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Anti-Instinctive Learning in Fruit Flies

A dissertation submitted in partial satisfaction of the
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in

Biology

by

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The dissertation of Ruichen Sun is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California San Diego

2019

DEDICATION

To my mom and dad.

EPIGRAPH

Well, I'm walkin' down the line,
I'm walkin' down the line
An' I'm walkin' down the line.
My feet'll be a-flyin'
To tell about my troubled mind.
—Bob Dylan, *Walking down the line*

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Chapter 2-4, in part, includes parts of a manuscript in preparation for publication. This dissertation author is the first author of the paper. The co-authors are: Joseph Delly, Emily Sereno, Sean Wong, Yan Huang, Xinyu Chen, Yuxuan Wang, and Ralph Greenspan.

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ABSTRACT OF THE DISSERTATION

Anti-Instinctive Learning in Fruit Flies

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Learning is a fundamental experience-dependent process involving permanent changes in behaviors. Among all learning processes, anti-instinctive learning, an ability to modify one's innate behaviors in ways that go against one's innate tendency, can confer great evolutionary advantages to animals and enable them to better adapt to the changing environment. Yet, our understanding of anti-instinctive learning and its underlying mechanisms is still limited. In this dissertation, I describe a new learning behavior of the fruit fly, which also identifies a second anti-instinctive learning behavior in the fly. This learning paradigm requires fruit fly to respond to a recurring, aversive, mild heat stress by modifying its innate locomotion behavior. Chapter 1 of this dissertation reviews definitions of behavior with a focus on locomotion, and the effects of learning on behaviors. Chapter 2 of this dissertation

describes the design of a novel behavioral apparatus, the *LaserSync* system, used in this study. Chapter 3 of this dissertation describes the wild type fruit fly's anti-instinctive learning behaviors. Chapter 4 of this dissertation describes the exploration of the molecular and cellular mechanisms underlying anti-instinctive learning behavior. We found that experiencing movement-triggered heat stress repeatedly significantly reduced walking activity in wild type flies, indicating that fruit flies are capable of anti-instinctive learning. Based on this new learning behavior, we explored potentially relevant molecular and neuronal mechanisms underlying anti-instinctive behavior. This work is an effort to develop an animal model of anti-instinctive learning behavior that allows for rigorous interrogation of the underlying biological mechanisms. Insights generated from this study will bring us closer in understanding the biology of learning, and perhaps higher cognitive functions in general.

Chapter 1

Introduction

1.1 What is Behavior?

More than 60 years ago, the Nobel-winning ethologist Nikolaas Tinbergen summarized four fundamental distinct aspects for the study of animal behaviors: survival value, ontogeny, evolution, and causation [137]. To illustrate, for a given behavior, one can ask four types of questions: 1) how does this behavior help the organism survive (survival value)? 2) How does this behavior change during the lifetime of the organism (ontogeny)? 3) How did individuals of previous generations affect this behavior of the individual in the current generation (evolution)? 4) What is the mechanism of the behavior (causation)? Note that among these four questions, the last question is perhaps the most difficult to answer. These questions provided a framework to understand specific animal behaviors. But before we can study any behavior, we must first answer: what is behavior?

It has been surprisingly difficult to define *behavior*. Tinbergen suggested that behavior is, "the total movements made by the intact animal" [138]. Yet, this definition is somewhat misleading – it suggests that a turtle, in being shifted by an ocean wave, is undertaking behavior of a sort. Nonetheless, no better definition of behavior has been proposed since Tinbergen wrote. A recent survey of definitions of *behavior* among 174 behavioral biologists showed no agreement [77]. The researchers, however, were able to synthesize a new definition

based on the respondents' varying answers: the internally coordinated responses (actions or inactions) of whole living organisms (individuals or groups) to internal and or external stimuli, excluding responses more easily understood as developmental changes [77].

Not only has the definition of behavior been fuzzy, but behavioral research itself also has made limited progress in the past several decades. One can use genome research as a comparison. If we apply Tinbergen's four questions to genomic studies, we can answer them more readily than we can in behavioral studies. First, the survival value of DNA is obvious: mutations that reduce an organism's fitness will be weeded out by natural selection. Second, changes in DNA can be compared and traced from individual to individual and from species to species. Third, DNA stays largely the same from generation to generation and from the beginning until the end of an individual's life. Lastly, the central dogma of molecular biology has already been verified as a valid framework of how DNA works [153, 28]. If we compare the timeline of progress in behavior research and that of genomic research, the contrast is even more telling: in 1953, the definition of species was as fuzzy as the definition of behavior; and today, progress in genomics has already helped to clarify confusion on speciations at the molecular level and facilitated the emergence of genetic engineering, while not much progress has been made in our understanding of behaviors. In terms of research methodology, today's ethologists, similar to ethologists in the past, are still focused on stereotypical behaviors that are robust to environmental changes or are part of fixed behavioral patterns [78].

An example of a behavior study can illustrate our limited understanding of behaviors. Animals closely related to one another phylogenetically sometimes show completely distinct forms of behavior, an example of which is the Nudibranch mollusk, commonly referred to as the sea slug. Nudibranchs are a group of aquatic shell-less mollusks found in the ocean. Many species within the Nudibranch group do not swim [75]. Among those which do, some swim by flexing their bodies in the dorsal-ventral direction, others by bending their bodies in the left-right direction [75]. *Tritonia diomedea* is a dorsal-ventral swimmer, while *Melibe leonina* is a left-right swimmer [75, 92]. While the genus of *Tritonia* and *Melibe* are monophyletic, they swim in starkly different ways. The difference can be traced to the neural circuits controlling

swimming [63, 136]. In *Tritonia*, neurons from both left and right sides fire synchronously during swimming, while in *Melibe*, neurons fire in alternation [63, 136]. Yet, apart from differences in neuronal functions, the flexibility of a fixed behavior may also contribute to the observed differences in swimming behaviors. *Melibe*, being a left-right swimmer, was found to possess dorsal-ventral neuronal homologs, although these neurons are mostly silent when *Melibe* is swimming (in a left-right way) [136]. It is unclear whether *Melibe* is capable of both dorsal-ventral and left-right swimming, and for unknown reasons, simply favors one to the other.

Several experiments could shed light on this question. As neurons on the left and right sides of the left-right swimmer *Melibe* fire in alternation during swimming, one might selectively activate these neurons (both on the left and right sides of the animal) in *Melibe* and see whether dorsal ventral swimming occurs. Another experiment would be to raise individual *Melibe* with groups of *Tritonia* from the day *Melibe* hatches, and see whether dorsal-ventral swimming occurs as a result of learning and influences from the surrounding environment. These Gedanken experiments, however, are challenging to perform in Nudibranchs, because they cannot be cultured and bred in the laboratory and that we do not have molecular markers to selectively target dorsal-ventral neurons in Nudibranchs.

In fact, these two issues, difficult to keep in laboratory and lack of available molecular markers, have been the major roadblocks for studying behaviors of most animals in the lab. Luckily, there is still hope, and it is from the fruit fly. Fruit flies, unlike the sea slug, offer an amenable test system for studying sophisticated behaviors and for elucidating the mechanisms underlying such behaviors [127, 43, 27, 85, 5]. Progress in genetics and molecular biology, pioneered by Seymour Benzer, has cemented the fruit fly as a powerful model organism in studying mechanisms underlying behaviors [16, 71, 38, 141, 107]. An example is aggression behavior. A recent study showed that female flies from different subspecies of *Drosophila* display different levels of aggression when feeding [118]. *Drosophila erecta* gathers around food sources much faster than *Drosophila melanogaster* does, and they show greater levels of aggression [145, 59]. Such behavior is food-specific and can be easily

observed during feeding [94]. When reared with *Drosophila melanogaster*, *Drosophila erecta* becomes less aggressive than when raised alone or with con-specifics. Such a difference in aggression and social behavior was found to be correlated with elevated expression of genes related to immune response and olfaction in the socialized flies [118]. These studies, together with other studies using fruit flies, revealed that a deeper understanding of animal behaviors is possible.

1.2 Moving Around in Nature

The response of an organism to external or internal stimuli is the hallmark of behavior [77]. In this sense, behaviors are algorithmic by nature: generating solutions to problems. The algorithmic nature of behaviors, however, does not require organisms to solve the same problem in the same way (e.g. different types of swimming behaviors of the closely related *Tritonia* and *Melibe*), nor does it impose upon the organisms uniform hardware in implementing these solutions. A good example to illustrate this, i.e. the diversity of behavioral solutions to a single problem, is how different species move in nature with or without a nervous system. For almost all organisms, the ability to move is a prerequisite for performing many behaviors. Having a nervous system certainly helps an organism to achieve a more sophisticated sequence of motions, but there remains room in animal life for much simpler solutions to movement. For example, organisms with no nervous system, such as the aneural *Trichoplax* and most bacteria, are capable of motion [125, 2]. Here, I briefly review and compare these locomotion solutions seen in different organisms.

Nature presents every organism, prokaryotes and eukaryotes alike, with the same problem: how to move? Consider a bacterial cell, suspended in liquid and attracted to certain stimuli. The flagellum is a structure consisting of a basal body, filament, and hook, driven by a proton motive force, and it represents a common bacterial solution to the problem of motion [17, 82]. Counterclockwise rotation forces the filaments in the flagellum to coalesce into a bundle along the long axis of the cell body; as a result, the bacterial cell moves forward.

Clockwise motion, on the other hand, makes the filaments less organized, causing the cell to tumble. Thus, the flagellar rotation direction is important in bacterial movements, and it is found that the rotational direction is controlled by a series of events inside the bacterial cell in response to extracellular stimuli [111].

In addition to bacteria, prokaryotes also include another major domain of life: archaea. It turns out that the flagellum is also the archaeal solution to the problem of motion [41]. Archaeal and bacterial flagella, however, are structurally different [60]. Many of the proteins that make up flagellum in archaea resemble the proteins that make up the bacterial type IV pili structure [93]. In bacteria, type IV pili are used for pulling the cell forward when anchored to a surface [48]. But in archaea, type IV pili-like flagella function by rotating their filaments without being attached to any surface, similar to bacterial flagella. Thus, despite the differences in molecular structures of their motor proteins, bacteria and archaea move in functionally the same way.

Regardless of the specific proteins used in moving, physics places constraints on how fast single cells move through a homogeneous environment. A bacterial cell can swim at 20–40μm/s, corresponding to 20-40 body lengths per second, or bps [53]. Some archaeal cells swim at anywhere between 3–380μm/s, or 3–380bps [53]. To put these numbers in context, one needs to compare it to the bacterial life cycle. *Escherichia coli* doubles every 40 minutes or less [26]. At 20μm/s, *E. coli* can move up to 48mm in its lifetime when there is no external force. Thus, it would be a challenge for *E. coli* to move on its own to food sources 100 meters away in one generation.

Some eukaryotes, such as protozoan *Paramecium* and *Trichoplax adhaerens*, also use flagella and cilia to move around. Eukaryotic flagella are structurally unlike those in bacteria [60, 72, 95], and may have evolved from pre-existing cytoskeletal components such as tubulin and dynein [90]. In eukaryotes, flagella and ciliary movements are rather inefficient. *Paramecium* propels itself at 50–200μm/s (1 bps) by moving its cilia attached to the outside of the cell membrane [73, 109, 147]. *Trichoplax* does not have any internal organs, tissues, nerves, or muscles. Unicellular species can propel themselves by synchronous ciliary movement, as

it is fairly easy to coordinate the cilia movement within a single cell. But for multicellular organisms, such as *Trichoplax*, coordinating ciliary movements across different cells becomes challenging. The cilia of *Trichoplax* can only beat asynchronously, resulting in *Trichoplax* moving at around 15μm/s, or 0.015bps — much slower than that of unicellular organisms (e.g. *E. coli*) [146, 125, 128].

Using cilia/flagella to move is better controlled by intracellular chemical signaling, and such processes are ill-suited for synchronizing multiple cells' cilia movements [82]. Thus, though efficient in moving prokaryotes and unicellular eukaryotes, the flagellum-like structures reach a bottleneck in moving multicellular organisms, and novel algorithms are needed for accomplishing their locomotion.

The emergence of muscles and their innervating nerves allow organisms to have more precise control of movements [32]. Derived from the mesoderm, muscles generate force with the sliding and contraction between two proteins, actin and myosin [55]. Most eukaryotes, including all bilaterians, such as vertebrates and insects and annelids, have striated muscles, suggesting that the emergence of muscle is an event in evolution possibly predating the rise of bilaterians [129]. As an example, jellyfish, one of the most basal animal phylum (Cnidaria), also use striated muscles to propel themselves [116]. It is likely that the striated muscles found in bilaterians evolved from ancient cells that were able to contract (as modern muscles do), but were unable to regulate their contractions (the way muscle-innervating neurons do) [129].

The Nervous system evolved to become the control system for muscle-coordinated movement [61]. The nervous system is a network of nerve cells, also called neurons, which can be divided into three categories: sensory neurons, interneurons, and motor neurons [65]. External stimuli activate sensory neurons, which transmit signals to downstream interneurons. Then, interneurons further transmit the signal to downstream motor neurons. Motor neurons synapse onto muscle fibers, forming what is called neuromuscular junctions. At neuromuscular junctions, motor neurons can elicit muscle contraction by transmitting signals to muscles via neurotransmitters, typically acetylcholine (in vertebrates) or glutamate

(in invertebrates) [69]. Endowed with such a system, organisms can move with more precision and greater speed compared to unicellular organisms.

1.3 Nature Alters Behaviors via Learning

The ways with which different organisms move around exemplifies how diverse the forms of a basic behavior can be. Their behaviors are products of the diverse environment the animals evolved in. One of the universal processes that the environment alters in behavior is learning. Learning, sometimes also referred to as acquisition or conditioning, is an experience-dependent biological process in which lasting changes occur in the individuals' behavioral potential [3]. Almost all organisms are able to learn, including aplysia, *C. elegans*, fruit flies, mammals, and of course humans [152, 104, 143, 19, 151].

Two major types of learning exist, non-associative and associative learning [65]. Non-associative learning refers to processes in which a behavioral response towards a single stimulus changes without an associated stimulus [65]. Non-associative learning includes habituation, where responses' intensity or frequency decreases upon repeated presentation of the stimulus, and sensitization, where a response' intensity or frequency increases with repeated stimuli [65]. Habituation and sensitization are viewed as the simplest forms of learning [25, 24].

In contrast to non-associative learning, associative learning can be broadly defined as a process in which a subject associates one stimulus with another or with a behavior [65, 151]. The process of associating two stimuli is referred to as classical conditioning, whereas the association of a stimulus and behavior is called operant conditioning [106, 124]. Classical conditioning is the process by which an animal acquires an automatic response to a particular environmental stimulus (in the presence of another stimulus), usually the kind of stimulus unable to elicit responses from the animal prior to the learning process [105, 97]. An example is Ivan Pavlov's study of dogs in which he showed that dogs can be conditioned to produce a salivation response to an otherwise neutral ringing of a bell if the bell ring is

repeatedly paired with food [98]. On the other hand, operant conditioning is a process by which an animal learns to associate certain behaviors of its own to a non-neutral outcome (either positive or negative) [124]. An example is B. F. Skinner's experiments on pigeons [39]. In the study, pigeons kept in an enclosed chamber received rewards (such as food pellets) if they perform a specific behavior (such as pecking a disc) [39]. This kind of training has been extensively used, either consciously or unconsciously, in training animals and even children [151].

