bristles, and possess multi-dendritic morphology that is atypical of gustatory sensory neurons (Thorne and Amrein, 2008; Shimono et al., 2009). Although we cannot eliminate the possibility that these ovipositor and vagina bristles are employed in other, non-bitter sensing gustatory processes, our findings argue that *Drosophila* can make taste-based evaluations about the quality of an egg-laying substrate by receiving input from non-abdominal pharynx neurons, while the female samples the appetitive quality of the surrounding food. We therefore demonstrate novel findings

regarding what anatomical structures are needed for taste-based *Drosophila* oviposition behavior.

Characterization of the *Drosophila* gustatory system presents challenges, notably because single sensory cells typically co-express multiple combinations of gustatory receptors (Weiss et al., 2011). In addition to defining sensory and central neurons important to the decision-making process employed during *Drosophila* oviposition behavior, our findings provide important insights into the complex nature of gustatory perception in the fruit fly. Surprisingly, we found that sensory cells expressing the same gustatory receptor, *Gr66a*, receive input for both the aversive and attractive pathways. Previous studies have shown that a single compound like carbon dioxide or acetic acid can induce opposing behavioral responses, however the divergence into distinct attractive and repulsive responses have been attributed to the compound being detected by independent sensory modalities, such as the olfactory versus the gustatory system (Joseph et al., 2009; Suh et al., 2004; Fischler et al., 2007), or by multiple classes of receptors that sense different qualities of a compound, such as odor versus acidity (Ai et al, 2010). In contrast, to the best of our knowledge we describe a previously uncharacterized phenomenon in *Drosophila*, in which both attractive and repulsive responses are simultaneously induced by activation of neurons expressing the same taste receptor complex within the same gustatory system.

Our findings that *Gr66a*-expressing neurons mediate an attractive preference are unique from previous studies, which have typically implicated bitter-sensing *Gr66a* cells in only aversive responses (Lee et al., 2010; Moon et al., 2006). It has been postulated that the broad expression of numerous gustatory receptors in bitter-sensing *Gr66a*-neurons allows *Drosophila* to detect a multitude of potentially toxic compounds, and then execute a general

aversion response that is only modulated by the intensity of bitterness from that particular compound (Masek and Scott, 2010). However, recent work demonstrates that combinatorial expression of gustatory receptors allows the fly to exhibit a remarkable degree of functional diversity in both the electrophysiological and behavioral responses toward distinct bitter compounds on the labellum (Weiss et al., 2011), suggesting *Drosophila* do not simply execute an indiscriminate avoidance program when activating bitter-sensing neurons. Similarly, we show that activation of *Gr66a* neurons can produce an attractive preference, suggesting that female *Drosophila* can utilize bitter-sensing neurons in behavioral capacities beyond a general aversion response.

Our results showing that *Gr66a*-expressing neurons concurrently mediate both positional aversion and egg-laying attraction to lobeline are intriguing, specifically regarding the question about how the *Drosophila* gustatory system can translate signal input from a single compound like lobeline into two distinct behavioral outputs. Given that the *Gr66a* taste-receptor complex is likely activated in all bitter-sensing neurons in the presence of lobeline, an additional cellular property must contribute to identifying lobeline-detecting neurons as belonging either to the repulsive or attractive preference pathway. Interestingly, activation of *Gr66a*-expressing neurons on the legs leads to avoidance of lobeline, while activation of the same taste-receptor complex in pharyngeal neurons leads to egg-laying preference for the same compound. This separation of responses based on *where* lobeline is being detected by *Gr66a* corresponds with the findings that leg and pharyngeal sensory neurons project axons to different regions of the SOG (Miyazaki and Ito, 2010). Thus, our results demonstrate that tissue-specific expression and activation of *Gr66a* provides one mechanism *Drosophila* use to differentially encode a single bitter input as both attractive and

repulsive, despite the compound being detected by neurons with overlapping gustatory receptor expression profiles.

Future studies will be important to ascertain the molecular mechanisms underlying how tissue-specific gustatory receptor expression leads to different wiring of taste-related neural circuitry, and hence different behavioral responses. For example, it has been postulated that *Gr66a, Gr32a, Gr33a, Gr39a.a,* and *Gr89a* form a core co-receptor complex required for bitter-signal transduction (Weiss et al., 2011), and this complex may form multimers with additional gustatory receptors that then confer ligand specificity to a compound in that particular sensory neuron, such as with *Gr93a* and caffeine (Lee et al., 2009). It will be interesting to investigate whether *Gr66a* neurons in the legs and the pharynx express distinct subsets of gustatory receptors, beyond the core co-receptor complex, and if the different gustatory receptor expression profiles reflect the differential wiring of these neurons to their respective neural circuits. Likewise, if the gustatory receptor profiles are identical between *Gr66a*-expressing leg and pharynx neurons, it will be interesting to investigate the developmental factors present in the different tissues that cause *Gr66a* axons to project into either the aversive or attractive pathways.

Appropriate perception of stimuli in the surrounding environmental is essential for organisms to make decisions that maximize their individual fitness and ensure the survival of their offspring. One such essential decision-making process occurs in *Drosophila* oviposition program, during which females select between an innate aversion for non-appetitive compounds and a specific attraction to these same compounds as egg-laying substrates. In this study, we characterize the sensory neurons and brain regions that are involved in this selection process, thereby providing an important step in understanding the gustatory-based

neural circuitry and complex selection process that female *Drosophila* employ when making an optimal decision to lay an egg. Furthermore, the results from this study elucidate key properties of the *Drosophila* gustatory system. Notably, we describe a novel strategy through which an organism utilizes a single sensory receptor complex in distinct anatomical locations to elicit opposing behavioral outputs from a single environmental cue. In summary, *Drosophila* provides an advantageous model system to study the selection process between mutually exclusive behavioral responses, and will likely provide novel insights into the basic neural and molecular mechanisms underlying decision-making in both invertebrates and vertebrates.

MATERIALS AND METHODS

Fly Stocks and Growth

Flies were reared on standard cornmeal/molasses/yeast/agar media under constant light at 25°C and 70% humidity. General behavioral characterization of positional aversion and egg-laying attraction responses to lobeline was typically performed in w^{1118} *Berlin* background, unless otherwise specified. *GAL4* lines from our P-element insertion library were also in the w^{1118} *Berlin* background. The *pox-neuro* lines were backcrossed at least four generations to w^{1118} *Berlin*, excluding the second chromosome which needed to be chromosome swapped to maintain the unmarked $poxn^{\Delta M22\text{-}B5}$ deficiency. Flies used in single female genetic mosaic experiments were also in a w^{1118} background (Gordon and Scott, 2009).

UAS-Shibire^{ts} flies contain two insertions of the transgene in a w^{1118} *Canton S* background. To ensure there was no variation in behavior due to mixed backgrounds, we

assayed w^{1118} *Berlin* controls with all *UAS-Shibire*^{ts} and *GAL80* trials. w^{1118} *Berlin* controls, mixed background w^{1118} *Berlin/UAS-Shibire*^{ts} flies, and w^{1118} *Berlin/GAL4* females exhibited similar behaviors in all tests at both 23°C and 30°C. Furthermore, our observations in Supplemental Figure 1B show that responses to 0.50mM lobeline are nearly identical in females with different genetic backgrounds, demonstrating that the presence of *Canton S* background should have minimal effects on positional and egg-laying preferences.

Two-Choice Assay of Egg-Laying and Positional Responses

The experimental assay to simultaneously measure egg-laying and positional responses to lobeline was performed as previously described **[12]**, with some minor modifications. Briefly, the base of plastic 6-ounce round bottom bottle (E & K Scientific, Santa Clara, CA) was cut off using a razor blade, and a 60-mm Petri dish lid was inserted into the removed portion of the bottle to facilitate scoring of female positional preference. Molten standard cornmeal/molasses/yeast/agar media was mixed with the appropriate volume of either aqueous (-)-lobeline hydrochloride (Sigma-Aldrich, St. Louis MO) or double-distilled water. 35-mm Petri dish lids (Becton Dickinson Labware, Franklin Lakes NJ) were divided in half using a razor blade, and either lobeline-containing or water-containing food was poured into each half in order to construct the two-choice plates.

Groups of 12-15 females, typically 1-2 days old, were collected and allowed to mate with three males for 2-3 days before being tested. Flies were gently knocked into bottles without CO_2 anesthesia to reduce behavioral perturbations; the bottle was capped with the two-choice plate, and then inverted for observation. Females were allowed to acclimate to the bottle apparatus for 1-2 hours, after which positional preferences were recorded. Bottles were

then placed in dark conditions to reduce environmental distractions, and allowed to lay eggs overnight, in order to accumulate enough eggs to obtain reliable OI indexes (Figure S1C). For temperature sensitive *UAS-Shibire^{ts}* assays, experimental procedures were conducted as described above, except that flies tested at the non-permissive temperature were put in a heated incubator with a transparent case allowing for visualization of positional behavior at 30°C.

To obtain positional preference indexes (PI), the number of flies on each half of the plate was scored at 10-minute intervals for 80 minutes. Values were totaled and a PI value was calculated: PI = (total flies on experimental food – total flies on control food) / (total flies on experimental food + total flies on control food). To obtain oviposition preference indexes (OI), the number of eggs on each half of the plate was counted after females laid eggs overnight: OI = (# eggs laid on experimental food - # eggs laid on control food) / # total eggs laid).

Extended 24 Hour Time Interval Assays

For behavioral assays that measured positional and egg-laying preferences for lobeline at times greater than 1-2 hours after initial bottle entry, experimental procedures conducted as described above, with the following modifications: (i) positional preferences were assayed at 3, 7, 11, 15, 19, and 23 hours after grouped females were first introduced to 0.50mM lobeline; (ii) two-choice dishes were collected and total eggs were counted immediately after the scoring of positional preferences; (iii) females were left in lighted conditions before and throughout testing.

Two-Choice Feeding Assay

To determine feeding preferences for food containing 0.50mM lobeline, the experimental assay and calculation of the feeding index (FI) was identical as previously described (Joseph et al., 2009; Chapter 2 of this thesis), with the following minor modifications: (i) we used 0.05% as the final dye concentrations of Erioglaucine (FD&C Blue #1) or Fast Green FCF dye (Green #3) (Sigma-Aldrich, St. Louis MO); (ii) females sampled lobeline-containing dye substrates for a longer time period (6 hours) to ensure a sufficient number of eggs were laid to check that egg-laying preference was not altered by the presence of Blue #1 or Green #3. Positional preferences were also scored, and females exhibited normal OI and PI values in the presence of dye.