A series of cellular and molecular events are involved in associative learning, and some of these events are shared across species [65]. One example is the classical conditioning of Aplysia's gill and siphon withdrawal reflex to gentle touches on its siphon using electric shocks to its tail[23]. A touch on an Aplysia's siphon activates its sensory neurons and result in a Ca^{2+} influx, whereas electric shocks to its tail trigger serotonin release to postsynaptic neurons, which include the siphon sensory neurons and motor neurons. Adenylyl cyclase in the sensory neurons respond to the co-occurrence of the Ca^{2+} influx and serotonin release (through serotonin receptors), and activates the postsynaptic motor neuron, resulting in the gill withdrawal [50, 65]. In other words, adenylyl cyclase facilitates the pairing of the siphon touch with electric shocks, allowing the former to elicit gill withdrawal reflex without the latter.

Important cellular and molecular events in the process of memory storage following learning have also been identified. Memory consists of short-term and long-term memory, the conversion of the former to the latter called consolidation [65]. During learning, electric shocks to the tail trigger serotonin release at the synapse between sensory and motor neurons. Serotonin then binds to postsynaptic receptors, activating adenylyl cyclase, which then converts intracellular ATP to cAMP. cAMP then activates protein kinase A (PKA), and this event leads to phosphorylation of existing intracellular proteins, resulting in the formation of short-term memory [65]. Converting short-term memory to long-term memory, however, requires new protein synthesis, which in turn requires gene expression. cAMP responsive element binding protein (CREB) is responsible for transcribing genes which have

cAMP response element sequences in their promoter regions [120]. Studies in fruit flies have shown that overexpression of CREB repressor blocks long-term memory formation, whereas overexpression of CREB activator leads to the formation of immediate long-term memory (also called photographic memory) [163, 164, 99, 142, 120].

1.4 Learning Against One's Innate Behaviors

Although the learning process modifies behaviors, not all behaviors can be modified in the same way and to the same extent. In particular, actions or tasks that conform to an animal's innate behaviors or confer immediate benefits (such as feeding) are easier to acquire than actions that go against an individual's innate behaviors [151]. It is estimated that most of the behaviors studied in laboratory conditions are between the two extremes mentioned [83, 65]. Conditioning subjects to perform behaviors that are against their instincts, which I call anti-instinctive learning, is perhaps one of the most difficult types of conditioning to study.

Yet, anti-instinctive learning is as important, if not more so, as other types of learning. An example is the Stanford Marshmallow study [89]. In the study, preschool children were presented with marshmallows and given a choice between eating the marshmallow immediately or waiting 15 minutes for another marshmallow. If the child eats the marshmallow before the end of 15 minutes, he or she would not be given another marshmallow. Some children in this study were able to wait 15 minutes for the bigger reward (a second marshmallow). These children's behaviors showed that humans are able to act against the instinct to consume food presented in front of us, a fact that at first glance seems to violate the principles of evolution. Yet, the ability to resist immediate rewards turns out to be positively correlated with a child's future scholastic achievements and better stress management ability as an adult, both of which are desirable traits in the human society [88].

In humans, behavior control, such as acting against one's instinct, usually requires prefrontal cortex activity in the brain [134, 4, 65]. In addition to behavior control, the

prefrontal cortex is also involved in higher cognitive functions, such as language, reasoning, and planning, resulting in its being often seen as the neurological foundation for higher cognition and intelligence [157]. Yet, the human brain consists of 120 billion neurons (with 21 - 26 billion neurons in the cortex) and understanding how these neurons interact and engender behavioral control is still technically infeasible [52], not to mention the issues inherent in human behavior studies. For example, in the Stanford Marshmallow experiment, age is a confounding factor positively correlated with the children's ability to wait [89]. This means that direct comparison between behaviors of 3-year-olds and those of 4-year-olds directly would not be appropriate, as the ability to act against their innate attraction towards sweets changes as a child grows older. Also, it is unclear if these children's previous experiences are controlled for in the study. It is possible that if a child has been rewarded for delay gratification prior to the study may affect how he or she behaved in the study. Yet, controlling for past experience in studies using human subjects is again challenging, and any attempt to control for past experiences in human subjects, such as keeping the subject isolated for days, would be deemed unethical.

In light of the importance of anti-instinctive learning and the current limitations in studying this phenomenon in humans, it is worth exploring the phenomenon in model organisms. Fruit flies, with their powerful genetics and rich behavior repertoire, are ideal for studying anti-instinctive learning behavior [42, 37]. Yet, one may wonder if the tiny fly indeed has the ability to perform behaviors as complex as learning to behave against its instinct. Studies on fruit fly courtship conditioning indicate that the answer to the previous question may be positive.

Courtship conditioning refers to the phenomenon in which the courtship behavior of a male fly is modified by its prior sexual experience [119]. Female virgin fruit flies are receptive to a male's courtship attempts [119]. After mating, the recently mated female becomes unreceptive to any new male's courtship attempts [119]. If a naive male has previously been rejected by an unreceptive female, the male fly would later court a receptive female with much less fervor [119]. As the drive to mate is one of the most basic instincts for animals,

a male fly's reluctance to court receptive females after being rejected by other females goes against their instincts. Therefore, the existence of courtship conditioning behavior in flies not only indicates that they are not automata born with only predetermined behavior programs but also suggests that they are able to perform anti-instinctive learning behavior.

However, it is challenging to study the mechanisms underlying anti-instinctive learning using courtship conditioning as a model. On the one hand, previous studies have suggested that experience-dependent changes in courtship behavior of the male fly are modulated by olfactory, visual, and tactile cues [139, 140, 1, 87]. On the other hand, a recent study showed that courtship conditioning was mainly due to an unsuccessful mating attempt per se instead of olfactory or visual cues [68]. These contradictory results can be partly attributed to the complexity of courtship conditioning behavior itself: the conditioning stimulus in this behavior is not one chemical or visual signal, but an intact female fly. For a male fly, a female fly is a very complex stimulus simultaneously containing olfactory, visual, and tactile signals. Therefore, although courtship conditioning is an example of anti-instinctive learning, it is not a suitable model to study the behavior. To study anti-instinctive learning behaviors in flies, one needs a simpler behavior model with simpler conditioning signals, preferably simpler than an actual fly.

No other anti-instinctive learning behavior in fruit flies have been reported since the discovery of courtship conditioning. In this dissertation, I describe a new learning behavior of the fruit fly, which also identifies a second anti-instinctive learning behavior in the fly. This learning paradigm requires fruit fly to respond to a recurring, aversive, mild heat stress by modifying its innate locomotion behavior. Chapter 2 of this dissertation describes the design of the experimental apparatus used in this study. Chapter 3 of this dissertation describes the wild type fruit fly's anti-instinctive learning behaviors. Chapter 4 of this dissertation describes the exploration of the molecular and cellular mechanisms underlying anti-instinctive learning behavior. This work is an effort to develop an animal model of anti-instinctive learning behavior that allows for rigorous interrogation of the underlying biological mechanisms. Insights generated from this study will bring us closer in understanding the

biology of learning, and perhaps higher cognitive functions in general.

1.5 Acknowledgment

Chapter 1, in part, includes an edited reprint of the material from the following review article. This dissertation author was the only author of the review article.

Sun, Ruichen. "The Origin of Behavior",Inference: International Review of Science, vol. 3, issue 4, 2018.

Chapter 2

LaserSync: A System for Studying Fruit Fly Anti-Instinctive Learning Behaviors

2.1 Introduction

Like all learning and memory processes, learning to behave against one's innate behavior requires training. Such a process requires the subject to form an unnatural association between an innate behavior of their own and an unpleasant outcome. One of the most basic innate behaviors of a fly is its walking behavior, while one kind of stresses that a fly strongly dislikes is heat [130, 110]. This allows one to build a new anti-instinctive learning model by leveraging these two coexisting traits in a fly.

To study the changes of fruit fly walking behavior before and after receiving recurring mild heat stress, a behavioral apparatus system called *LaserSync* was designed and built. The *LaserSync* system is capable of delivering precise heat stress to a single fly while continuously monitoring its walking behavior. The *LaserSync* system consists of 4 LaserBoxes, an adapter board, a myRIO FPGA system, and a computer (Figure 2.1, 2.2, 2.3). Up to 4 flies can be assayed independently in each of the 4 LaserBoxes at the same time. Data for these flies'

walking behaviors are recorded in the LaserBoxes and transferred via the adapter board and the myRIO FPGA system to the computer for storage. Detailed descriptions of these components are introduced in the following sections.

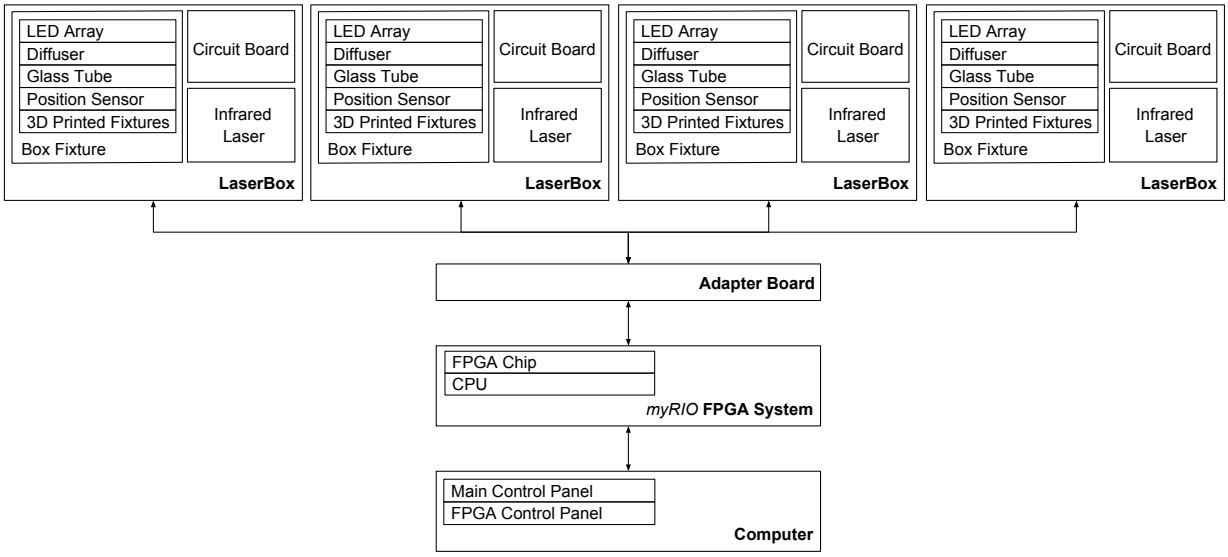


Figure 2.1: Overview of the components in the *LaserSync* system and their relationships. In the first row are the 4 LaserBoxes. In the second row is the adapter board. In the third row is the myRIO FPGA system. In the fourth row is the computer. Data flows from the first row to the fourth row

2.2 LaserBox

LaserBox is both the experiment chamber and the starting point of data collection in the *LaserSync* system. A total of 4 LaserBoxes were built in the *LaserSync* system. Each of the 4 LaserBoxes consists of a box fixture, an infrared laser emitter and a LaserBox circuit board (Figure 2.4, 2.5). Inside the box fixture are a 3D-printed fixture, a position sensor, a glass tube, a diffuser, and a red LED array. The 4 LaserBoxes are identical in design and can operate independently and simultaneously.

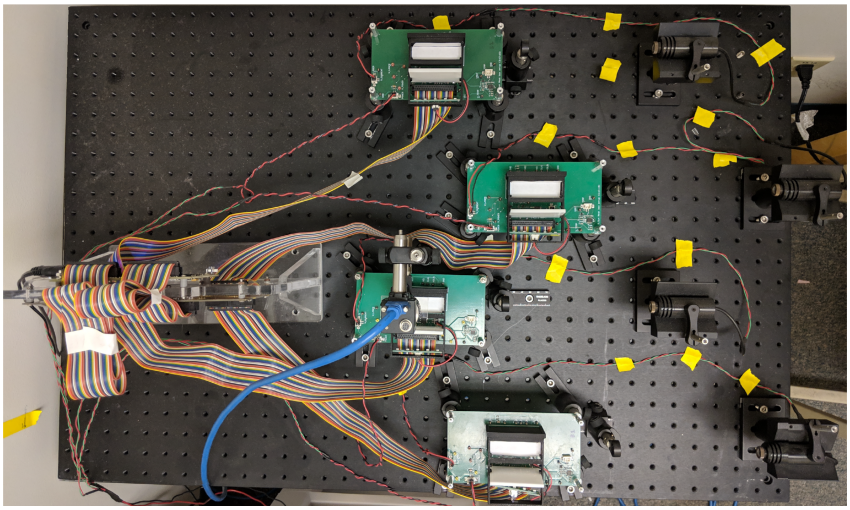


Figure 2.2: A top view of the *LaserSync* system. The 4 black-colored parts on the right hand side of the figure are infrared lasers with associated supporting parts. The 4 green stations at the center of the figure are 4 LaserBoxes. The transparent fixture with rainbow-colored ribbon cables connectors attached on the left side of the figure is the adapter board and myRIO FPGA system.

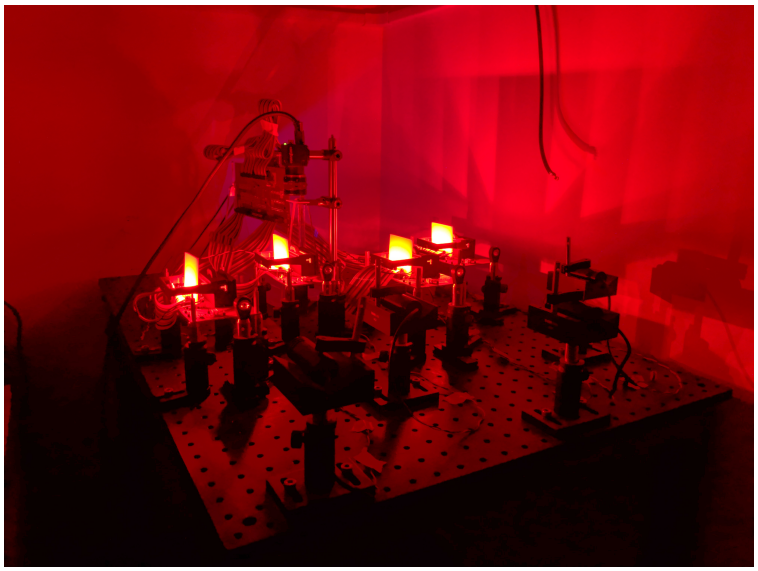


Figure 2.3: A side view of *LaserSync* system during an experiment. The red light comes from the 4 red LED arrays in each of the 4 LaserBoxes.