Surgeries

To impair olfaction, females were anesthetized with $C0_2$ and the third antennal segment was removed with sharp forceps. To impair gustation on the legs, sharpened forceps were used to make a cut at the junction between the first and second tarsal segments on either the anterior, medial, or posterior pairs of legs (see Figure 3C for position of the cut). After both types of surgeries, females were allowed to recover and mate for 2-3 days before being tested.

Imaging and Immunohistochemistry

Representative imaging of the *Gr66a*^{GAL4} expression pattern (Figure 3) and clonal analysis experiments (Figure 5) was performed by directly visualizing the fluorescence of *GAL4/UAS-CD8-GFP, UAS-T2-GFP* or *GAL4/UAS-CD8-GFP* using a Leica confocal microscope (Leica Microsystems Inc., Bannockburn, IL). The green channel detects *GFP*

expression induced by *Gr66a*^{GAL4}, while the red channel was utilized to independently detect autofluorescence of the *Drosophila* cuticle. We subsequently overlayed the green channel and red channel to distinguish between cuticle autofluorescence and specific fluorescence induced by *GFP* expression; any autofluorescence from the cuticle that is being detected by both the green and red channels appears yellow when overlayed, while specific *GFP* expression is only detected and imaged by the green channel. Immunostaining of *5-120*^{GAL4}*/+*; *UAS-CD8-GFP/+* and *5-120*^{GAL4}*/MB{GAL80}*; *UAS-CD8-GFP/+* fly brains (Figure 6) was performed with antibodies against GFP and the nc82 neuropil marker, as described in our previous study (Joseph et al., 2009; Chapter 2 of this thesis).

Single Fly Clonal Analysis and Dissections

To generate transgenic females that possessed silenced clones restricted to a limited number of cells within the *Gr66a* expression pattern, we crossed *tubulin-FRT-GAL80-FRT; UAS-TNT; heat shock-FLP* (see Gordon & Scott, 2009 for strain construction **[57]**) to *Gr66a*^{GAL4}*; UAS-CD8-GFP* flies. Resulting *tubulin-FRT-GAL80-FRT/+; UAS-TNT/ Gr66a*^{GAL4}*; heat shock-FLP/UAS-CD8-GFP* progeny were then heat shocked for 1 hour 15 minutes at the pupal stage to generate clones. In summary, heat shock activation of *hs-FLP* randomly causes *FRT* sites to recombine *GAL80* away from its promoter, thereby halting *GAL80* repressor production. As a result, the *UAS/GAL4* system is de-repressed (i.e. activated), inducing *UAS-TNT* neuronal silencing and *UAS-CD8-GFP* labeling in these *Gr66a* neurons that underwent a stochastic recombination event. Single females were then collected after eclosion, and allowed to mate with three males for 2-3 days before being assayed for both positional aversion and egg-laying attraction responses to 0.50 lobeline.

Experimental protocols and preference index calculations for single fly assays were identical to those described above for the two-choice assay of egg-laying and positional responses, except that bottles only contained individual females. After behavioral analysis, individual flies were immediately collected and dissected to ascertain which *Gr66a*-expressing tissue regions contained *UAS-CD8-GFP* labeled, and thus *UAS-TNT* silenced clones. Briefly, we separated the head, anterior legs, and abdomen from the thorax using a razor blade, and whole mounted the tissues on a microscope slide with two bridging cover slips, to prevent compression of dissected samples. Of note, the abdomen was placed ventral surface facing up, to facilitate imaging of *Gr66a*-expressing cells. The slide was then sealed and tissue regions were imaged using a confocal microscope. After obtaining z-stacks of each dissected specimen, individual flies were assigned as either *GFP* positive or *GFP* negative for each particular tissue region.

After obtaining useable expression data for 89 single clonal females, and 19 individual control flies of the same genotype that did not undergo heat shock, we divided the assayed females into two groups for each different tissue region: (1) flies possessing *GFP* positive, and hence *UAS-TNT* silenced clones in a particular tissue region within the $Gr66a^{GAL4}$ expression pattern, and (2) flies that were *GFP* negative, and thus lacked *UAS-TNT* activity. We then performed unpaired t-tests comparing the mean OI values of each group to see if there was a significant decrease in egg-laying attraction in the *GFP* positive females when compared to the *GFP* negative females. Specifically, if *Gr66a* neurons in a particular tissue region were responsible for the egg-laying attraction, then the mean OI value of *GFP* positive, *UAS-TNT* silenced flies should differ significantly from *GFP* negative

siblings, since *GFP* positive grouping should be enriched with flies exhibiting disrupted egg-laying preference. Meanwhile the *GFP* negative grouping should primarily contain individuals with wild-type egg-laying preference, and therefore exhibit a mean OI value very similar to the no heat shock controls. This OI comparison analysis was performed on the following tissue region groupings of the same 89 clonal females: labellum, legs, abdomen, LSO, and VCSO.

Statistics

Statistical analyses are as described in figure legends and the main text, and unless otherwise specified the data is presented as means ± standard error of the mean (SEM). All statistical analyses were performed using GraphPad Prism, Version 4.0 (GraphPad Software, Inc., San Diego CA).

ACKNOWLEDGEMENTS

The authors thank Kristin Scott and Michael D. Gordon for the *TSH{GAL80}* and *tubulin-FRT-GAL80-FRT; UAS-TNT; heat shock-FLP* flies necessary for the clonal analysis experiments, as well as very helpful advice. We also thank Scott Waddell for the *MB{GAL80}* lines, and Werner Boll and Markus Noll for both the *pox-neuro* flies and very useful input. Funding was provided by grants from National Institutes of Health/National Institute on Alcohol Abuse and Alcoholism.

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FIGURE LEGENDS

Figure 1: Bitter-tasting lobeline concurrently induces aversive positional and attractive egg-laying responses in female *Drosophila*.

(A) Dose-response curve for positional and egg-laying responses to increasing concentrations of lobeline. Values for the positional preference index (PI) and oviposition preference index (OI) were collected from the same groups of flies (see Materials and Methods for calculation of PI and OI). Significant differences between 0.00mM no-lobeline control assays and 0.50mM two-choice dishes were observed (*, $P<0.05$; **, $P<0.01$; 1-way ANOVA, Bonferroni's post-test; $n\geq9$). **(B)** Bar graph representation of average PI and OI values demonstrated with 0.50mM lobeline; subsequent experiments were performed at the 0.50mM dose. **(C)** PI and OI values of females assayed 3, 7, 11, 15, 19, 23 hours after being introduced to 0.50mM lobeline. Both positional aversion and egg-laying attraction remained constant between different time-intervals ($P>0.05$; non-zero linear regression test; $n\geq6$). Linear regression plots for PI values (blue line) and OI values (red line) had slopes=0.002 and 0.001, respectively. No significant differences were observed between average PI or OI values across different time-intervals ($P>0.05$; 1-way ANOVA; $n\geq6$).

Figure 2: Silencing *Gr66a*-neurons disrupts both aversive positional and attractive egg-laying responses.

(A) Behavioral responses to lobeline in females expressing *UAS-Shi^{ts}* in *Gr66a* neurons. *Gr66a^{GAL4}/UAS-Shi^{ts}* flies exhibit a loss of positional aversion and egg-laying preference for 0.50mM lobeline when shifted from permissive (23°C) to non-permissive (30°C)

temperatures. PI and OI preferences of experimental *Gr66a*^{GAL4}/*UAS-Shi*^{ts} flies (color bars) were significantly different from *UAS-Shi*^{ts}/+ and *Gr66a*^{GAL4}/+ controls (gray bars) at 30°C. (*, P<0.05; **, P<0.01; 1-way ANOVA, Bonferroni's post-test for comparison between columns within the 23°C or 30°C groups; 2-way ANOVA, Bonferroni's post-test for comparison between temperatures within same genotypes; n≥9). In addition, *Gr66a*^{GAL4}/*UAS-Shi*^{ts} flies also exhibited a significant loss of aversion at 25°C, likely due to leaky activity of the *UAS-Shi*^{ts} transgene. **(B)** When compared to *Gr66a*^{GAL4}/+ and *UAS-TNT*/+ controls, *Gr66a*^{GAL4}/*UAS-TNT* also possessed a significant loss of both positional aversion and egg-laying attraction to 0.50mM lobeline (***, P<0.001; 1-way ANOVA; n≥28). Of note, positional aversion in the *UAS-TNT*/+ control was also significantly greater than *Gr66a*^{GAL4}/+ (***, P<0.001), however the above average repulsion associated with the *UAS-TNT* construct in the heterozygote control did not affect *Gr66a*^{GAL4}/*UAS-TNT* flies, since they demonstrated essentially no positional repulsion to lobeline. **(C)** *Gr66a*$^{\Delta ex83}$/*Gr66a*$^{\Delta ex83}$ flies exhibited a loss in positional aversion and egg-laying attraction to 0.50mM lobeline when compared to *w*^{1118} *Berlin* controls (***, P<0.001; unpaired t-test (two tailed); n≥10).

Figure 3: *Gr66a*^{GAL4} expresses in gustatory neurons present in the *Drosophila* proboscis and legs.

(A) *Gr66a*^{GAL4} is expressed in sensory neurons in the labellum (large arrow), lateral sensory organ (LSO; small arrow), and ventral cibarial sensory organ (VCSO; arrowhead) of the *Drosophila* proboscis. Image in (A) was taken from the posterior side of the head. **(B)** Image of the LSO taken from the anterior side of the head, such that *Gr66a*^{GAL4} expression can be better visualized. **(C)** *Gr66a*^{GAL4} is expressed in the first tarsi of the anterior forelegs in

female *Drosophila*. Dashed line represents the location where cuts in tarsal ablation experiments were performed (Figure 4B). In (A-C), *Gr66a*^{GAL4} was visualized with *UAS-CD8-GFP* (green channel); cuticle autofluoresence was used to define boundaries of the head and the leg (red channel). For (A-C): Scale bar = 40μm.

Figure 4: *Gr66a* neurons on *Drosophila* legs receive sensory input for positional aversion response to lobeline.

(A) Restoration of positional aversion to lobeline using thorax-specific *TSH{GAL80}* to suppress *Gr66a*^{GAL4}/*UAS-Shi*^{ts} silencing in leg sensory neurons. Females expressing *TSH{GAL80}, Gr66a*^{GAL4} and *UAS-Shi*^{ts} exhibited normal positional aversion at the non-permissive temperature (30°C) when compared to flies with only *Gr66a*^{GAL4} and *UAS-Shi*^{ts}, as well as the *UAS-Shi*^{ts}/+, *Gr66a*^{GAL4}/+, and *TSH{GAL80}/+; UAS-Shi*^{ts}/+ controls. (*, $P<0.05$; 1-way ANOVA, Bonferroni's post-test; $n\geq15$). Egg-laying attraction remained disrupted at 30°C in females expressing *TSH{GAL80}, Gr66a*^{GAL4} and *UAS-Shi*^{ts}, when compared to relevant controls. (*, $P<0.05$; **, $P<0.01$; 1-way ANOVA, Bonferroni's post-test for comparison between columns within the 23°C or 30°C groups; 2-way ANOVA, Bonferroni's post-test for comparison between temperatures within same genotypes; $n\geq15$).

(B) Behavioral responses to lobeline in females with the first tarsi removed on either the anterior, medial, or posterior pairs of legs. A loss of positional aversion to 0.50mM lobeline was only observed in flies lacking first tarsi gustatory bristles from the anterior legs (**, $P<0.01$; 1-way ANOVA, Bonferroni's post-test; $n\geq10$). Egg-laying responses were unaffected by tarsal ablation ($P>0.05$; 1-way ANOVA, $n\geq10$).

Figure 5: *Gr66a* neurons in the pharynx receive sensory input for egg-laying attraction to lobeline.

(A) Average oviposition indexes of females grouped as either lacking or possessing *GFP*-labeled, *UAS-TNT* silenced clones (blue and green bars, respectively) in the following *Gr66a*-expressing tissue regions: labellum, legs, abdomen, lateral sensory organ (LSO) and ventral cibarial sensory organ (VCSO); vertical gray lines separate the different groupings of the same 89 flies. A significant disruption in egg-laying preference to 0.50mM lobeline was only observed when comparing females that were grouped as –GFP or +GFP for silencing of neurons in the VCSO (**, $P<0.01$; unpaired t-test (two tailed); n-values for –GFP versus +GFP mean OI values for each tissue grouping are listed within respective blue and green bars). Scale bar = 20μm. **(B)** Representative image of a single GFP labeled, *UAS-TNT* silenced clone within the VCSO. Genotype of the representative female is: *tubulin-FRT-GAL80-FRT/+; Gr66a^{GAL4}/UAS-TNT; heat shock-FLP*/*UAS-CD8-GFP*. Live imaging of *UAS-CD8-GFP* is shown with the green channel; cuticle autofluorescence was recorded with the red channel. **(C)** Comparison between: females with *+GFP*, *UAS-TNT* silenced clones in the VCSO (green bar), females of the same genotype that did not undergo heat shock (gray bar), and *–GFP, UAS-TNT* suppressed females that underwent heat shock but did not possess silenced neurons within the VCSO (blue bar) (*, $P<0.05$; **, $P<0.01$; 1-way ANOVA, Bonferroni's post-test; $n\geq19$).

Figure 6: Silencing the mushroom body simultaneously disrupts positional and egg-laying responses to lobeline.

(A) At the non-permissive temperature of 30°C, *5-120^{GAL4}* females expressing *UAS-Shi^{ts}* in the mushroom body lose both positional aversion and egg-laying attraction to 0.50mM lobeline when compared to relevant controls (gray bars). In contrast, *5-120^{GAL4}* females expressing both *UAS-Shi^{ts}* and *mushroom body-GAL80 (MB{GAL80})* exhibit normal behavioral responses to 0.50mM lobeline (**, P<0.01; ***P<0.001; 1-way ANOVA, Bonferroni's post-test for comparison between columns within the 23°C or 30°C groups; 2-way ANOVA, Bonferroni's post-test for comparison between temperatures within same genotypes; n≥13). **(B)** Confocal imaging of *5-120^{GAL4}/+; UAS-CD8-GFP/+* females reveal strong *GAL4* expression in the mushroom body, as well as some other neurons within the brain. **(C)** Inclusion of *MB{GAL80}* in *5-120^{GAL4}/MB{GAL80}; UAS-CD8-GFP/+* females suppresses *GAL4* expression specifically in the mushroom body, while maintaining expression in other extraneous neurons. In (B) and (C), *GAL4* was visualized in immunostained brains using antibodies against *CD8-GFP* (green channel) and nc82 neuropil marker (red channel). For (B) and (C): Scale bar = 20μm.

Figure 7: A model for the neural circuits mediating positional aversion and egg-laying attraction to lobeline.

Sensory input for lobeline is simultaneously received by *Gr66a* neurons in the legs that receive signals for the positional aversion pathway (blue lines), and by *Gr66a* neurons in the pharynx that receive signals for the egg-laying attraction pathway (red lines). Both types of sensory neurons project into distinct sub-regions of the SOG, where some separation of signals is likely maintained during first-order processing. Our data suggests that lobeline

signals are relayed to the mushroom body, where they are integrated into a combinatorial signal (purple lines) that is evaluated before an appropriate motor output is selected.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1: Additional characterization of bitter-induced behavioral responses in female *Drosophila*.

(A) Female *Drosophila* demonstrate aversion to lobeline in two-choice feeding assays. Feeding preference indexes (FI) were obtained for dishes containing either (i) blue dye + 0.50mM lobeline / green dye + water, or reciprocal (ii) blue dye + water / green dye + 0.50mM lobeline two-choice combinations. Variation due to day-to-day preferences for dye alone was corrected for using paired FI values obtained from blue dye + water / green dye + water controls to normalize indexes. FI values for the reciprocal two-choice dye + lobeline experiments were then pooled and averaged for comparison to the mean FI of the corrected no-choice blue dye + water / green dye + water controls (*, $P<0.05$; paired t-test (two tailed); n=16). **(B)** Females also exhibited positional aversion and egg-laying attraction to 10mM quinine, another bitter tasting compound, when compared to no-quinine controls (*, $P<0.05$; **, $P<0.01$; unpaired t-test (two-tailed); $n\geq7$). **(C)** Average number of total eggs laid at different time-intervals by females assayed in experiments from (Figure 1C). Groups of females needed to lay more than 10 eggs per assay for reliable oviposition indexes; thus groups were allowed to lay eggs overnight. **(D)** Females from the *Canton S, Oregon R*, and w^{1118} *Berlin* genetic backgrounds exhibit similar positional aversion and egg-laying attraction

responses to 0.50mM lobeline (P>0.05, 1-way ANOVA; n≥7). Additionally, w^{1118} *Berlin* males are equally repulsed to 0.50mM as w^{1118} *Berlin* females.

Supplemental Figure 2: Females with disrupted olfactory sensory input exhibit normal lobeline induced behavioral responses.

w^{1118} *Berlin* females lacking antenna, mixed background *UAS-Shi*^{ts}/+ females lacking antenna, and w^{1118} *Berlin Or83b*^{1}*/Or83b*^{1} mutant flies exhibit positional aversion and egg-laying attraction for 0.50mM that is not significantly different from wild-type w^{1118} *Berlin* control females (P>0.05; 1-way ANOVA, Dunnett's multiple comparison post-test; n≥10).

Supplemental Figure 3: *pox-neuro* mutants lacking taste-bristles on legs only lose positional aversion to lobeline.

pox-neuro (*poxn*) flies lacking taste bristles on their legs, namely the deficiency $poxn^{\Delta M22\text{-}B5}$ homozygotes and Δ*XBs6;* $poxn^{\Delta M22\text{-}B5}$ partial rescue, demonstrated a loss of positional aversion when compared to $poxn^{\Delta M22\text{-}B5}$*; full-152* and $poxn^{\Delta M22\text{-}B5}$*; SuperA158* rescue lines that have functional gustatory bristles on their tarsal segments (*, P<0.05; **, P<0.01; 1-way ANOVA, Bonferroni's post-test; n≥6). Egg-laying attraction to 0.50mM lobeline was normal in all lines tested, including $poxn^{\Delta M22\text{-}B5}$ homozygotes (P>0.05; 1-way ANOVA; n≥6), suggesting that the *Gr66a* gustatory neurons responsible for the egg-laying behavior are not transformed by the *poxn* developmental defect.

Supplemental Figure 4: Single females with silenced *Gr66a* neurons in the legs have diminished positional aversion to lobeline.

(A) Mean PI values for females grouped as either *–GFP* heat shocked females that lacked clones in the legs (blue bar), possessing a *+GFP*, *UAS-TNT* silenced clone on a one leg (teal bar), possessing *+GFP*, *UAS-TNT* silenced neurons on both legs (green bar), or controls of the same genotype that did not undergo heat shock (gray bar). Females that possessed silenced *Gr66a* neurons on both legs trended towards a loss of positional aversion, but a significant difference was only seen when compared to the no heat shock controls, likely due to the increased variability associated with obtaining PI values in single fly assays. Of note, the no heat shock controls were significantly more repulsed than *–GFP* females, suggesting that the heat shock itself could have some effects on positional responses in our assay. (*, $P<0.05$; ***, $P<0.001$; 1-way ANOVA, Bonferroni's post-test; n=59 for –GFP, n=21 for +GFP 1-leg, n=9 for +GFP 2-legs, n=18 for no heat shock).

Supplemental Figure 5: Silencing the mushroom body in *$30Y^{GAL4}$* females disrupts aversive positional and attractive egg-laying responses.

(A) At the non-permissive temperature of 30°C, *$30Y^{GAL4}$* females expressing *UAS-Shi^{ts}* in the mushroom body lose both positional aversion and egg-laying attraction to 0.50mM lobeline when compared to relevant controls (gray bars). (*, $P<0.05$; 1-way ANOVA, Bonferroni's post-test for comparison between columns within the 23°C or 30°C groups; 2-way ANOVA, Bonferroni's post-test for comparison between temperatures within same genotypes; $n\geq8$). Of note, the positional aversion between *$30Y^{GAL4}$/UAS-Shi^{ts}* females at 25°C and 30°C were not significantly different in the 2-way ANOVA Bonferroni's post-test, likely due to the fact that

leaky activity of the *UAS-Shi^{ts}* transgene also caused a decrease of positional aversion at the permissive temperature. Additionally, *30Y^{GAL4}*/+ females demonstrated a significant increase in positional aversion at 23°C, when compared to *UAS-Shi^{ts}*/+ (*, P<0.05). However, this increase in positional aversion resulting from *30Y^{GAL4}* construct in the heterozygote did not affect the *30Y^{GAL4}/UAS-Shi^{ts}* females, which still lost positional aversion.

Supplemental Figure 6: Silencing of neurons in additional mushroom body *GAL4* lines causes disruption in positional and egg-laying responses to 0.50mM lobeline.