2.2.1 Box Fixtures of the LaserBox

As mentioned earlier, the main components of a box fixture are a 3D printed fixture, a position sensor, a glass tube, and an LED array. The 3D printed fixture itself is made of acrylonitrile butadiene styrene. The relative positions for each of these components inside

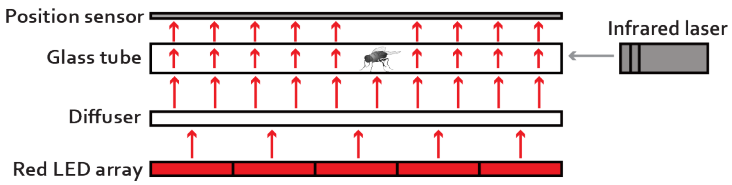


Figure 2.4: A schematics of the LaserBox, which consists of a position sensor, a glass tube, a diffuser, a red LED array, and an infrared laser. The red color of the red LED array represents where the red light is emitted. The red arrows pointing away from the LED array indicates the direction of the red light, which arrives at the position sensor. The gray arrow pointing away from the infrared laser indicates the direction of the infrared light. The black shape inside the glass tube represents a single fly, which blocks a small portion of the red light, resulting in less light arriving at the sensor in the location where the fly is.

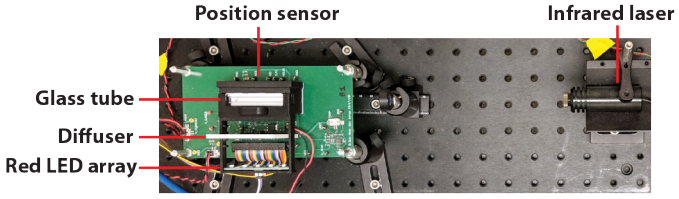
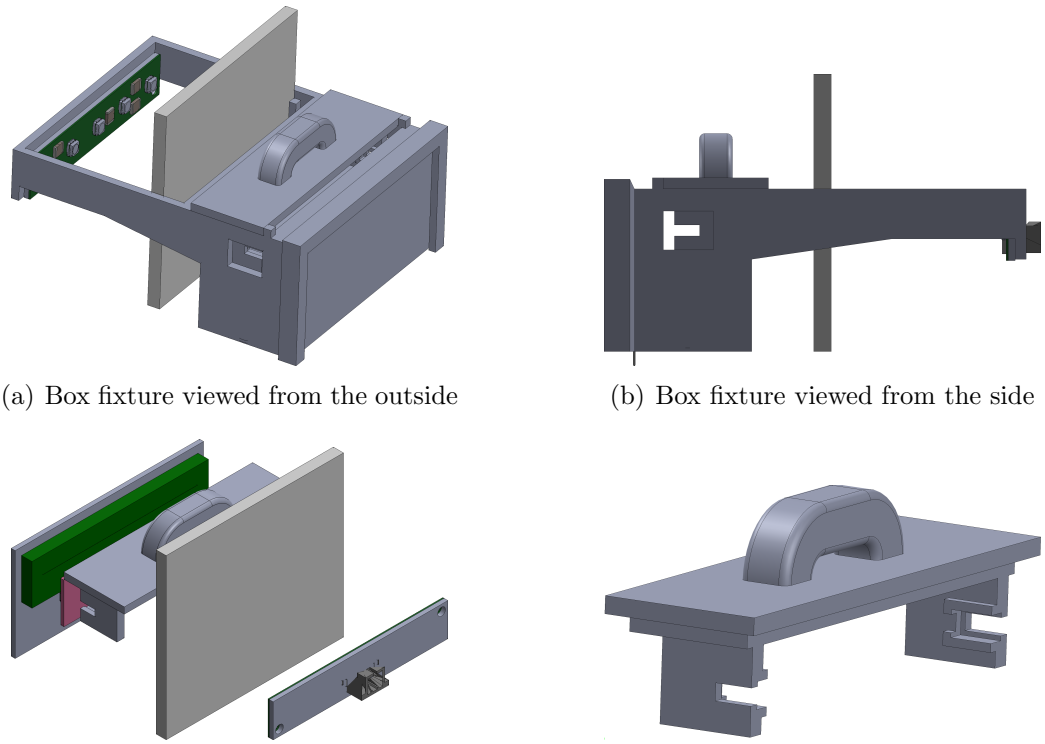


Figure 2.5: A top view of a LaserBox. Each sub-components of the LaserBox are labelled with a red line. The green board underneath the 3D-printed black fixture is the LaserBox circuit board.

the 3D printed fixture are a position sensor, a short-pass optical filter, a glass chamber, a diffuser and a red LED panel (Figure 2.4, 2.6). When viewed from outside of the LaserBox, most of the components are hidden except for the LED array and the diffuser between the LED array and the holder (the green panel is the LED array and the grey square is the diffuser, Figure 2.6(a)). The position sensor is a linear sensor array (AMS-TAOS USA Inc., Cat. No. TSL1406R, Plano, Texas, USA) with a custom-made 645nm short-pass filter placed in front of it (dimension: $55 * 12.7 * 1$ mm^3, Midwest Optical System, Inc., Cat. No.SP645-R55X12.7mm). The glass chamber is a custom-made $48.7*4*2$ mm^3 transparent borosilicate tube (Friedrich & Dimmock Inc., Cat. No.BRT-2-4-50, Millville, New Jersey, USA) with two custom-made detachable $4*2*1$ mm^3 glass windows (Fisher Scientific, Cat. No.12-550-100) at both ends of the holder to provide complete enclosure. The assembled chamber is held in a custom-made 3D-printed holder, which an experimenter can quickly place in the LaserBox (Figure 2.6(d)). The light emitted by the red 630nm LED panel (Figure 2.7) is diffused by

an acrylic diffuser and then passes through the glass chamber and short-pass filter, and then reaches the position sensor (Figure 2.4). The relative positions of these components can be found in Figure 2.6.



(a) Box fixture viewed from the outside

(b) Box fixture viewed from the side

(c) Box fixure sub-components

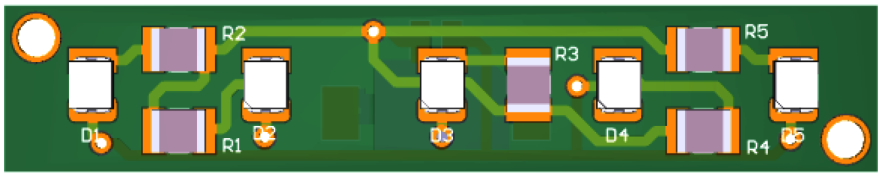
(d) The 3D-printed holder for securing the position of the glass tube

Figure 2.6: LaserBox' box fixture and its sub-components.

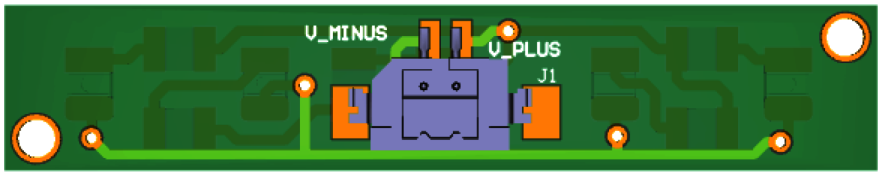
2.2.2 Infrared Laser Emitter of the LaserBox

The second main component of the LaserBox is an 808nm infrared laser emitter (Roithner Lasertechnik GmbH, Cat. No.RLDB808-120-3, Vienna, Austria), which is placed on the right side of the box fixture (Figure 2.5). In between the box fixture and the laser is a condenser (Thorlabs Inc., Cat. No.ACL12708U, Newton, New Jersey, USA), which condenses the light emitted by the laser emitter before it reaches the box fixture.

Previous fruit fly behavioral apparatuses used Peltier elements or an electric board as a heating source [8, 160, 123, 33, 15, 162]. We found that infrared laser is a better source for



(a) A top view of the LED board. The 5 white rectangular parts are the LED diodes. The 5 purple rectangular parts are resistors. The light green lines are circuits connecting all the parts on this board.



(b) A bottom view of the LED board. The blue irregularly-shaped part at the center of the board is the connector connecting to power sources.

Figure 2.7: LED array circuit board.

delivering heat stress compared to Peltier elements or an electric board. Compared to Peltier elements, which delivers heat stress to the animal by warming up the surrounding air, laser emitters increase the fly's body temperature faster and allows for faster cooling, as infrared light heats up the animal directly [122, 160]. Compared to electric boards, which delivers heat stress to the animal only when the animal is in contact with the wires on the circuit board, the laser emitter can deliver heat stress continuously to the fly regardless of where it stands [15]. The laser emitter (wavelength: 808 nm) delivers collimated infrared light of intensity between 0 mW and 270 mW. The maximum intensity of 270 mW can increase the fly's body temperature from room temperature ($20 - 22°C$) to 45°C and maintain it at 45°C (Figure 2.8). Therefore, the laser emitter can warm up the fly's body temperature from room temperature to up to 45°C. This is validated by measuring body temperature when the fly is being irradiated by the infrared light. To do so, one inserts a thermocouple data acquisition module-connected mini hypodermic probe (OMEGA Engineering, Inc., Cat. No. TC-08 and No.HYP1-30-1/2-T-G-60-SMP-M) in an adult fly's abdomen and placing the fly in the center of the glass chamber irradiated with the infrared light. The fly's body temperature can be readily measured as long as the probe is inside the fly's body. This

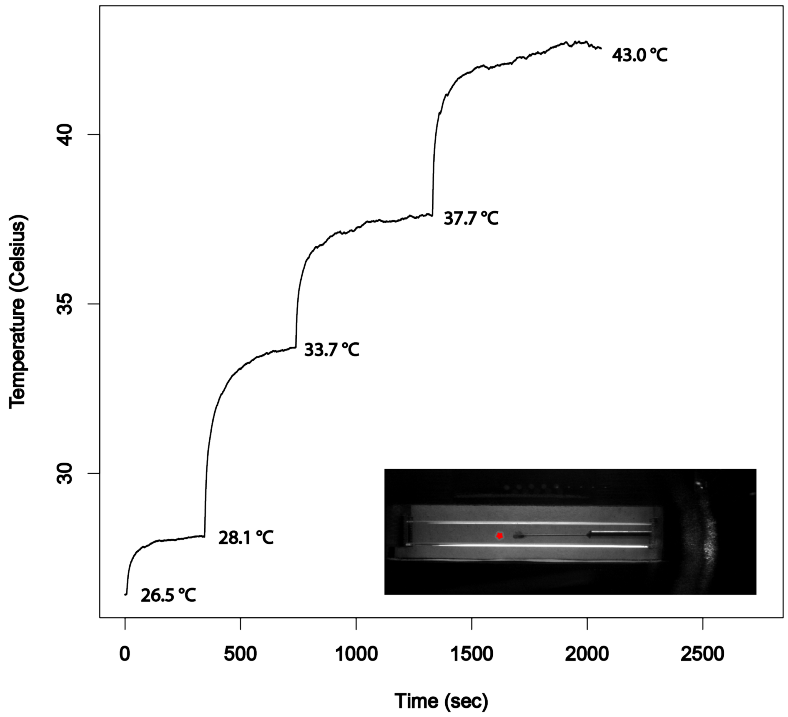


Figure 2.8: Infrared laser can warm up a fly and maintain its body temperature at any level between room temperature and 45°C. The inset shows a fly, indicated by the red star, with its body temperature being measured by a metal temperature probe inside the glass tube.

invasive temperature measurement is a separate validation step and is not conducted during behavior experiments.

Of the 4 LaserBoxes, one has an external monochromatic camera (FLIR Integrated Imaging Solutions, Inc., Cat. No.Flea3 1.3 MP Mono USB3 Vision (e2v EV76C560)) positioned at the top of the box fixture. This camera provides visual information for experimenters to deliver prompt heat stress to the fly during experiments.

2.2.3 Circuit Board of the LaserBox

The LaserBox circuit board, located at the bottom of the box fixtures of each LaserBox, is responsible for 1) integrating optical signals received by the position sensor and transmitting the optical signals downstream to the adapter board for further processing, and 2) changing the ON/OFF status of the LED array and the infrared laser emitter based

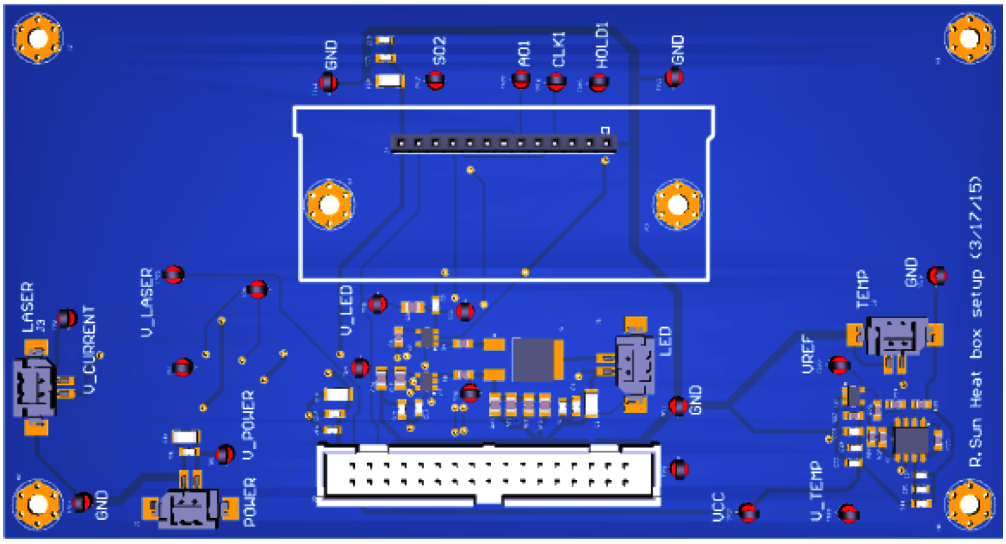


Figure 2.9: A top view of LaserBox circuit board. The white lines at center indicates the location of the box fixtures. Other small components are labeled directly on the board.