(A) Diagram of the α/β, α'/β', and γ neuronal lobes that comprise the mushroom body. Expression of *GAL4* in the candidate mushroom body lines described in (B)-(F) is represented by the corresponding α/β, α'/β', or γ lobe being shaded blue in the diagram accompanying the behavioral data. **(B)** *5-120^{GAL4}/UAS-Shi^{ts}* females exhibit both a loss of positional aversion and egg-laying attraction at the non-permissive temperature (30°C), when compared to *5-120^{GAL4}*/+ and *UAS-Shi^{ts}*/+ controls. *5-120^{GAL4}* expresses in the α/β and γ lobes. **(C)** *30Y^{GAL4}/UAS-Shi^{ts}* females exhibit both a loss of positional aversion and egg-laying attraction at 30°C when compared to heterozygote controls. *30Y^{GAL4}* flies express *GAL4* in the α/β, α'/β', and γ lobes. **(D)** *5-98^{GAL4}/UAS-Shi^{ts}* females only exhibit a loss of egg-laying attraction at 30°C when compared to heterozygote controls. *5-98^{GAL4}* expresses *GAL4* strongly in the γ lobes and weakly in the α/β lobes, suggesting that the egg-laying response to lobeline is mediated in part by neurons in the γ lobes. **(E)** *4-59^{GAL4}/UAS-Shi^{ts}* females only lose positional aversion at 30°C when compared to heterozygote controls. *4-59^{GAL4}* expresses *GAL4* in the α'/β' lobes, suggesting that the α'/β' lobes play a role in positional aversion response to lobeline. **(F)** *17D^{GAL4}/UAS-Shi^{ts}* females only lose positional

aversion at 30°C when compared to heterozygote controls. *17D*^{GAL4} expresses *GAL4* in the α/β lobes **[51]**, suggesting that the positional aversion to lobeline is also mediated by neurons in the α/β lobes. (*a* = P<0.001; *b* = P<0.01; *c* = P<0.05; 1-way ANOVA, Bonferroni's post-test for comparisons between columns within the 23°C or 30°C groups; **, P<0.01; *, P<0.05; ***, P<0.001; 2-way ANOVA, Bonferroni's post-test for comparison between temperatures within same genotypes; n≥6).

Figure 1

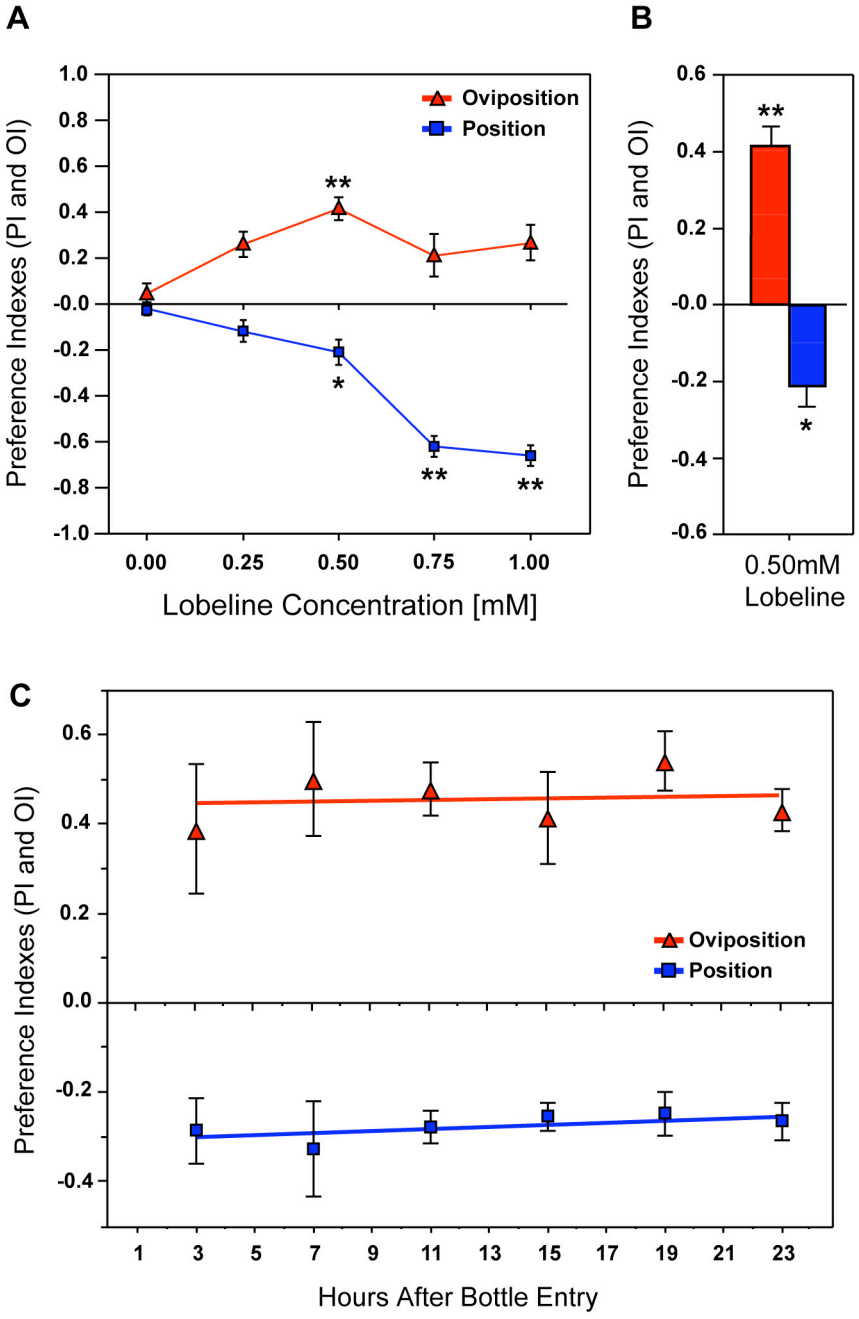

A
B
C
Oviposition
Position
Preference Indexes (PI and OI)
Lobeline Concentration [mM]
0.50mM
Lobeline
Hours After Bottle Entry

Figure 2

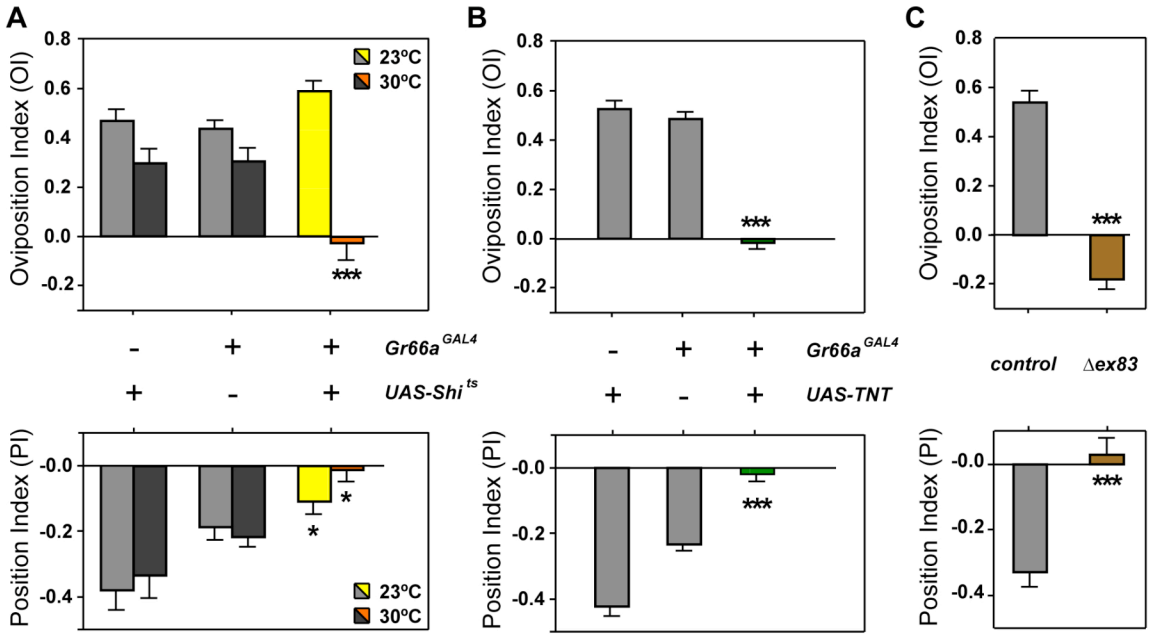

A
B
C
Oviposition Index (OI)
0.8
0.6
0.4
0.2
0.0
-0.2
23ºC
30ºC
***
-
+
+
Gr66a GAL4
+
-
+
UAS-Shi ts
Position Index (PI)
-0.0
-0.2
-0.4
*
*
***
control
Δex83
UAS-TNT

Figure 3

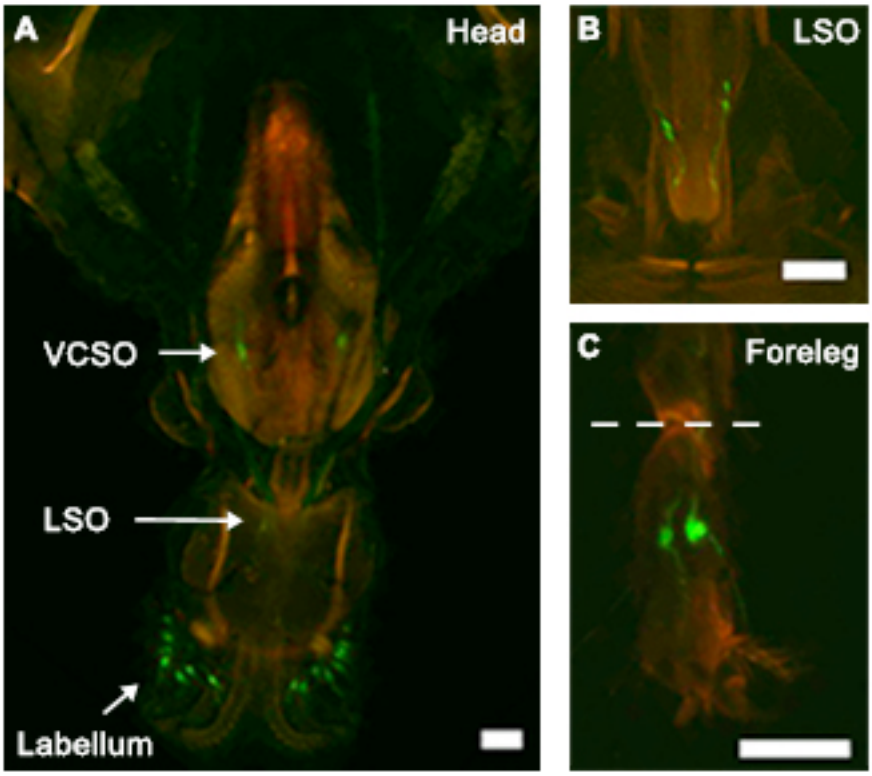


Figure 4

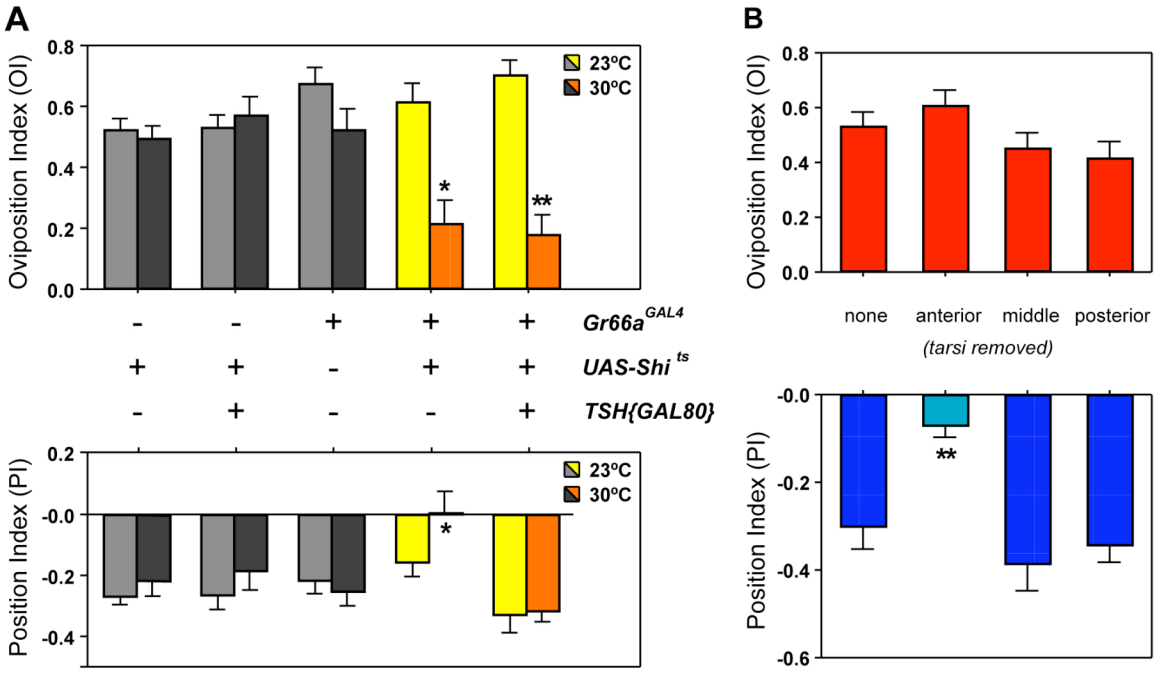

A
Oviposition Index (OI)
0.8
0.6
0.4
0.2
0.0
23°C
30°C
*
**
-
-
+
+
+
Gr66a GAL4
+
+
-
+
+
UAS-Shi ts
-
+
-
-
+
TSH{GAL80}
Position Index (PI)
0.2
-0.0
-0.2
-0.4
23°C
30°C
*
B
Oviposition Index (OI)
0.8
0.6
0.4
0.2
0.0
none
anterior
middle
posterior
(tarsi removed)
Position Index (PI)
-0.0
-0.2
-0.4
-0.6
**

Figure 5

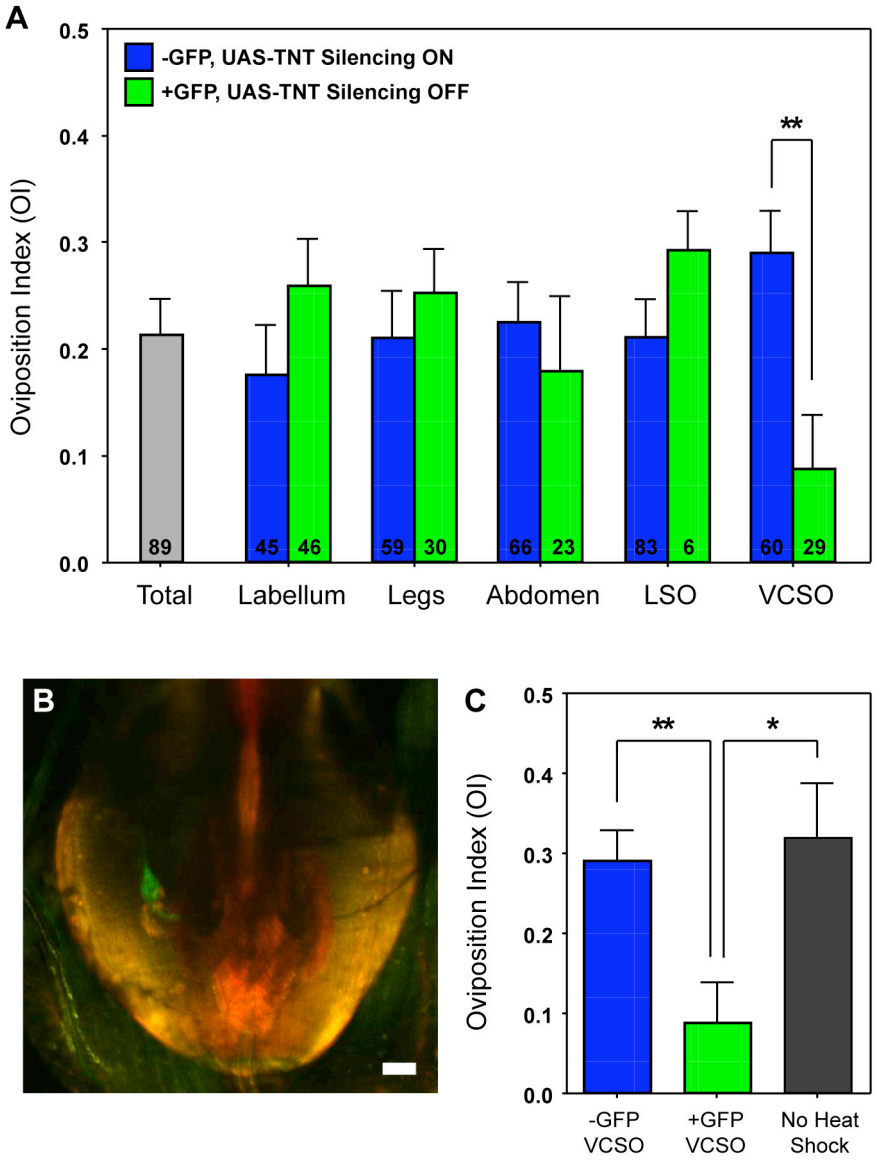

A
-GFP, UAS-TNT Silencing ON
+GFP, UAS-TNT Silencing OFF
Oviposition Index (OI)
0.5
0.4
0.3
0.2
0.1
0.0
**
89
45
46
59
30
66
23
83
6
60
29
Total
Labellum
Legs
Abdomen
LSO
VCSO
B
C
Oviposition Index (OI)
**
*
-GFP VCSO
+GFP VCSO
No Heat Shock