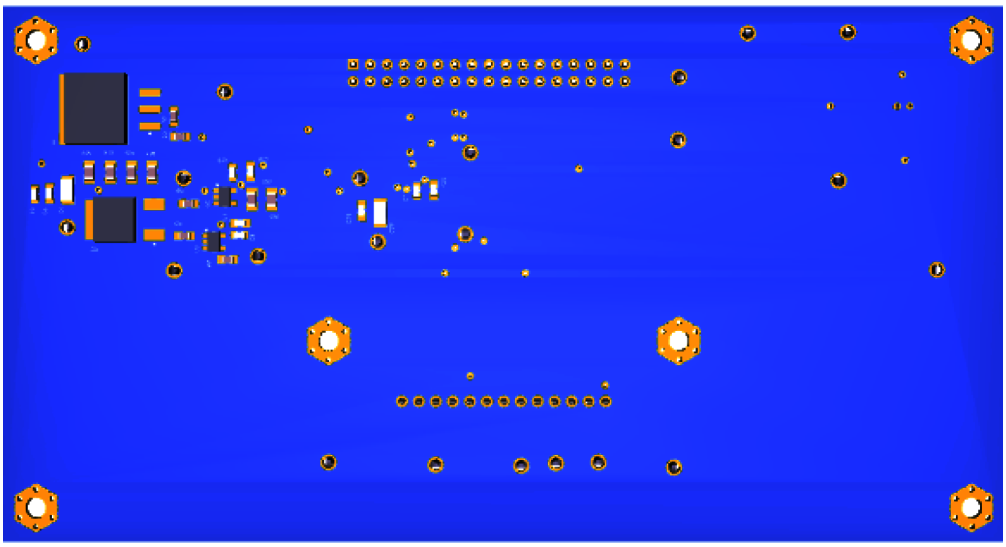


Figure 2.10: A bottom view of LaserBox circuit board.

on experimenter's command. The top and bottom of the circuit board are shown in Figure 2.9 and 2.10. Refer to Figure 2.11 and 2.12 for detailed circuitry design.

2.3 Adapter Board

The adapter board of the *LaserSync* system receives optical signals from the position sensors in each of the 4 LaserBoxes and relays them to the myRIO FPGA system for real-

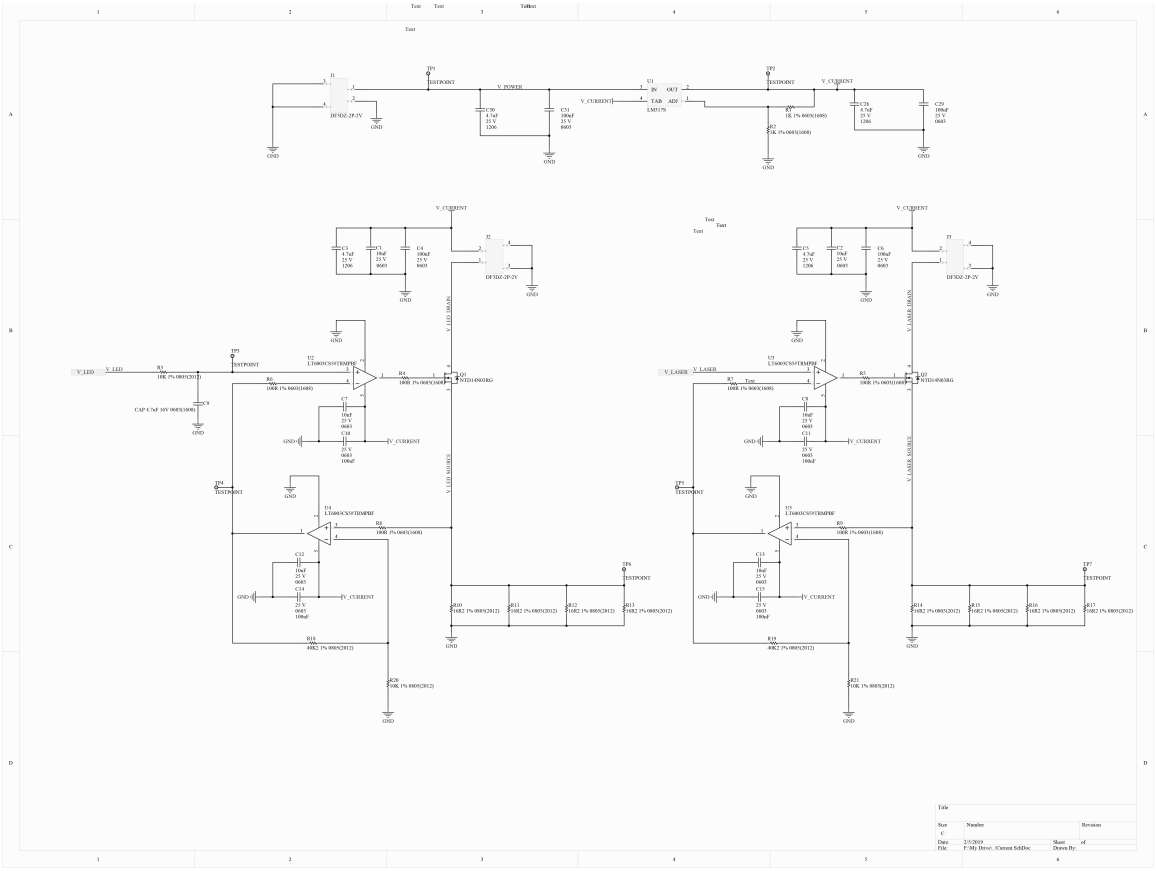


Figure 2.11: Circuitry design of the circuit board of the LaserBox, page 1/2

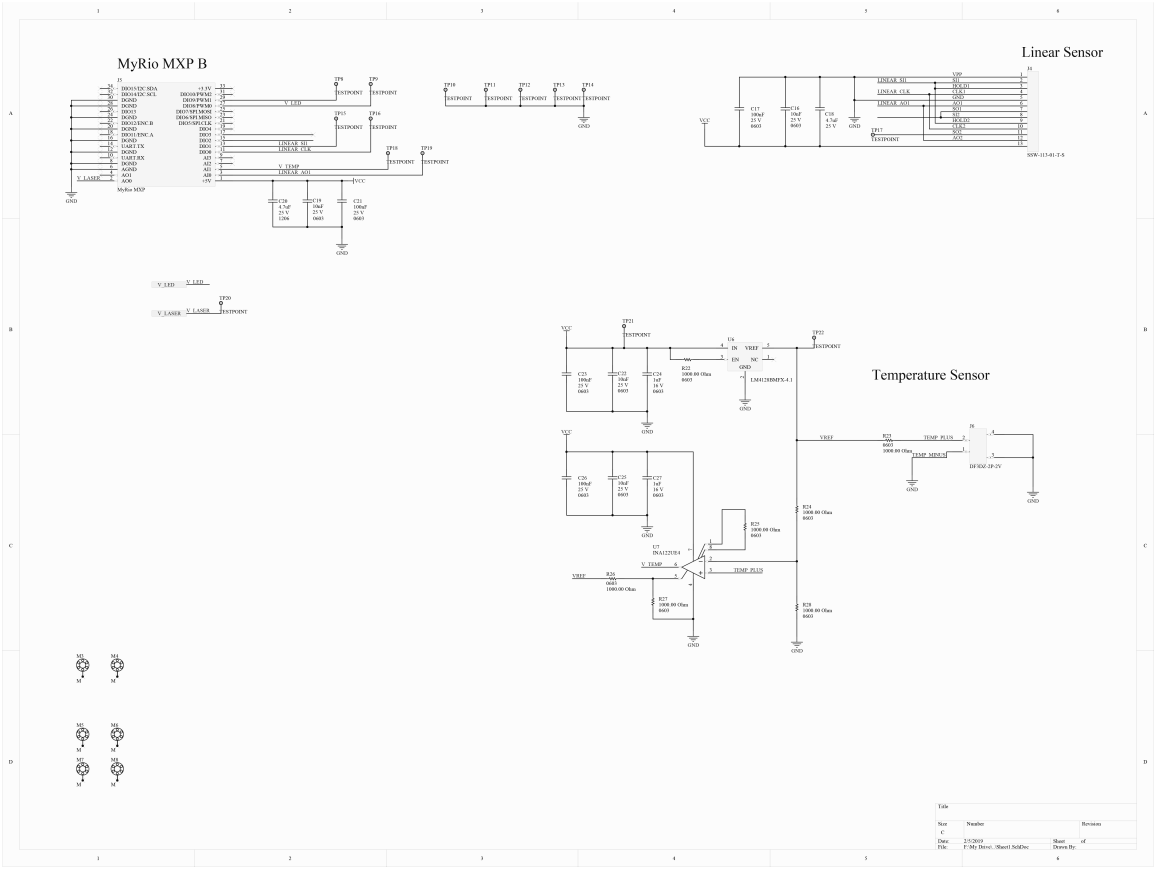


Figure 2.12: Circuitry design of the circuit board of the LaserBox, page 2/2

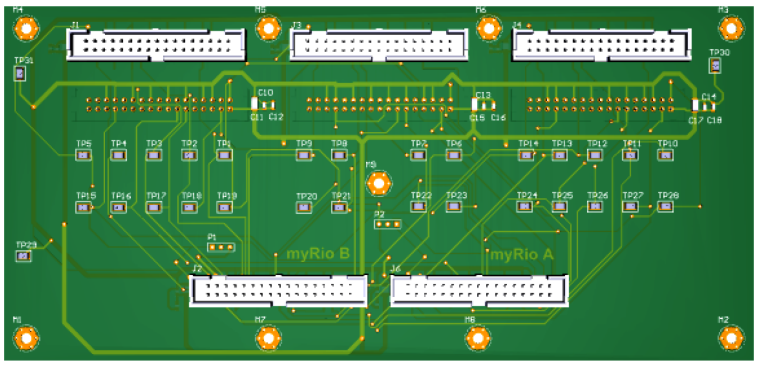


Figure 2.13: A top view of the adapter board.

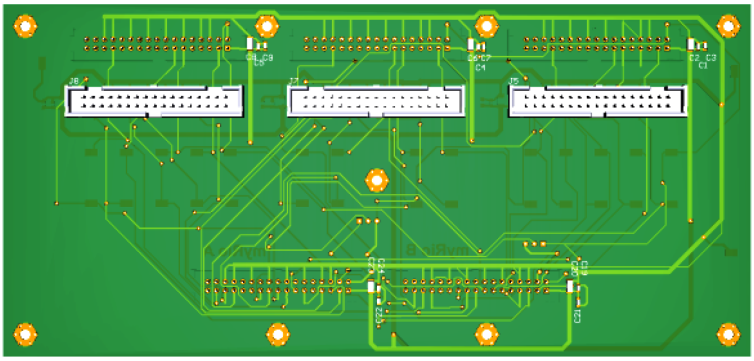


Figure 2.14: A bottom view of the adapter board.

time data processing. Also, the adapter board passes the experimenter's command to change ON/OFF status to LED arrays and laser emitters in each of the 4 LaserBoxes. The top and bottom of the adapter board are shown in Figure 2.13 and 2.14. Also, refer to Figure 2.15 and 2.16 for detailed circuitry design.

2.4 MyRIO FPGA System and End-User Computer

MyRIO FPGA system is a device embedded with re-configurable input and output ports (hence the acronym RIO) manufactured by National Instrument, Inc (Cat. No.myRIO-1950). FPGA stands for field programmable gate arrays, a kind of device re-programmable by the user. The *LaserSync* system's software control systems are deployed to the myRIO FPGA system.

The myRIO FPGA system performs the initial data processing during an experiment

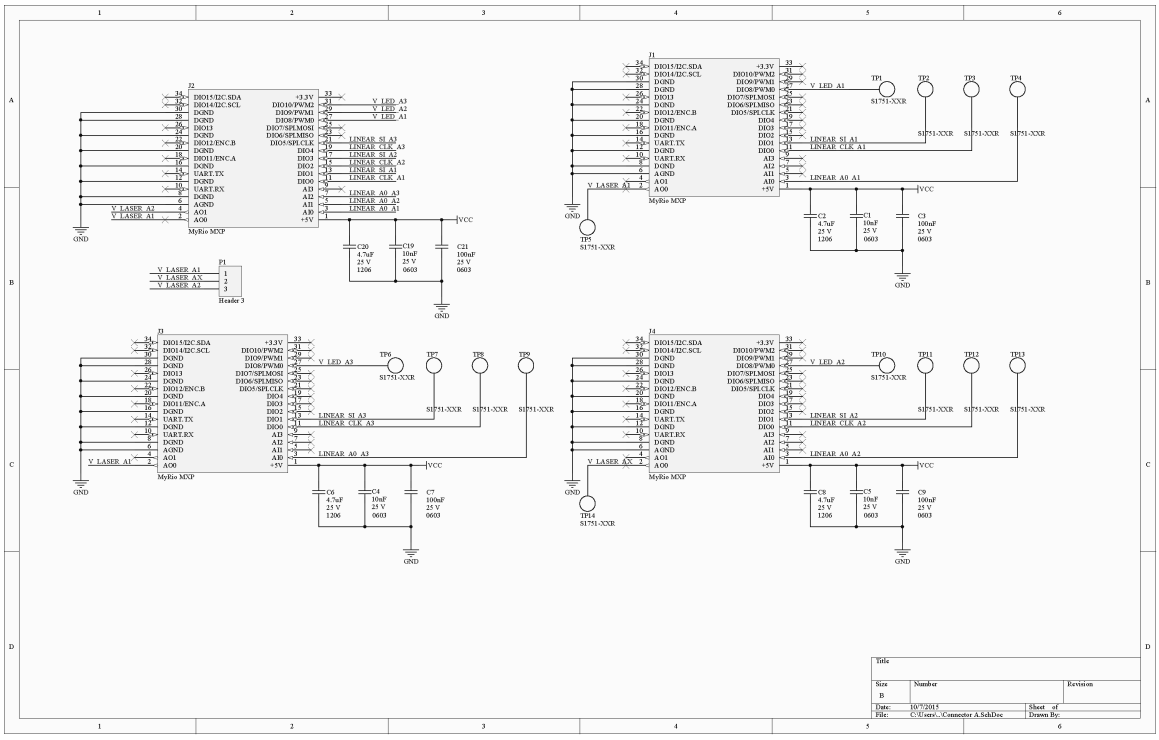


Figure 2.15: Circuitry design of the adapter board, page 1/2

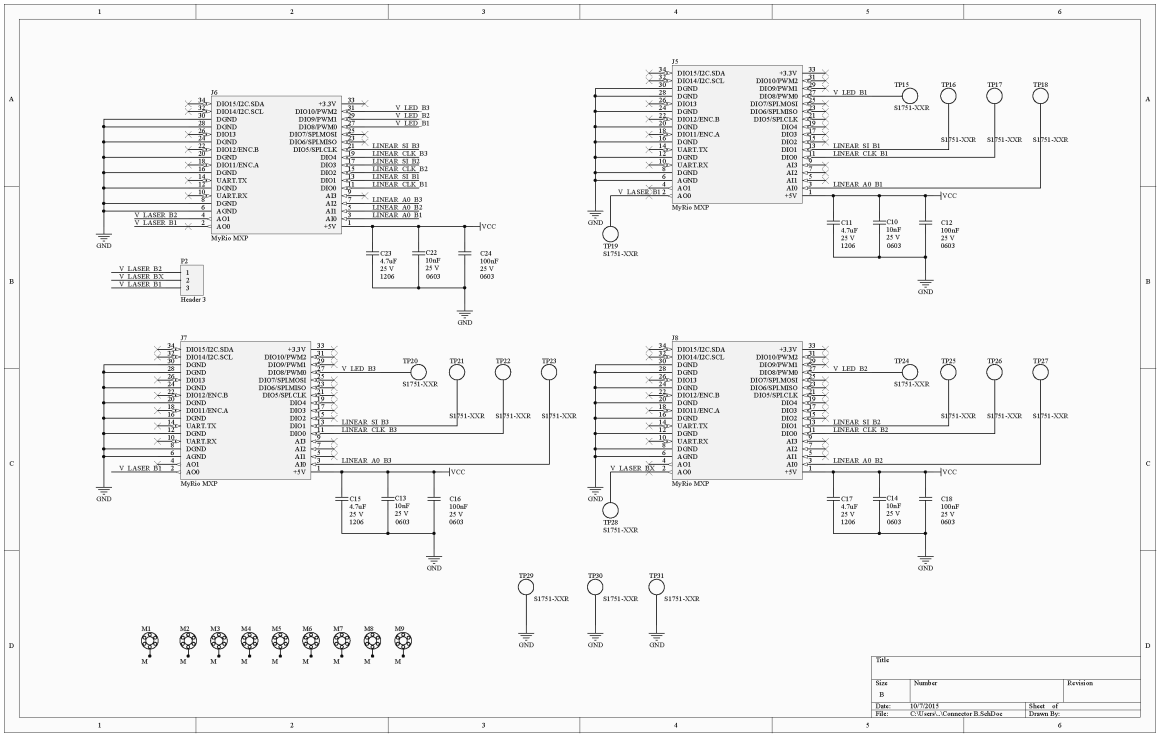


Figure 2.16: Circuitry design of the adapter board, page 2/2

(i.e. in real-time). The real time data pre-processing is enabled by the two microchips, one of which is an FPGA chip, and the other is a regular dual-core ARM CPU. The FPGA CPU enables data acquisition to be done at 40MHz at the pixel level, allowing the position sensor to record the fly's locations at a frame rate of 50Hz.