Figure 6

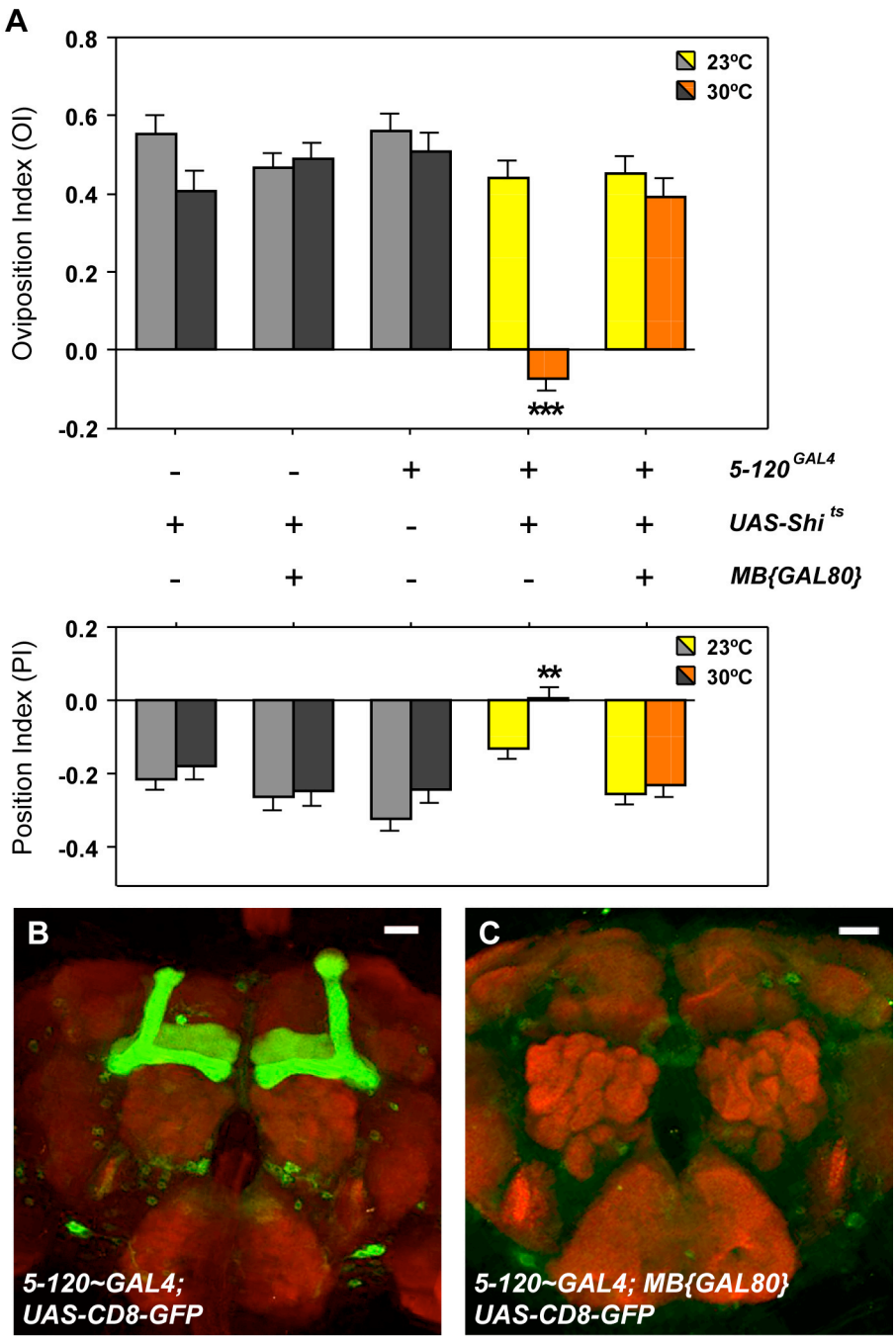
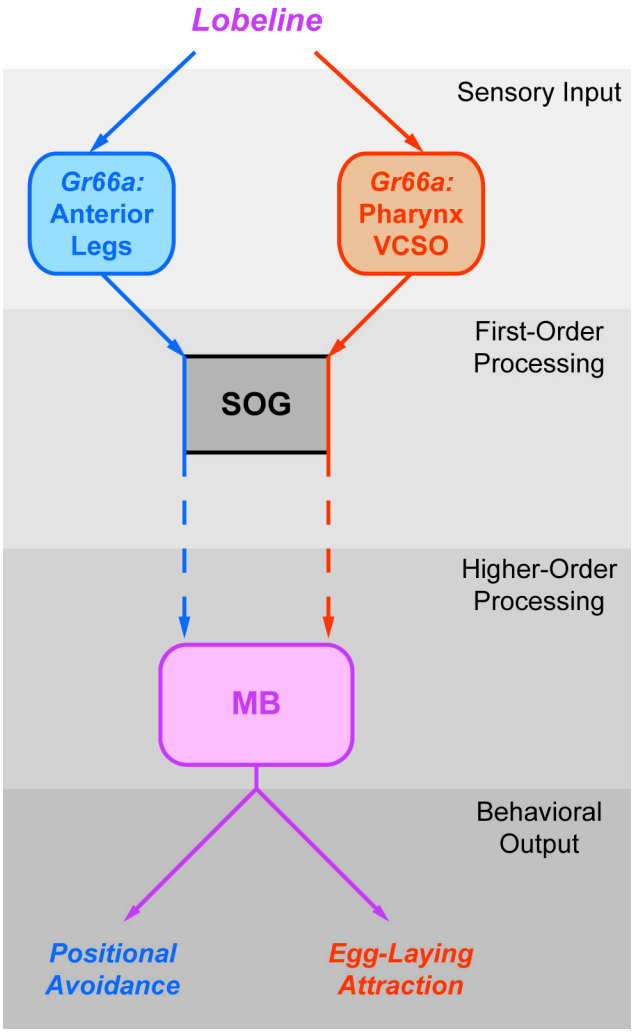
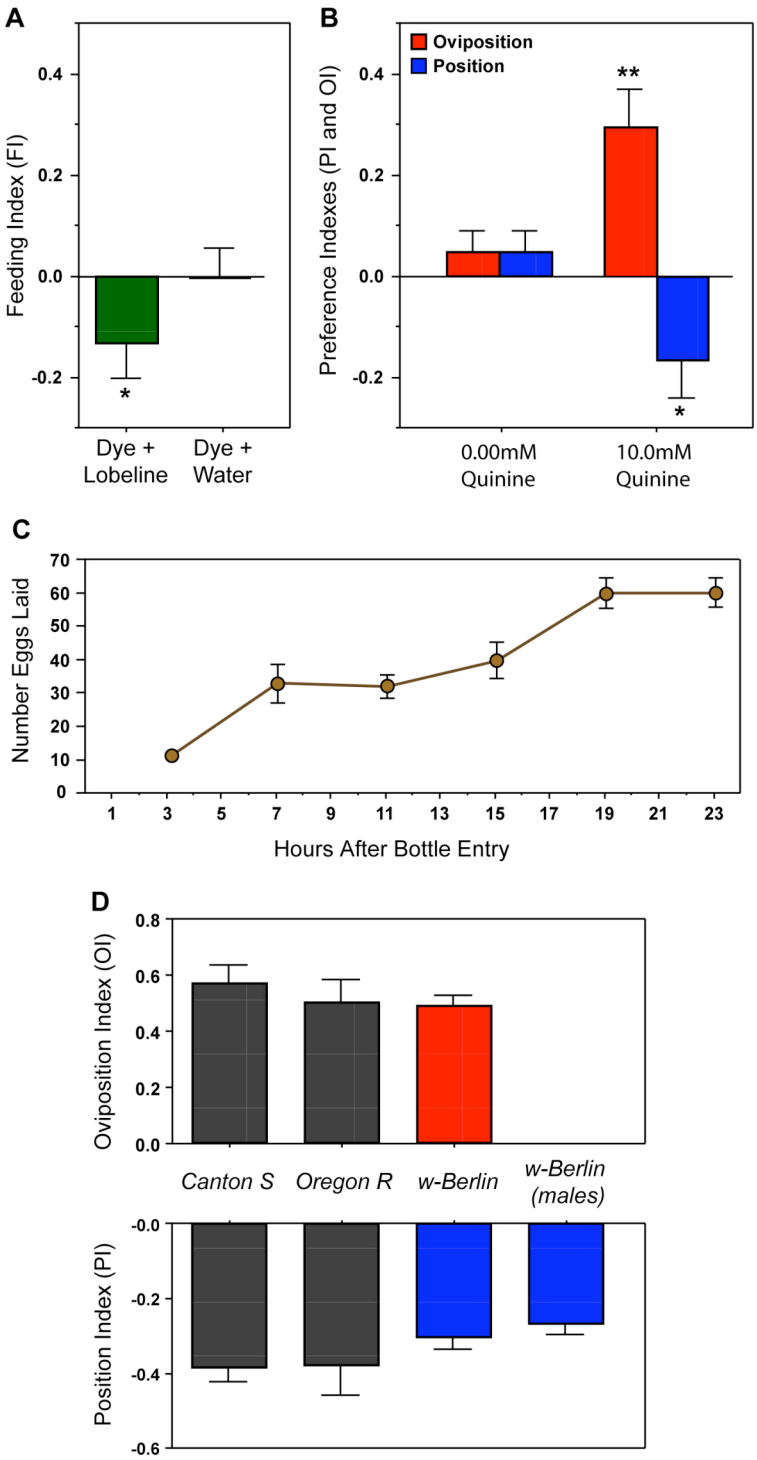

A
Oviposition Index (OI)
0.8
0.6
0.4
0.2
0.0
-0.2
23°C
30°C
***
- - + + + 5-120 GAL4
+ + - + + UAS-Shi ts
- + - - + MB{GAL80}
Position Index (PI)
0.2
0.0
-0.2
-0.4
**
B
5-120~GAL4;
UAS-CD8-GFP
C
5-120~GAL4; MB{GAL80}
UAS-CD8-GFP

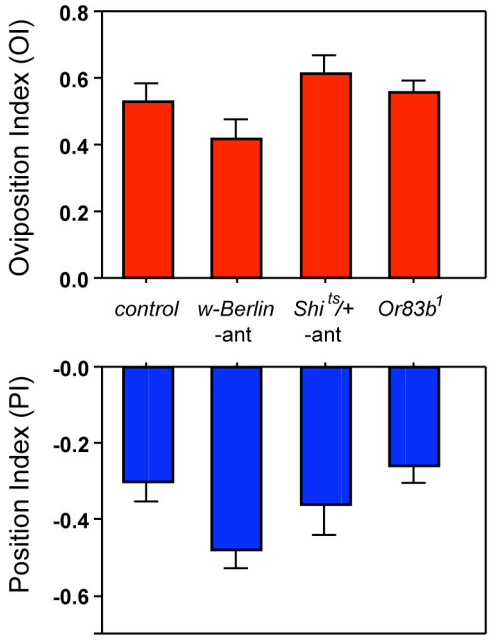
Figure 7


Lobeline
Sensory Input
Gr66a: Anterior Legs
Gr66a: Pharynx VCSO
First-Order Processing
SOG
Higher-Order Processing
MB
Behavioral Output
Positional Avoidance
Egg-Laying Attraction