During experiments, fruit fly' walking behaviors are recorded by position sensors (in all 4 LaserBoxes) and live-streamed through the arena-view camera (in 1 of the 4 LaserBoxes). The position information of the flies is transferred from individual position sensors via the adapter board and myRIO FPGA system to the end-user computer. In the meantime, the laser emitters' ON/OFF status is changed from time to time by an experimenter operating the software system installed on the end-user computer (see Figure 2.21 for a user interface of the software system installed on the computer). For details of the criteria for turning the laser emitter ON or OFF, see Chapter 3. For more details about the software, see section 2.5.

2.5 Software Systems

The *LaserSync* system is programmed in LabVIEW (Laboratory Virtual Instrument Engineering Workbench), a visual programming language developed by National Instruments, Inc. An individual program written in LabVIEW is called a Virtual Interface, or VI. The *LaserSync* software consists of two parts: an FPGA VI and a main VI. During experiments, the fly's locations and the laser emitter status are recorded and stored initially in the format of technical data management streaming (TDMS), a file format native to the LabVIEW environment. For the complete FPGA VI and main VI's source code, see `https://github.com/ruichensun/RS_LabVIEW`. TDMS format is not commonly used in data analysis pipelines, therefore, after each experiment, I convert these TDMS raw data files to the CSV format to facilitate downstream data analysis. See `https://github.com/Ruichensun/TDMS2CSV` for complete conversion source code.

2.5.1 FPGA VI

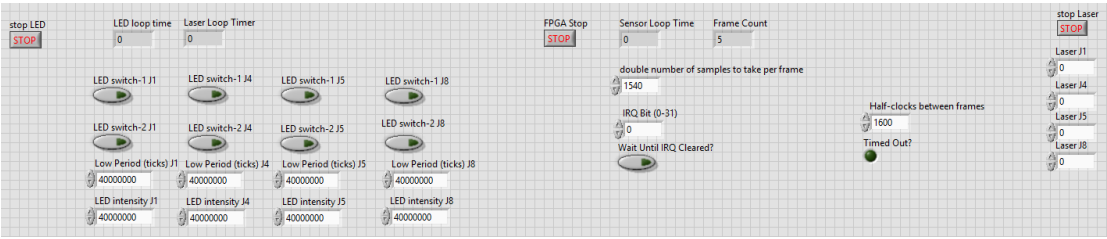


Figure 2.17: User interface of the FPGA VI. J1, J4, J5, J8 represent the 4 LaserBoxes. IRQ stands for interrupt request line, which is physical connection in the program that can send interrupt signals to the FPGA chip. All buttons are labeled according to its function.

The raw data are recorded as the intensity of light received by each pixel of the position sensor. The frames generated from the position sensor are not collected frame by frame, but rather, pixel by pixel, while the output of FPGA VIs is grouped by frames (768 pixels a frame). In other words, during experiments, the sensor collects $1 * 768$-pixel frame at 50Hz. At this frame rate, the minimum required CPU speed is $768 * 50$Hz, equivalent to 38.4KHz. Since the 4 sensors are generating data simultaneously, the minimal required CPU speed quadrupled to 153.6KHz. Although the myRIO FPGA system has two chips, only the FPGA CPU can handle a sampling rate of 153.6KHz. Therefore, in order to achieve reliable data acquisition (without any data loss) at 50Hz sampling frame rate from 4 independent position sensors located in each LaserBoxes simultaneously, the core components of the control system were programmed and deployed in the FPGA CPU to achieve the 153.6KHz requirement at the back-end of the system. Figure 2.17 shows the user interface of the FPGA VI (graphical codes of the program on the FPGA chip). In normal circumstances, an experimenter would not need to interact with this VI, as all of the experimental procedures can be done by interacting with the Main VI (see section 2.5.2). The core functionalities built inside this FPGA VI are monitoring of the raw data from the position sensors (graphical codes shown in Figure 2.18), control of the red LED arrays (graphical codes shown in Figure 2.19), and control of the infrared laser emitters (graphical codes shown in Figure 2.20).

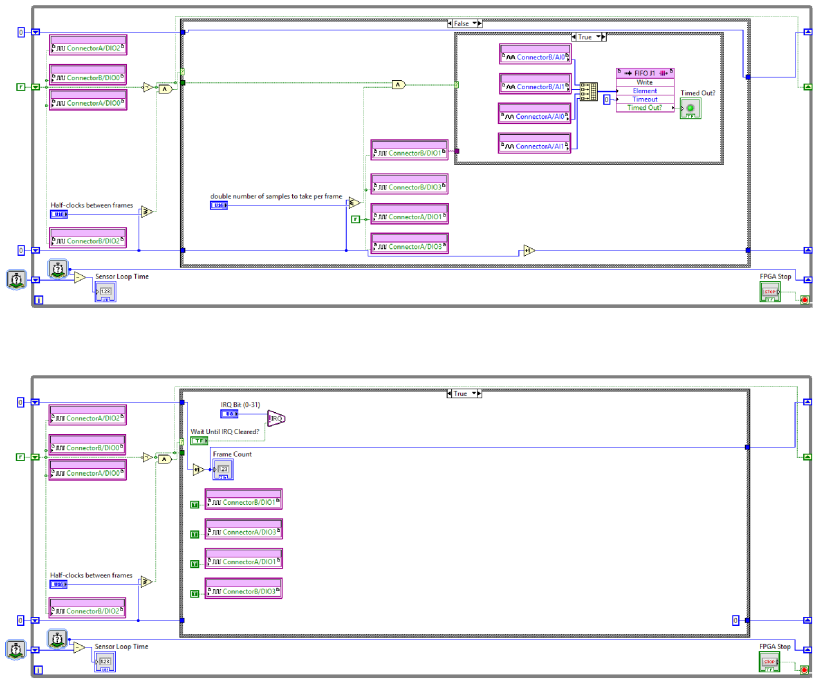


Figure 2.18: Graphical code of position sensor data monitoring. The two panels shown here correspond to the code's two core functions: 1) continuously collect data from each pixel until 768 pixels have been collected (top panel), 2) terminate the previous frame with 768 pixels and initiate a new frame (bottom panel).

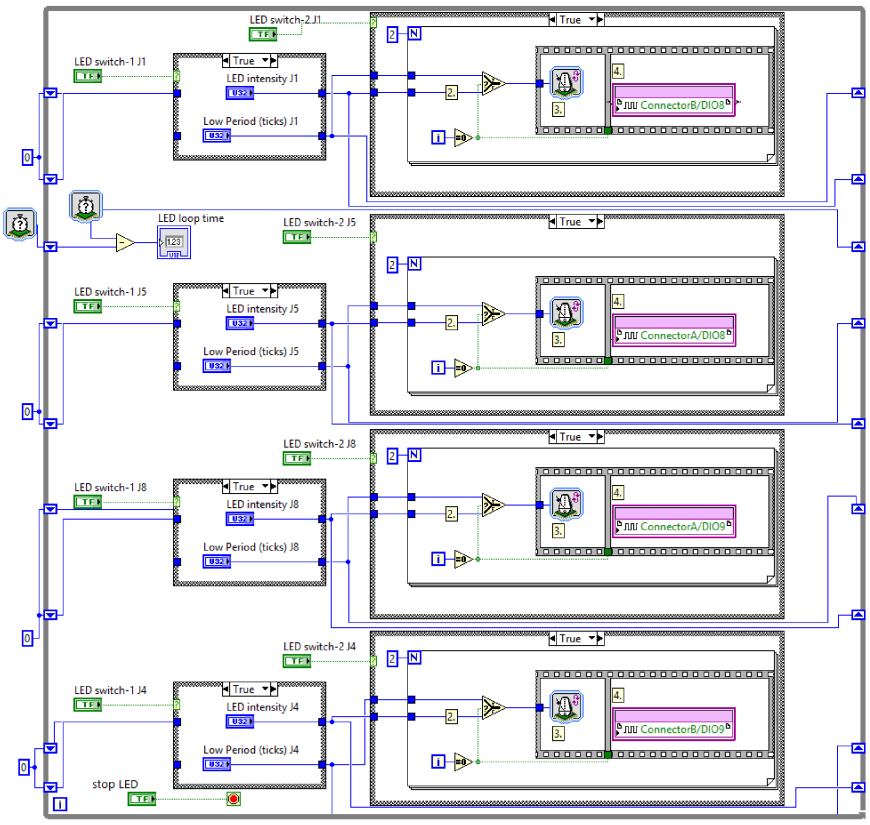


Figure 2.19: Graphical code of LED array control function. The code contains 4 identical blocks, corresponding to the 4 LaserBoxes.

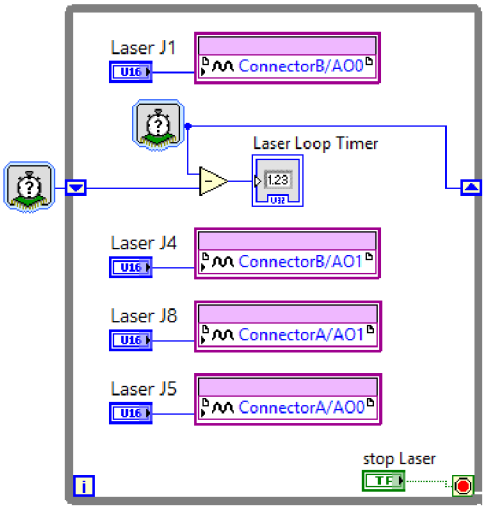


Figure 2.20: Graphical code of infrared laser emitter control function. J1, J4, J5, J8 refer to the 4 LaserBoxes.

2.5.2 Main VI

Main VI is an interface between an experimenter and the *LaserSync* system during experiment. It is designed to receive data from FPGA VI, conduct the first step of data pre-processing, and store the processed data on a hard drive (Figure 2.21, 2.22).

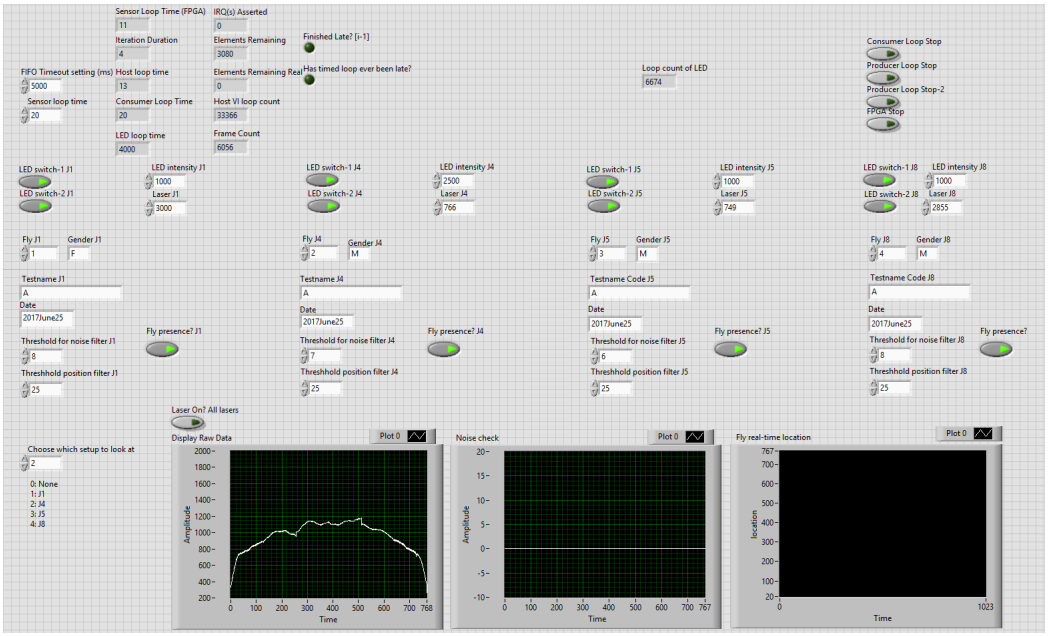


Figure 2.21: User interface of the main VI. J1, J4, J5, J8 represent the 4 LaserBoxes. All buttons are labeled according to its function. The 3 plots stream the light intensity of each pixel, the difference between the current frame and a dynamic reference frame, and the difference time series representation of the fly's location (from left to right)

Data from the FPGA are pixels in groups of 768 (see section 2.5.2). Main VI takes these data and performs initial data pre-processing, which is finding the fly's location and storing it in a USB flash drive connected to the MyRIO system.