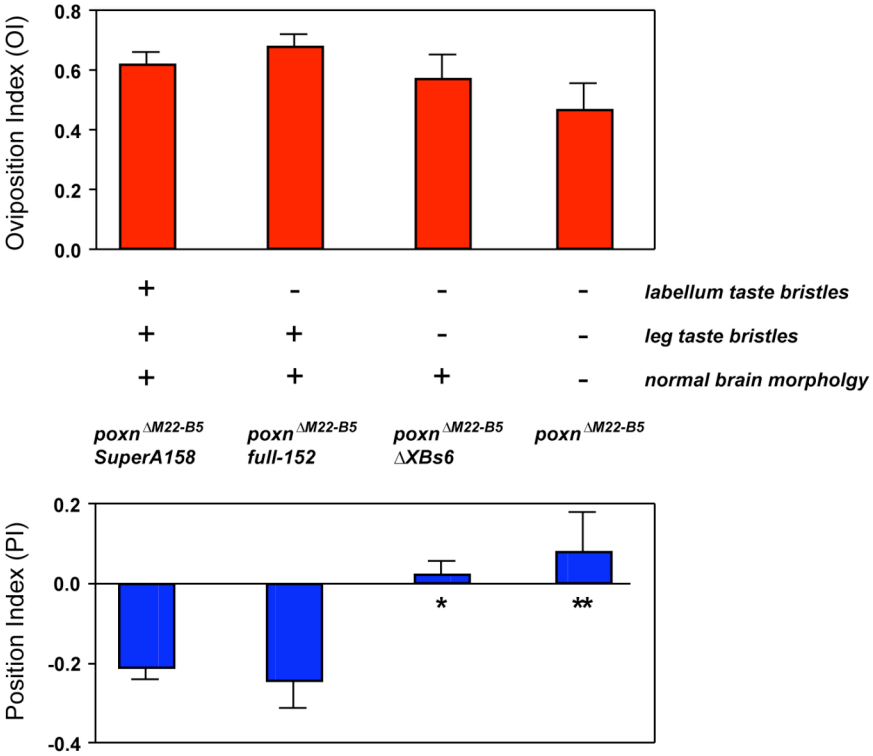
Supplemental Figure S1



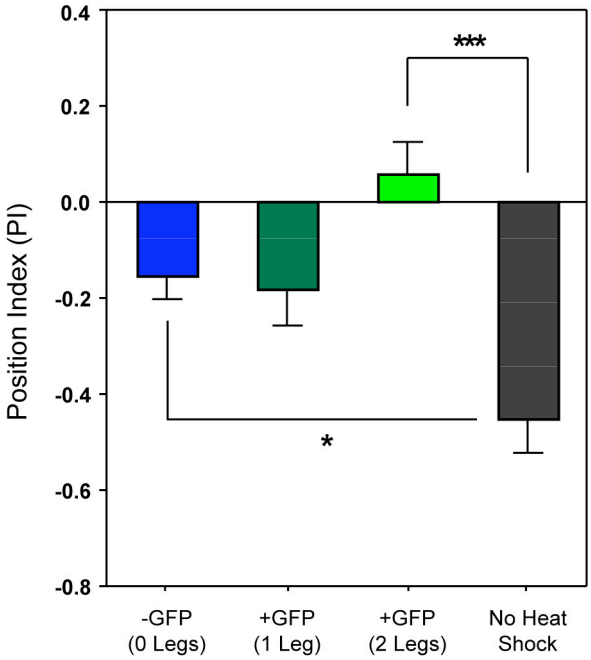
Supplemental Figure S2



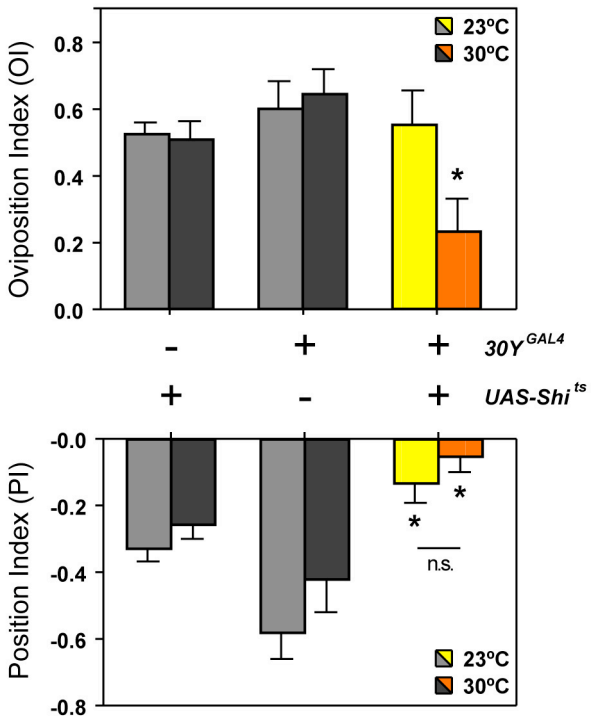
Supplemental Figure S3



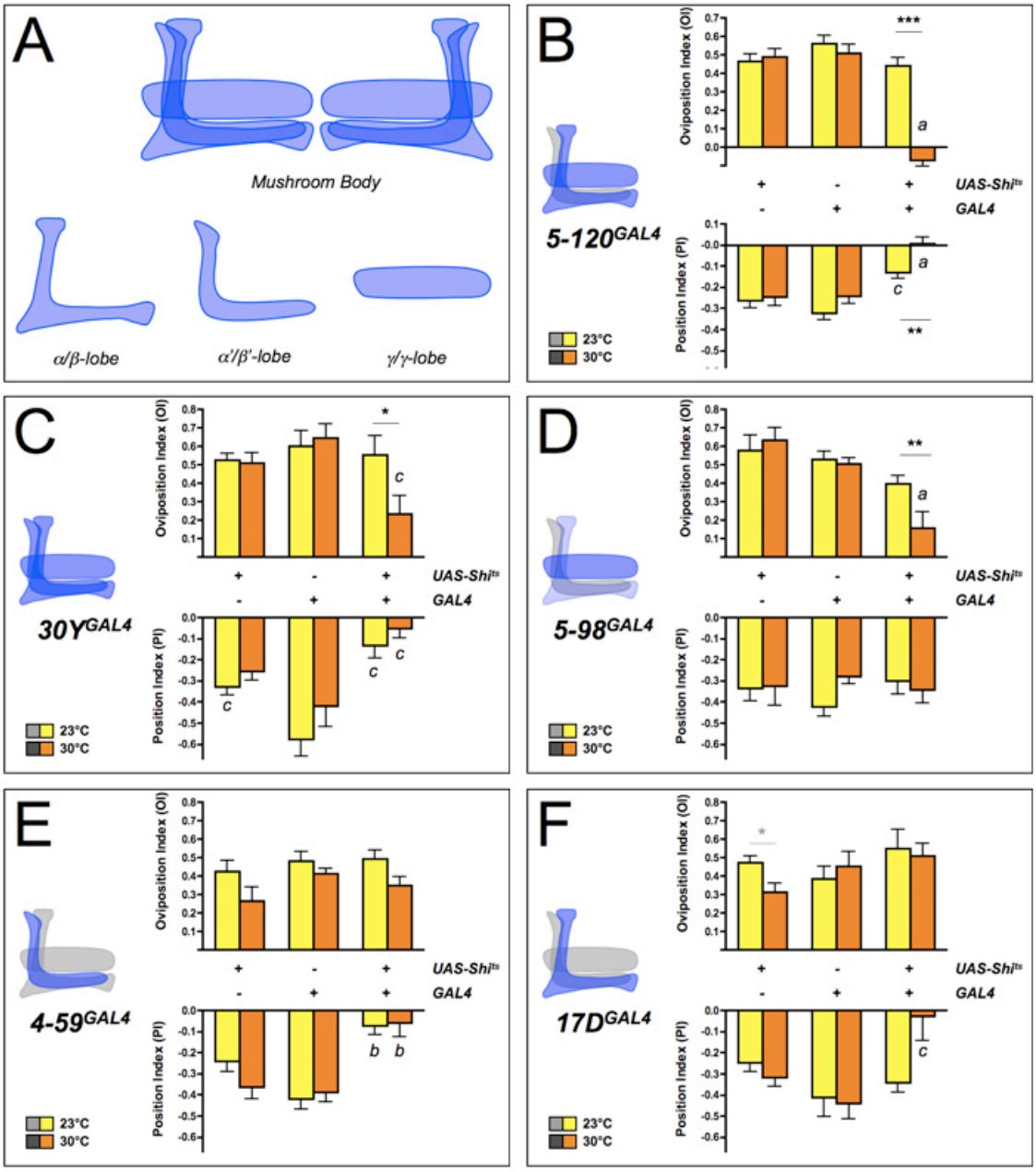
Supplemental Figure S4



Supplemental Figure S5



Supplemental Figure S6



Conclusion

FUTURE DIRECTIONS

During the development of this project, two goals were established: (1) substantiate oviposition site-selection of *Drosophila melanogaster* as an effective model for simple decision-making, and (2) utilize the genetically amenable fruit fly to identify the neural circuitry mediating the sensory detection, signal valuation, and response selection stages of the decision-making process. Over the course of the experiments detailed in this thesis, the questions associated with both goals were addressed, and several important answers to these scientific queries were obtained. As such, significant progress was made towards completing these goals, with regard to this study as a dissertation project. However, research into *Drosophila* oviposition behavior as a simple model for decision-making is by no means completed—numerous potential investigative paths can be initiated or continued, based on the results from this study.

With regard to questions surrounding substantiating *Drosophila* oviposition as a decision-making process, a significant amount of interesting behavioral and evolutionary questions remain. Specifically, what is the evolutionary basis for this peculiar behavioral paradigm, in which female fruit flies lay eggs on substrates that at first glance appear to be non-appetitive or even detrimental to the survival of both adults and progeny? Given our observations with acetic acid (AA), we offer a few hypotheses worth testing in future. Experiments in which fertilized eggs were left on the boundary between AA-containing and AA-lacking food in the two choice assay revealed that, after hatching, larvae would typically avoid the AA-containing food (Ryan M. Joseph; unpublished observations). Similarly, when eggs deposited on AA-containing food were allowed to hatch on the two-choice dishes, the larvae fled the AA-containing half for the AA-lacking half (Anita V. Devineni; unpublished

observations). These results demonstrate that compounds like AA can be used to effectively separate eggs from larvae and/or adults, allowing us to postulate a few evolutionary explanations. First, larvae are constantly ingesting their environment; the AA-induced positional aversion of larvae and adults versus the AA-induced egg-laying attraction could therefore have evolved as a mechanism to separate immobile eggs from being buried in liquefied media that older generations of larvae have already ingested. Likewise, this competition between AA-based behaviors could be used to prevent intra-species competition for resources between adults, larvae, and eggs. Second, this separation may have instead evolved for inter-species separation between *Drosophila melanogaster* and other insect conspecifics that also utilize fermenting fruit as a feeding and oviposition resource; indeed, studies have demonstrated that when compared to other sibling species *Drosophila melanogaster* has a higher tolerance of acetic acid (Parsons, 1979; Parson, 1982). Thirdly, in addition to providing division between resource competition of different insect species, the separation afforded by attraction to AA could also be beneficial to developing embryos by keeping *Drosophila* eggs in an more acidic environment that is less attractive to predators or less vulnerable to microbial overpopulation and/or infection. Thus, investigating the evolutionary importance of this decision-making process between positional aversion and egg-laying attraction to the same compound should provide an interesting study into how species balance their own innate adult preferences with the responses that are beneficial to the survival of their progeny.

Further questions remain regarding the general characterization of the *Drosophila* oviposition program, which could also provide interesting investigative inroads into how female flies valuate their surroundings and predict the benefits of a particular environment

for their offspring. Specifically, recent studies have demonstrated that egg-laying preferences for non-appetitive substrates can be affected by the size of the surrounding testing chamber and/or environment (Yang et al., 2008; Miller et al., 2011). If females are searching for oviposition sites in a small environment like our two-choice assay (Joseph et al., 2009), they deposit eggs on the non-appetitive alternative. However, when searching for oviposition sites in a larger environment, they deposit eggs directly on nutritive substrates (Miller et al., 2011). One can speculate that the female is balancing two predicted needs for her offspring: (1) the need to ensure hatching progeny have nearby food sources available, versus (2) the need to separate eggs from adults and larvae in a crowded environment. It will be interesting to see what parameters of the surrounding environment are ranked as most valuable, and to test what specific environmental cues alter these predicted perceptions that female *Drosophila* generate when deciding to lay an egg. For example, recent observations from our laboratory have suggested that the population density can affect egg-laying preferences to bitter-tasting compounds (Norma Velazquez Ulloa; unpublished observations). These results provide one potential mechanism females use during the *valuation* step of oviposition-related decision-making to rank the different behavioral output options, and offer an interesting line of investigation into this particular stage of the decision-making process.

Additionally, some of the genes uncovered in the screen for AA-based egg-laying mutants should likely become interesting subjects of study with regard to better understanding the *detection-valuation-selection* processes involved in oviposition-based decision-making in *Drosophila.* For example, if the *3-41b* insertion does in fact disrupt expression of *jing*, informative research about the development of the neural circuits required for egg-laying preference can be conducted. Notably, *jing* seems to be involved in the development of

central nervous system neurons (Sedaghat et al., 2002: McClure and Schubiger, 2008); by tracking which neurons in *jing* mutants have defects, we may be able to isolate additional neurons—notably those between the sensory cells and the higher-order brain structures in the circuit map—that are necessary for egg-laying preference responses. The *3-21a* insertion that mapped to *Rab6* may also have similar developmental affects, at least with regard to the peripheral nervous system (Purcell and Artavanis-Tsakonas, 1999), as well as the closely related *Rab5*, which has been shown involved in dendrite morphogenesis (Satoh et al., 2008). Thus, testing of *Rab6* and *Rab5* alleles could also prove useful when looking for developmental phenotypes in the nervous system that affect egg-laying, which may subsequently lead to the identification of additional neurons in the circuit maps of the competing positional aversion and/or egg-laying attraction preference pathways.

amnesiac may also be an interesting gene of future study, given its co-localization to the mushroom body, which we showed to be important to decision-making processes in *Drosophila*. *amn* has been implicated in learning and memory (Keene et al., 2006) and attention-related processes (van Swinderen, 2007); it will be interesting to see how learning and memory are factored into the female fly's evaluation process when deciding where to lay an egg. Notably, we discovered that *Gr66a* neurons in the pharynx receive bitter-tasting sensory input that is relayed to the egg-laying preference pathway. Does the female fly sample the appetitive qualities of food while foraging, but then "remember" that she encountered a non-appetitive substrate earlier, then return to that particular substrate to deposit an egg? Subsequently, do learning and memory mutants like *amn* exhibit defects in egg-laying preference because they cannot recall that they previously encountered a nearby attractive egg-laying substrate? Future testing of *amn* alleles and additional learning and

memory mutants can potentially answer these questions. Furthermore, it has recently been shown that octopamine is directly involved in *Drosophila* ovulation (Lee et al., 2009), while neurons that produce insulin-like peptide have been implicated in egg-laying preference (Yang et al., 2008). *amn* has sequence similarity to a neuropeptide hormone (Feany and Quinn; Korzus, 2003), and thus merits molecular study because it would provide an additional neurochemical target that potentially regulates oviposition preference.