The main VI locates the fly using the following method. First, a dynamic reference frame based on the average of 10 previous consecutive frames, each frame containing 768 pixels, is created. Then, the dynamic reference frame will be subtracted from a current frame (Figure 2.23). The result of the subtraction shows the difference between the current frame and the dynamic reference frame (Figure 2.24). If the fly has moved during the past 10 frames (200 ms), the regions that it currently occupies will show up as positive values in the difference frame, as the fly is blocking the red light's pathway and casting a shadow

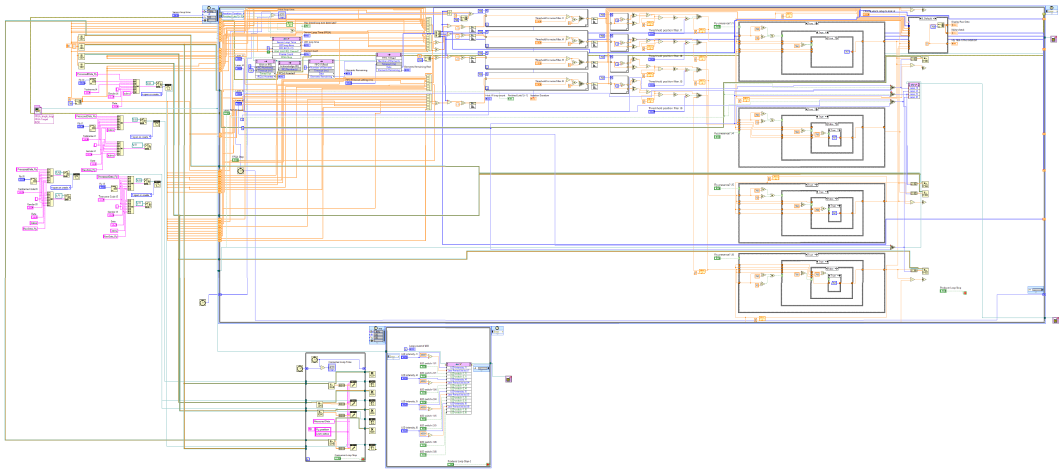


Figure 2.22: Graphical code of the main VI. It consists of four parts: file initiation (at the left of the image), position sensor monitoring (the big rectangle at the center of the immage), red LED array control (the smaller rectangle underneath the position sensor monitoring function), and infrared laser emitter control (the smaller rectangle underneath the position sensor monitoring function).

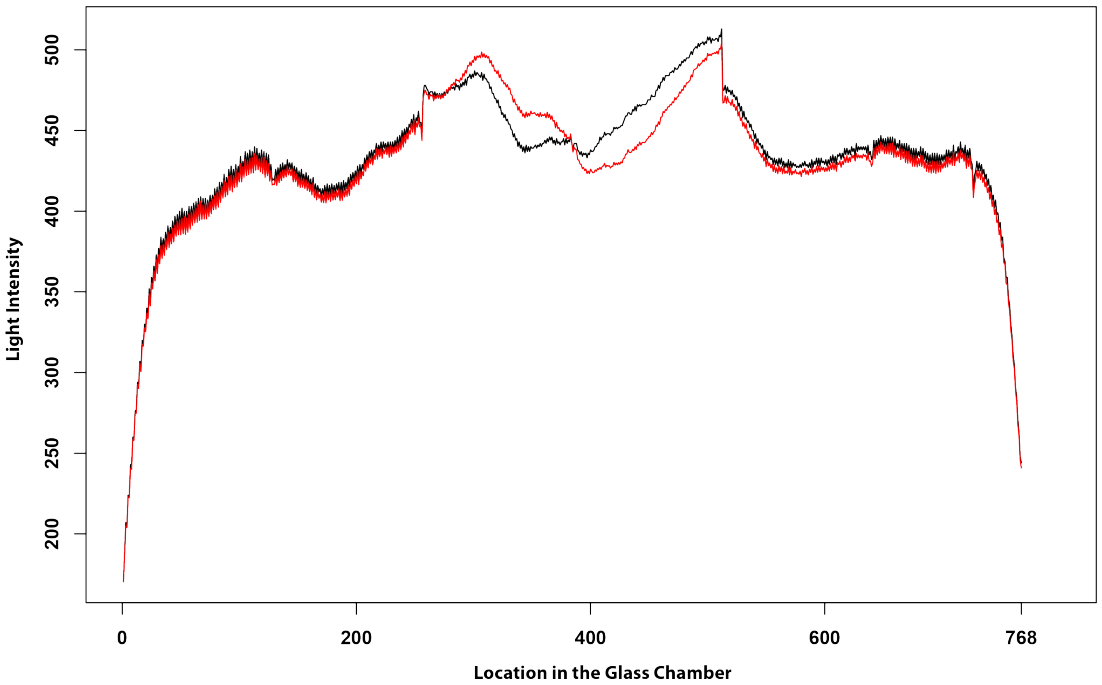


Figure 2.23: Visualization of light intensity at all 768 pixels in a 1D current frame (black line) and a 1D dynamic reference frame (red line).

on the sensor. This results in a lower intensity at this position in the current frame than that in the reference frame (whose value is the average of the past 10 frames). If the fly has not moved during the past 10 frames, every pixel in the current frame will have the same

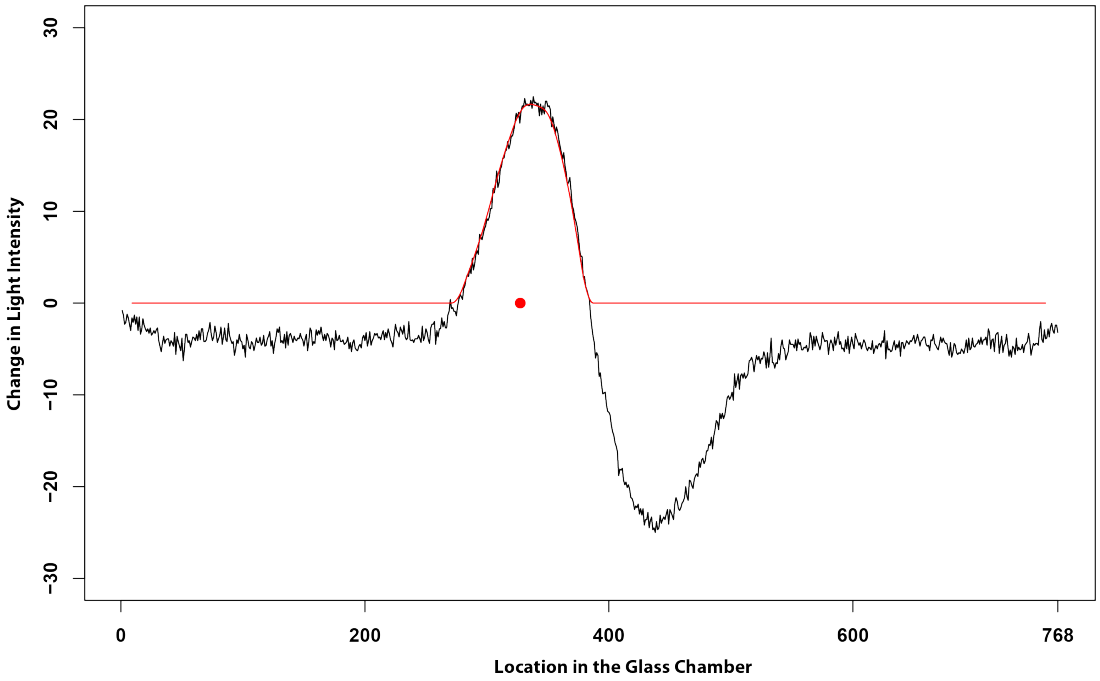


Figure 2.24: Visualization of the fly location (red dot) deduced from the difference frame (black line). As the original 1D difference frame contains noise at all pixels (black line), and the fly is located at regions with positive value of light intensity change, the smoothed 1D difference frame with only non-negative value retained (red line) was used to deduce fly's location (red dot).

value as its corresponding pixel in the reference frame, hence no difference exists between the current frame and reference frame. Thus, the resulting difference frame will have values of zero across the entire 768 pixels. Using this method, one can interpret the regions with positive values in the difference frame as the fly's position (Figure 2.24). Once the regions with positive values in the difference frame is identified, the center of this region is used to represent the fly location in that image. Such a computation is carried out for every frame at a frame rate of 50Hz during each experiment.

During experiments, the computed location of each fly is temporarily stored on a USB flash drive on the MyRIO FPGA system. After the end of the experiment, the stored location information of the fly is transferred to the end-user computer for permanent storage and further data analysis.

2.6 Merits of the *LaserSync* system

Previous studies of fruit fly reinforcement learning behaviors were enabled by several custom-made systems, such as flight simulator and heat box [155, 160]. The flight simulator and heat box use heat as the stressor to train the flies to perform associative learning behaviors, such as flying in certain directions in the flight simulator, or avoiding specific locations in the heat box enclosure [155, 160]. However, both setups have limitations. In the flight simulator, the fly is tethered. This creates an unnatural posture which limits the fly's range of motion, making it easier for the fly to become exhausted and rendering the fly unwilling to perform its usual behaviors. Additionally, extra stimuli such as an air puff, are often needed to keep the fly engaged in the task, which may confound the measurement of responses to the heat stressor. Moreover, in order to get a very strong learning outcome, previous studies chose punishingly high temperatures to stress the flies in the flight simulator setup – sometimes strong enough to kill it within a couple of seconds. In the heat box apparatus, Peltier elements are used to deliver heat [8, 160, 123, 33, 162]. This method relies on heating air inside the chamber in order to transfer heat from the Peltier elements' surface to the fly's body. Therefore, such heat delivery is inherently slow and inefficient, creating a delay in the reinforcing signal. Also, this way of delivering heat stress often requires higher temperature due to the low heat transfer efficiency.

The *LaserSync* system is designed and built to overcome the above issues. It has three main advantages. First, it can deliver precise and instantaneous heat stressors, which not only ensures the consistency of reinforcement feedback but also prevents the fly from being overheated. Secondly, the laser emitter's status can be accurately synchronized across 4 LaserBoxes for experiments requiring control groups. In particular, the LaserBoxes can be configured in such a way that the flies in the control group receive exactly the same heat stressor as flies in the experimental group, allowing the study of learning behavior in a controlled manner. Thirdly, inside the glass chamber, a fly can walk freely while its location is accurately tracked.

2.7 Acknowledgment

Chapter 2 includes materials from the following article, currently being prepared for submission for publication. This dissertation author is the first author of the paper.

Sun, Ruichen; Delly, Joseph; Sereno, Emily; Wong, Sean; Huang, Yan; Chen, Xinyu; Wang, Yuxuan; Greenspan, Ralph. "Anti-Instinctive Learning Revealed by Locomotion-Triggered Heat Stress in *Drosophila*".

Chapter 3

Anti-Instinctive Learning in Wild Type Fruit Flies

3.1 Introduction

The relationship between innate and learned behaviors has attracted a lot of attention since the mid-20$^{\text{th}}$ century [137, 21, 78, 79]. Innate behaviors have long been thought to be fixed and robust [137]. Recent studies, however, have shown that innate behaviors are fluid and can be modified by internal states, environmental cues, and learning [131, 133, 117, 144, 132, 9, 159].

The ability to modify one's innate behaviors via learning can confer great evolutionary advantages to animals and enable them to better adapt to changing environments. Among the different ways learning can modify innate behaviors, anti-instinctive learning is perhaps one of the most challenging types of learning (see Chapter 1 of this dissertation and [83]). Understanding the degree to which a subject is capable of anti-instinctive learning and the underlying mechanisms of anti-instinctive learning have the potential to answer the question of how innate and learned behaviors are related. Yet, progress in this area has been limited.

Previous works on fruit fly courtship conditioning have indicated that their innate behavioral repertoire may be fluid and that flies may possess the capability for anti-instinctive

learning (see Chapter 1). However, studies on courtship conditioning have not agreed on what specific signals were learned during the conditioning phase, as conditioning a male fruit fly with a mated female would always involve a mixture of olfactory, visual, tactile, auditory, and gustatory cues [139, 140, 1, 87, 68]. Thus, a simpler behavior paradigm of anti-instinctive learning is called for. In light of the limited progress in this area, I designed and built the *LaserSync* system (see Chapter 2) to study a novel anti-instinctive learning behavior of fruit flies.

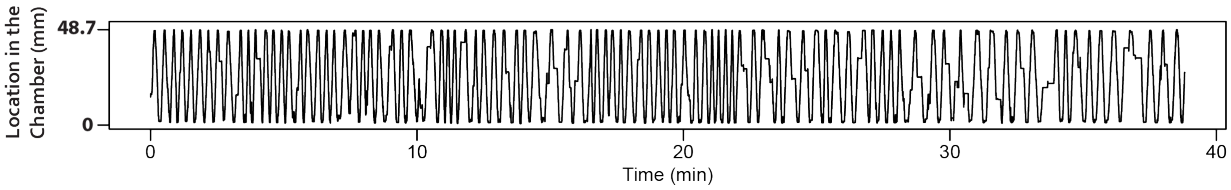


Figure 3.1: A fly's walking behavior inside the glass chamber of LaserBox

When placed inside a LaserBox of the *LaserSync* system, a fly shows robust walking behavior: pacing the glass chamber back and forth tirelessly for 40 minutes (or longer) (Figure 3.1). This robust locomotion behavior allows us to study how this innate behavior can be perturbed by learning. In addition to its active nature, fruit flies, as poikilothermic creatures, naturally prefer ambient temperature over an extremely hot enviro-https://www.overleaf.com/project/5c1abb087118db71cbf51d82nment [84, 35]. When facing a sharp temperature increase, innately heat-aversive flies become more active and try to escape from the warming environment [35, 12, 40, 45, 110]. Therefore, learning to stop walking when heated is against a fly's innate tendency. If a fly is capable of anti-instinctive learning, experiencing recurring movement-triggered heat stress will reduce its walking. In this chapter, I describe a novel behavior paradigm using the *LaserSync* system to investigate if wild type fruit flies can reduce walking after experiencing recurring movement-triggered heat stress.

3.2 Methods

3.2.1 Fly Rearing

Adult virgin female and male Canton-S flies of 2-7 days old were used in this study. Flies were kept in 23°C with 50 - 80% humidity and 12:12 light-dark (LD) cycles. All flies were assayed during the circadian time (CT) 1 - 6 and CT 7 - 10 (lights are turned on at CT 0 and turned off at CT 12). After behavioral assays, all flies were returned to their original isolation vials and were kept until death. All flies were isolated at eclosion and reared individually in 2.5 ml plastic isolation vials containing 150mg food (Caplugs Cat. No.214-2002-010, Rancho Dominguez, California). Isolating flies at eclosion allows us to study an individual fly's learning ability without the complication of group rearing, where peer exposure may affect a fly's behavior in learning tasks. The fly food used in this study was made of dark corn syrup (30 mL/L), yeast (35 g/L), nipagin (1.125g/L), propionic acid (7.5mL/L), ampicillin (50mg/L), chloramphenicol (50mg/L), sucrose (15 g/L), and agar (10 g/L).

3.2.2 Behavioral Experiment Setup

The *LaserSync* system was used in the study. For details about the *LaserSync* system, see Chapter 2.

Before each experiment, an experimenter transfers a fly into the glass tube (chamber) by placing the chamber inside the isolation vial and allowing the fly voluntarily to enter the chamber. No anesthetic was used in the process. After the fly enters the chamber, the experimenter closes both ends of the chamber using a glass window (window) on each side. The chamber, with both ends closed, is now secured inside a holder (for an image of the holder, see Figure 2.6(d)). Then, the experimenter places the holder into the LaserBox's holder slot and is ready for experiments.