In addition the forward-genetic screens, the reverse-genetic neuroanatomical screens also provide additional candidate brain regions for future study. Of particular note, a number of fan-shaped body (FSB) lines also exhibited simultaneous disruption of positional aversion and egg-laying attraction to lobeline when silenced with *UAS-Shibire^{ts}* (Chapter 3), which suggests that this structure could be another integration center in the decision-making process associated with the *Drosophila* oviposition program, beyond the mushroom body. These results are intriguing, since this structure is very interconnected synaptically with the ellipsoid body (EB) (Hanesch et al., 1989), but the EB only showed defects in positional aversion when silenced with *UAS-Shibire^{ts}* (Chapter 2 and Chapter 3). Thus, in addition to identifying more possible intersection points and/or integration centers in the neural circuitry that mediates *Drosophila* oviposition behavior, investigations into the FSB and EB could shed additional light on the neuroanatomical and behavioral interactions affiliated with these central complex structures.

With regard to the mushroom body (MB), future study is also merited. In particular, initial investigations revealed that positional aversion and egg-laying attraction responses could be—for the most part—be mapped to specific neuronal subsets, or lobes, of the MB (Chapter 4: Figure S6). Specifically, lines with silencing in both the γ-lobe and γ'-lobe of the

MB exhibited egg-laying defects when responding to lobeline. Notably, *5-120*, *5-98*, *30Y*, and fru^{GAL4} have *GAL4* expression in both these lobes, and exhibit losses in preference for lobeline as an egg-laying substrate. On the other hand, *mb247* only expresses in the γ-lobe and displays wild-type egg-laying preferences, thereby suggesting that inactivation of both the γ-lobe and γ'-lobe neuronal subsets is required to disrupt egg-laying attraction to lobeline (Chapter 3: Figure 8 and Chapter 4: Figure 6,S5,S6). In contrast, *MB-GAL4* lines with silencing in the α/β-lobes or α'/β'-lobes typically lost the positional aversion response to lobeline, but possessed normal egg-laying attraction preferences (Chapter 3: Figure 8), suggesting that these lobes are required for the avoidance preference pathway. However, some *GAL4* lines that expressed in the α/β-lobes or α'/β'-lobes did not lose positional aversion to lobeline, including: *5-98, 10-229,* and *5-66a*. Recent work has shown that the α/β-lobes or α'/β'-lobes can be broken down into further subsets of neurons (Aso et al., 2009). Future study will be needed to determine if the silencing in these particular *MB-GAL4* lines was in fact complete or partial, with regard to the subsets within the α/β-lobes and α'/β'-lobes, and should therefore provide useful information about which particular regions of the MB lobes are critical to positional aversion response to lobeline. Thus, numerous questions remain regarding the MB and its role in decision-making within the *Drosophila* oviposition program, in particular with respect to whether the structure is merely an intersection point between the competing preference pathways, or if it is truly performing the *evaluation* and/or *selection* steps in a decision-making process.

With regard to the field of gustatory sensory perception, this study also opens potentially fruitful avenues of investigation. In particular, our results demonstrating that tissue-specific activation of a single *Gr66a*-receptor complex can induce opposing behavioral

outputs reveal one mechanism in which *Drosophila* can use the same sensory receptor machinery to encode and elicit very different behavioral outputs. However, what about the specific tissue causes differences in these *Gr66a*-expressing neurons? Do *Gr66a*-expressing neurons in the pharynx possess an additional gustatory receptor that *Gr66a*-expressing neurons on the legs do not possess? Indeed, some gustatory receptors like *Gr2a* have been shown to only express in the pharynx (Wang et al., 2004; Isono and Morita, 2010), and may have projections that overlap in the SOG. It will be interesting to see if *Gr2a* and *Gr66a* are co-expressed in the same cells present in the *Drosophila* pharynx, and if so whether *Gr2a* is the gustatory receptor needed for the lobeline-based egg-laying response. Alternatively, are the gustatory receptor expression profiles in leg and pharynx *Gr66a* neurons identical, and is the differential wiring into separate behavioral pathways achieved by other tissue-specific developmental factors? Future investigations into both the gustatory receptor profiles of these neurons and the associated accessory cells will be required to distinguish between these distinct possibilities.

Surprisingly, we observed that *Gr66a*-expressing neurons in the abdomen do not play a primary role in determining *Drosophila* egg-laying attraction to bitter compounds like lobeline. These results are in contrast to morphological studies classifying sensilla on the ovipositor and vaginal plate as gustatory bristles important to determining the suitability of a substrate as a potential egg-laying site. Our findings demonstrate that these abdominal gustatory bristles are likely performing another taste-based task pertaining to oviposition, which is not related to the selection between positional aversion and egg-laying attraction responses to bitter chemicals like lobeline. Gustatory sensory neurons also detect additional modalities, such as sweet, low-salt, high-salt, and osmolarity (Weiss et al., 2011); it will be

interesting to see in future investigations what particular taste-modalities these gustatory bristles on the ovipositor and vaginal plate mediate, since they do not appear to be *Gr66a* neurons that detect bitter-compounds. In accordance with this line of investigation into additional taste-modality contexts as they pertain to egg-laying behavior, it will be interesting to see how broadly this decision-making paradigm for the selection between egg-laying attraction and positional aversion responses can be applied. Notably, although we additionally demonstrate bitter-tasting quinine also induces both egg-laying and positional responses similar to lobeline-induced preferences (Chapter 4: Figure S1), expanding our paradigm with a larger panel of tastants, including salts-containing substrates and more bitter compounds, could provide additional evidence that establish our model as a decision-making process in *Drosophila.*

CLOSING REMARKS

This study originally began as a rotation project looking for a simple way to detect preference in *Drosophila melanogaster*, notably for the ecologically relevant drug, ethanol. At the time, less was known about *Drosophila* oviposition, and we hypothesized that egg-laying could be used as a simple marker for adult feeding and positional preferences, reasoning that female fruit flies would simply lay an egg wherever they were residing, based on these dominant preferences. However, we quickly observed that such a straightforward relationship between egg-laying preferences and positional responses did not exist. We had simply selected acetic acid as one compound to validate our two-choice assay simply by virtue that it is the other chemical product generated by fermentation in ripening fruit, which *Drosophila* frequent for nutrient ingestion, mating location, and egg deposition. Little did we

know that the capacity of acetic acid to generate contradictory egg-laying attraction and positional aversion responses in our two-choice assay would open a can of L3 larva (i.e. metaphoric worms), so to speak, and lead us into the investigative realm of behavioral switches, choice-like processes, and ultimately decision-making.

Thus, our experimental design did not test what we had originally intended it to test. However, rather than abandon the project, we recognized the unique situation generated by acetic acid in our two-choice assay: an incredibly simple yet robust paradigm in which we could provide two competing and mutually exclusive behavioral outputs to *Drosophila* females with a single environmental compound. We potentially had a very easy to use model for decision-making, and subsequently sought to characterize and validate the paradigm. As the project progressed, both our research and studies by our peers (Yang et al., 2008 and Miller et al., 2011) indeed confirmed that *Drosophila* oviposition could be employed as a model for decision-making. As such, we wanted to know more about this selection process between egg-laying attraction and positional aversion, and delved into the neural circuitry governing *Drosophila* oviposition behavior. We identified sensory neurons and higher-order brain structures that were independently important to these AA-induced preference pathways, but did not immediately identify neuronal central integration centers that would provide solid neurophysiological evidence that the situation we were observing indeed required a decision-making event.

However, persistence sometimes works in scientific research. To improve our chances of identifying overlapping and/or intersecting neural circuitry that mediate competing positional and egg-laying responses, we expanded our two-choice paradigm to include bitter-tasting compounds. Using lobeline as a representative bitter tastant, we

identified a central brain region, the mushroom body, which is important to *both* positional aversion and egg-laying attraction to bitter compounds. These results demonstrated that the mushroom body is likely an intersection point in the neural circuits governing these contradictory preference pathways, and given the role of the mushroom body in numerous other *Drosophila* decision-like behaviors, suggest that this brain region is a decision-making integration center in the *Drosophila* oviposition program.

Taking a step back to view the larger picture of a Ph.D. project, it is interesting to note that the origins of the study do not always reflect the destination. However, one interesting aspect of science is how unexpected results, if carefully considered and pursued, can often take a researcher down unintended yet fruitful investigate roads. In particular, with regard to this study, we made numerous important discoveries about decision-making in *Drosophila* and the neurons that mediate the oviposition program, even though that was not the original intent. Furthermore, a number of important findings with impact to fields outside of the decision-making field also resulted from work detailed in this dissertation project. Our results showing the tissue-specific activation of a single *Gr66a*-receptor complex can induce opposing behavioral outputs are particularly important to the field of sensory perception, since it demonstrates one way in which *Drosophila* can use the same sensory receptor machinery to encode and elicit very different behavioral outputs. Our findings that female fruit flies do not necessary employ taste-bristles on the abdomen when making choices about where to lay an egg is important as well, since past studies have often predicted that abdominal bristles would be indispensable for these responses; our results thus bring to light some limits of these previous assumptions. Our observations that the mushroom body is

involved in taste-related behavior is also novel, since this structure typically processes olfactory input.

Thus, if a researcher is observant towards unexpected yet potentially interesting experimental results, important findings can be made. Fitting to the topic of research detailed in this dissertation, this author has realized that the choices and decisions we make—regarding scientific investigation or life in general—do not always produce the expected responses and/or results. However, if one notices the interesting properties in the unexpected results, we can make even more interesting discoveries in areas that we never originally thought possible.

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