During experiments, the entire *LaserSync* system is kept in the dark, except for the red light emitted from the LED array, whose peak wavelength is 630 nm (Figure 2.3). As

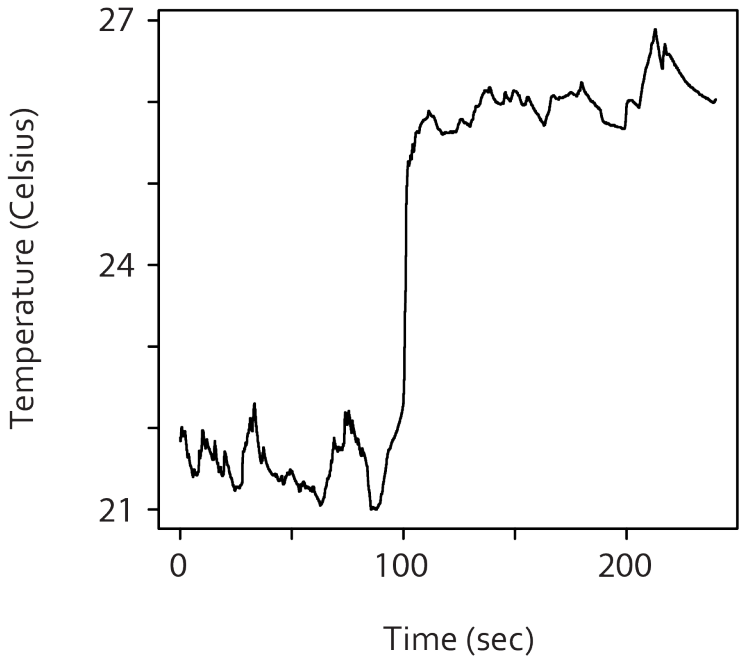


Figure 3.2: Mild heat stress was used as the stressor. Laser emitter can quickly elevate a fly's body temperature from room temperature to $26 - 27°C$ and maintain the fly's body temperature at that level (black line).

fruit flies cannot perceive red and infra-red light, it is reasonable to assume that the fly receives no visual stimuli during experiments [67]. In addition to no visual signals, the flies experience few or no olfactory or gustatory cues during experiments, as all chambers are cleaned thoroughly after each experiment to prevent any odor or body secretion being carried over from one experiment to another.

A mild punitive temperature of $26 - 27°C$ was chosen as the stressor (Figure 3.2). Behavioral studies on freely moving fruit flies frequently used $37°C$ or higher temperature as stressor [9, 162, 22, 33, 14]. Using $37°C$ or higher temperature as a stressor, however, may cause physiological damage to the fly as it can die after more than a few seconds of exposure to high heat (personal communications with Dr. Bjoern Brembs). As the behavioral experiment in this study involves recurring heat stress and lasts one hour, and as the laser emitter in the *LaserSync* system is able to warm up the body temperature of the fly to any level below $45°C$, using conventional high heat as the stressor is not ideal and not necessary. Therefore, in order to study anti-instinctive learning behavior with minimal

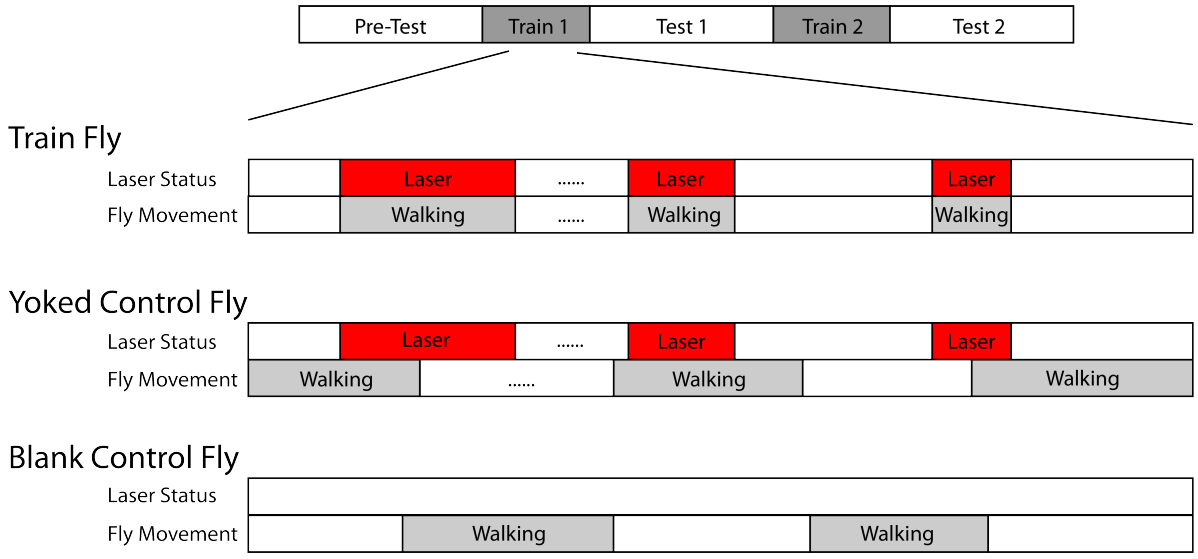


Figure 3.3: A 5-session fruit fly anti-instinctive learning behavior assay. The top row represents the 5 session assay, consisting of the Pre-Test, Train 1, Test 1, Train 2, and Test 2 sessions. The training given to each fly during experiments are visualized in the area extended from "Train 1". For each of the "Laser Status" row, red cells indicate that the Laser emitter's status is ON, white cells indicate that the Laser emitter's status is OFF. For each of the "Fly Movement" row, gray cells indicate that the fly is walking, white cells indicate that fly is not moving.

potential physiological damage, a mild heat stressor was chosen.

3.2.3 Behavioral Assay

To investigate whether flies are capable of anti-instinctive learning, a 5-session behavioral assay was designed (Figure 3.3).

Every fly in the experimental group (training fly) receives 5 sessions in one experiment: Pre-test, Train 1, Test 1, Train 2, and Test 2. Each of the 3 test sessions (Pre-Test, Test 1, and Test 2) are 10 minutes long, during which its walking behaviors are recorded. No heat stress was given during the 3 test sessions. Each of the two training sessions (Train 1 and Train 2) consists of up to 20 episodes of mild heat stress treatments which are only given to the fly when it moves. The mild heat stress treatment stops when the fly stops walking. If the fly has received 20 episodes of mild heat stress treatments, or if it has been stationary for 8 minutes, the Train session concludes and the experiment moves on to the next session.

The cutoff at the 8-minute was based on the observation that 1) different flies need different amounts of mild heat stress to finish the Train 1 and Train 2 session, and not setting a cutoff time would result in the duration of different experiments inconsistent and overly variable, and 2) the fact that prolonged inactivity during the Train sessions in itself is an indicator of having learned to inhibit its walking activity.

In addition, the training fly receives the mild heat stress treatment when it is at either end of the chamber, the ends defined as the left and right most 3mm segment of the chamber, during Train 1 and Train 2 sessions. This is due to the fact that flies are naturally attracted to edges [126]. If a fly is allowed to stay still at either end of the chamber during the training session, the edge-attraction instinct will confound the measurement of learning effect.

Two types of control groups are used: a yoked control group (yoked fly) and a blank control group (blank fly). Flies in both groups were assayed simultaneously individually in a LaserBox that is different from the LaserBox the training fly is in. The yoked fly receives identical mild heat stress treatment to that of the training fly, regardless of whether the yoked fly is moving or not. The blank fly does not receive any mild heat stress treatment throughout the entire duration of the experiment.

Flies were assigned to the training group, yoked control group, and blank control group randomly. In this chapter and 4, if not stated otherwise, training fly group data is color-coded with red, while yoked fly group data and blank fly group data are color-coded with blue and gray, respectively.

3.2.4 Data Analysis

Data Collection and Pre-processing

Before experiments, every fly is given a number as its unique identifier. During each experiment, a fly's real-time location information along with the ON/OFF status of laser emitters is recorded in individual technical data management streaming (TDMS) files

(see Chapter 2 for details about the TDMS file and the LabVIEW software system). The 5 sessions of data from one fly are stored in 5 separate files. After each experiment, these TDMS files are converted into comma-separated value files (CSV files) for downstream processing (see Chapter 2 for details). R, a programming language and a free software environment for statistical analysis, is used for downstream processing. In addition to the CSV files storing the walking behaviors of the fly, each fly's basic information, such as eclosion dates and gender, are recorded in a separate CSV file ("fly info data file"). The fly info data file is used as a reference to match each fly's basic attributes with its behaviors over the course of 5 sessions in an experiment.

For each fly, the percentage of time active during the Pre-Test session and the likelihood of receiving mild heat stress for training flies during the Train 1 session are used for quality control. Flies that are inactive more than 90% of the time are removed from further analysis. The lack of robust walking behavior before the fly receives any heat stress signals that this fly may be physiologically different from healthy flies. For the training flies, they would receive more mild heat stress during walking than during resting. Therefore, for these flies, the likelihood of receiving mild heat stress during walking is much higher than that during resting. If a training fly likelihood of receiving heat stress during walking is less than 20% higher than its likelihood of receiving heat stress during a pause, the fly will also be removed from further analysis. The 20% difference requirement is only used for screening the Train 1 sessions, as in the Train 2 session many training flies have shown complete lack of activity and did not trigger any mild heat stress.

After quality control, each fly's overall percentage of time active ("activity level") during Pre-Test, Test 1, and Test 2 sessions is computed and analyzed. After the quantification, activity levels of training flies are compared to yoked flies and blank flies. In Train 1 and Train 2, a fly's cumulative activity levels were used for comparison.

For details about customized functions written for analysis, refer section 3.5 at the end of this chapter. For complete scripts written for this study, see `https://github.com/Ruichensun/fly_source`.

Statistical Analysis

The Kruskal–Wallis test followed by pairwise Wilcoxon rank sum tests (with Benjamini Hochberg correction) is used for all statistical comparisons. Confidence intervals are calculated using permutation tests with 10,000 permutations of the raw data. Interpretation of p values are shown in Table 3.1.

Table 3.1: P Value Interpretation

Category	Symbol
$0.0500 \leq p \leq 1.0000$	n.s.
$0.0100 \leq p < 0.0500$	*
$0.0010 \leq p < 0.0100$	**
$0.0001 \leq p < 0.0010$	***
$0.0000 \leq p < 0.0001$	****

3.3 Results

3.3.1 Training Flies and Yoked Flies Experience the Same Heat Stress in Different Ways

Training flies and yoked flies receive the same heat stress during Train 1 and Train 2 session. However, what the flies are doing when experiencing mild heat stress are different. We measure both groups of flies' likelihood of receiving mild stress during walking and during pause (Figure 3.4, 3.5, Table 3.2). The likelihood of receiving heat stress during walking is the percentage of time the fly is receiving mild heat stress out of the total time the fly is walking. Similarly, the likelihood of receiving mild heat stress during pause is the percentage of time the fly is receiving mild heat stress out of the total time the fly is in pause.

During the Train 1 session, the training flies receive heat stress $88.5\% \pm 2.0\%$ of the time when they walk, while these flies receive the stress only $29.0\% \pm 5.9\%$ of the time when they are not walking (Figure 3.4). During the same Train 1 session, however, yoked flies

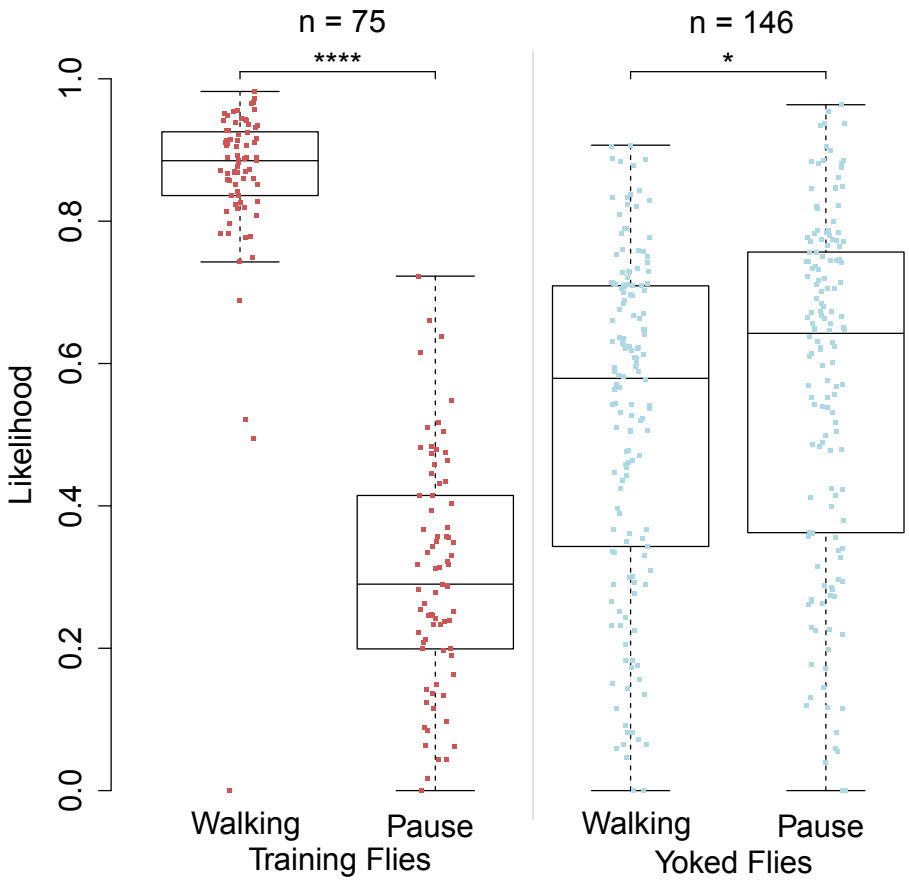


Figure 3.4: Likelihood of receiving heat stress during Train 1 session.

receive heat stress with comparable likelihood during both walking and resting: 57.9%±4.6% during walking, and 64.2% ± 4.0% during resting (Figure 3.4), indicating that their walking behaviors are not preferentially punished as those of the training flies are.

For the training flies, the difference in the likelihood of receiving heat stress during walking and resting is significant during the Train 2 session. These training flies experience mild heat stress 83.7% ± 5.0% of the time during walking and 15.1% ± 5.8% during resting (Figure 3.5). On the other hand, during the same Train 2 session, yoked flies has comparable likelihood of receiving mild heat stress when they are walking or in pause. During walking, yoked flies' median likelihood of receiving heat stress is 31.3% ± 16.1%. When staying still, a yoked fly's likelihood of receiving mild heat stress is 29.1% ± 11.8% (Figure 3.5). As

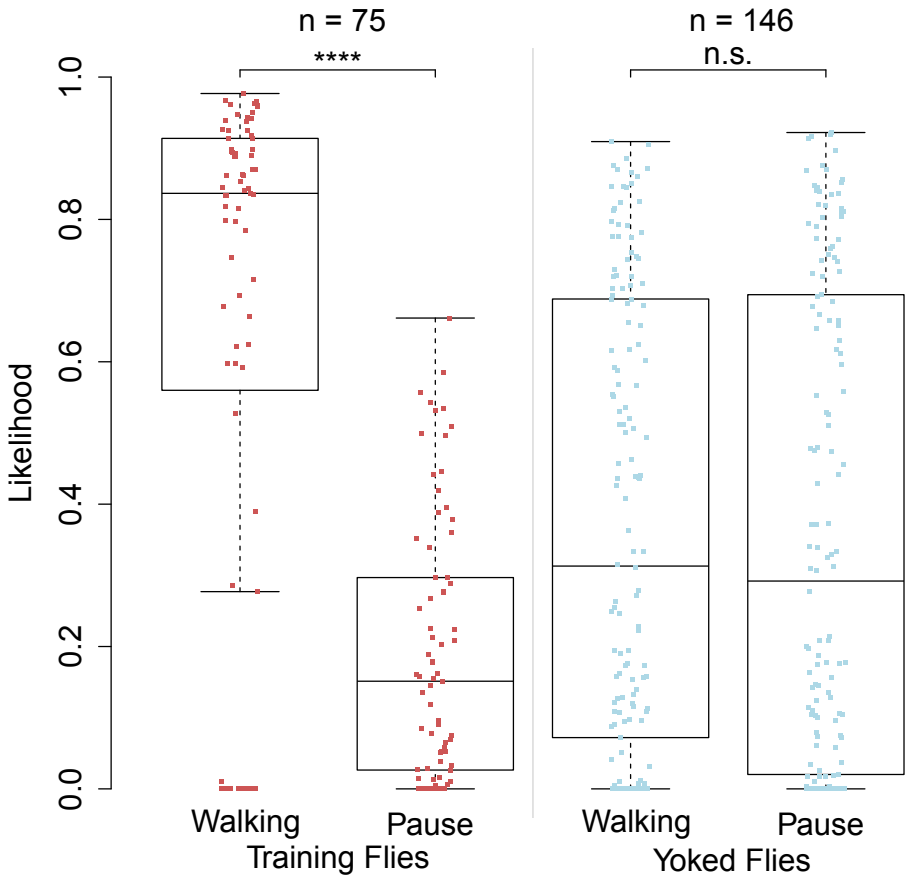


Figure 3.5: Likelihood of receiving heat stress during Train 2 session.

Table 3.2: Estimates of Likelihood of Receiving Mild Heat Stress During Train Sessions

Fly Group	Median	CI (Lower)	CI (Upper)	Sample Size
Training Flies in Train 1, Walking	0.885	0.865	0.905	75
Training Flies in Train 1, Pause	0.290	0.231	0.340	75
Yoked Flies in Train 1, Walking	0.579	0.533	0.636	146
Yoked Flies in Train 1, Pause	0.642	0.602	0.696	146
Training Flies in Train 2, Walking	0.837	0.787	0.890	75
Training Flies in Train 2, Pause	0.151	0.093	0.229	75
Yoked Flies in Train 2, Walking	0.313	0.152	0.461	146
Yoked Flies in Train 2, Walking	0.292	0.174	0.455	146

the likelihood of receiving mild heat stress is comparable between when the fly is walking and when it is standing still, this indicates that the mild heat stress the yoked flies receive are random. Thus, the experience of heat stresses inside the LaserBoxes are fundamentally different for training flies and yoked flies, allowing us to use behavioral changes in the yoked flies as a control group for behavioral changes in the training flies.

3.3.2 Training Flies Reduce Activity During the Training Sessions

Table 3.3: Wild Type Flies' Ending CAD (Unit: seconds) of Each Train Session

Session	Group	Median	CI (Lower)	Median CI (Upper)	Sample Size
Train 1	Training	52.24	44.01	60.37	75
	Yoked	63.10	57.97	68.38	146
	Blank	66.56	55.69	76.95	73
Train 2	Training	31.20	22.22	41.24	75
	Yoked	57.52	51.42	63.76	146
	Blank	60.88	48.87	69.56	73

If flies are capable of anti-instinctive learning, training flies would show less movement during the Train 1 or Train 2 sessions compared to either the yoked flies or the blank flies. To test this hypothesis, we measured the cumulative active duration (CAD) of all flies during the two Train sessions (Figure 3.6, 3.7). The time a fly requires to complete one training session varies from fly to fly due to the operant nature of the experiment. As a result, the

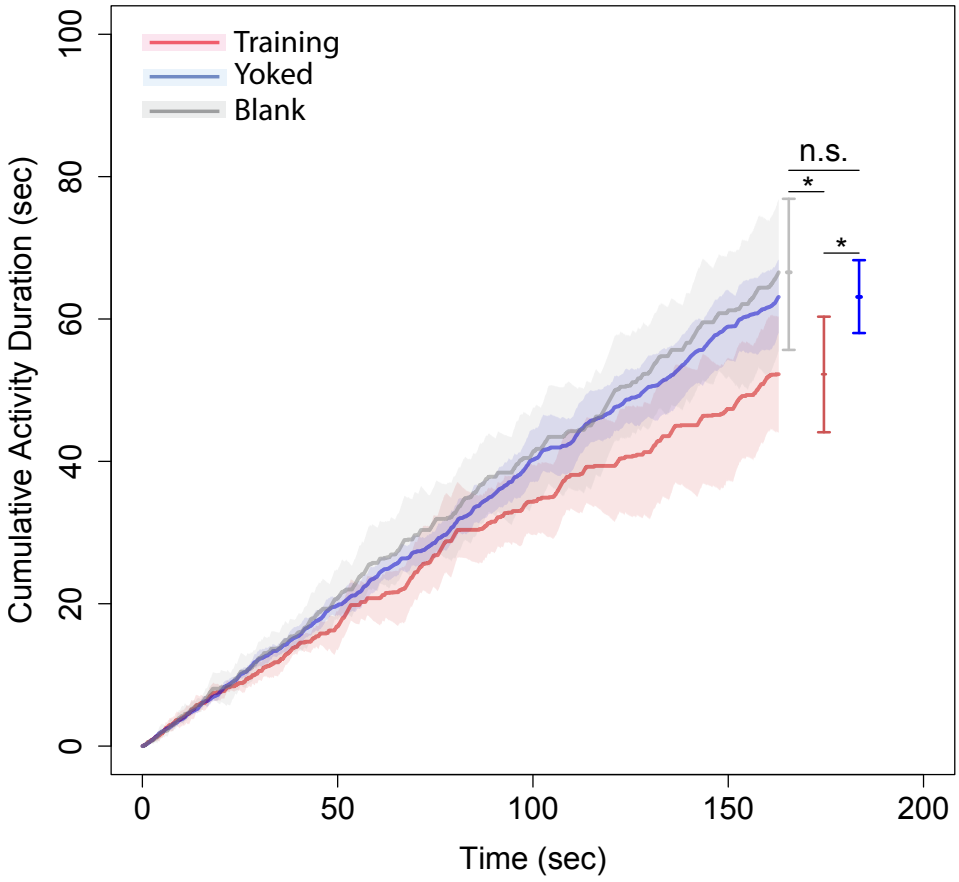


Figure 3.6: CAD of wild type flies during the Train 1 session. The solid lines indicate the median of CAD of each group at each time point. The shaded areas indicate bootstrapped 95% confidence interval at each time point. Kruskal-Wallis test followed by Mann-Whitney U test with BH correction was done on all three groups at the end of the session.

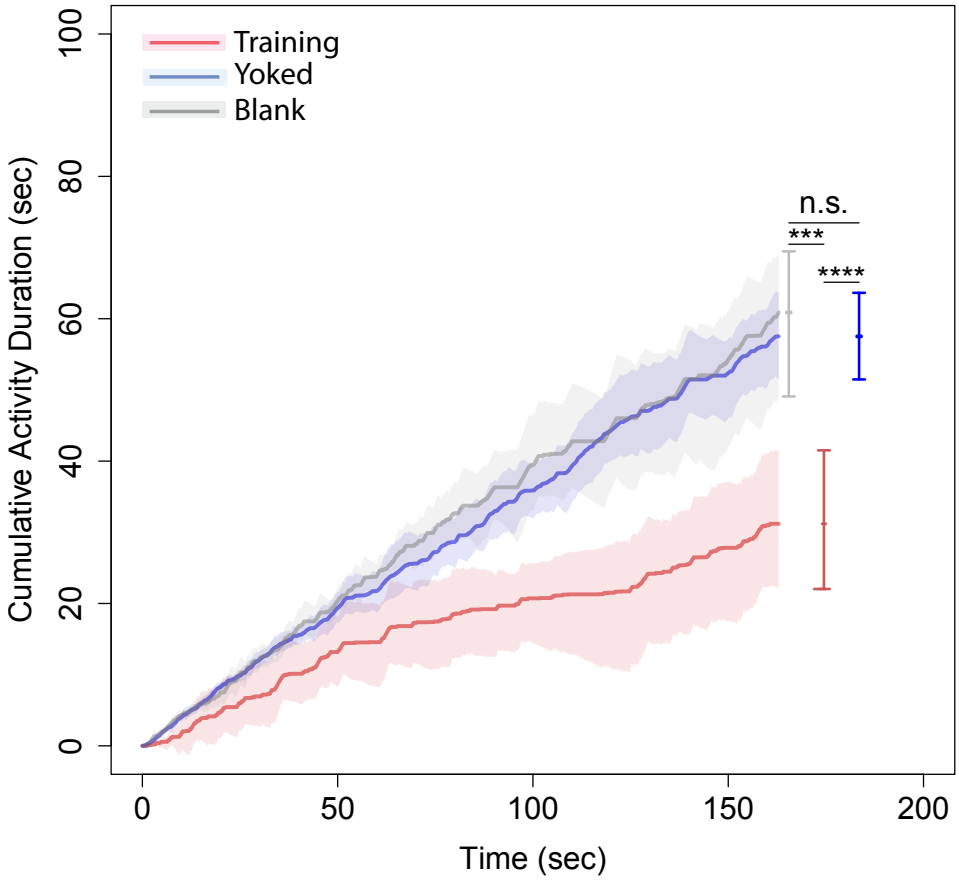


Figure 3.7: CAD of wild type flies during the Train 2 session. The solid lines indicate the median of CAD of each group at each time point. Training flies' CAD are significantly lower than the other groups. The shaded areas indicate bootstrapped 95% confidence interval at each time point. Kruskal-Wallis test followed by Mann-Whitney U test with BH correction was done on all three groups at the end of the session.

Table 3.4: P Values from Wild Type Fruit flies Activity Levels Kruskal-Wallis Test, Followed by Mann-Whitney U Test with Benjamini-Hochberg correction

Test Groups	Pre-Test P Values	Test 1 P Values	Test 2 P Values
Krusal-Wallis Test	0.1821	0.005071	3.826e-07
Training vs Yoked	N/A	0.0083	1.5e-05
Training vs Blank	N/A	0.0090	6.9e-07
Yoked vs Blank	N/A	0.7886	0.19

minimum length of both training sessions, 163 secs, was used when comparing the CAD across three groups of flies. This time point is referred as the end of Train 1 and Train 2 sessions. At the end of Train 1 session, the CAD of flies in the training group (CAD: 52.24 ± 8.18 sec) is smaller than that of the yoked flies (CAD: 63.10 ± 5.20 sec) and the blank flies (CAD: 66.56 ± 10.63 sec) (Figure 3.6, 3.3). At the end of the Train 2 session, the training flies move significantly less (CAD: 31.2 ± 9.51 sec) compared to those in the yoked flies (CAD: 57.52 ± 6.17 sec) or the blank flies (CAD: 60.88 ± 10.34 sec) (Figure 3.7, 3.3). These results, together, indicate that flies in the training group have gradually learned to stop moving while flies in the two control groups fail to do so, a sign of learning.

3.3.3 Training Flies' Reduced Activity Level Persist After Training Ends

As training flies showed a clear sign of anti-instinctive learning during both Train 1 and Train 2 sessions, the next question is whether their learned inhibition of walking persists after each training session ends. To answer this question, we measured each animal's percentage of time active, also called activity level, during each of the 3 test sessions (Pre-Test, Train 1, Train 2). During the Pre-Test session, flies are active most of the time (Figure 3.8, 3.5). Specifically, the median activity levels for flies in each group are $58.0\% \pm 3.0\%$ for the training group; $56.0\% \pm 2.5\%$ for the yoked flies; and $53.0\% \pm 5.0\%$ for the blank flies. During the Test 2 session, which is after two training sessions, flies from the training group showed significantly lower activity than the flies in the two control groups.

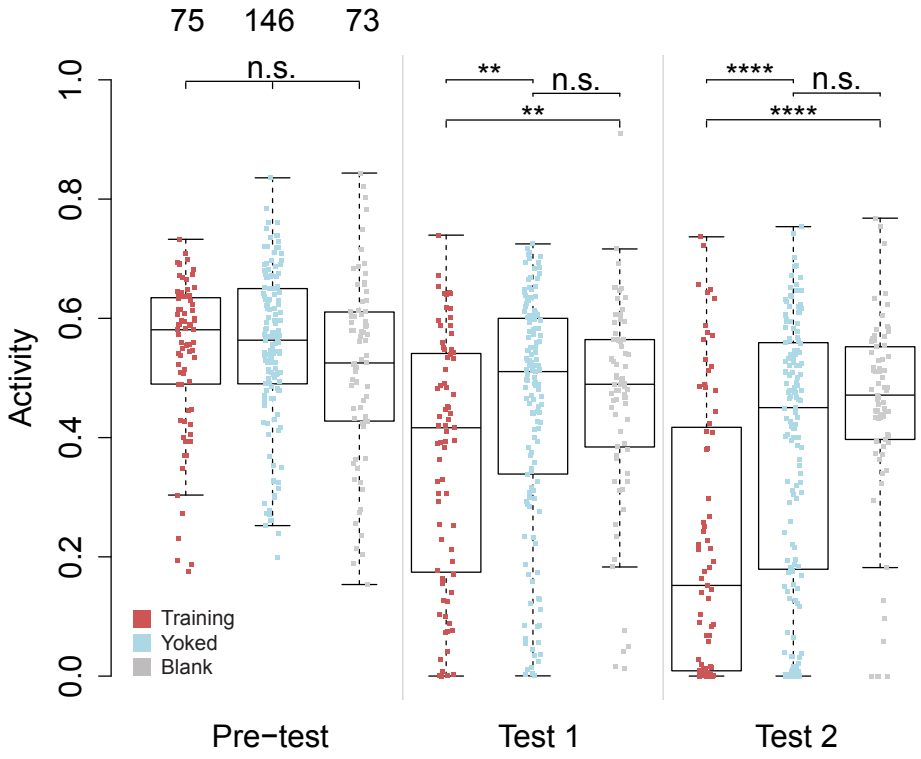


Figure 3.8: Wild type flies' percentage time active during test sessions

Table 3.5: Wild Type Flies' Activity Level in Each Test Session

Session	Group	Median	CI (Lower)	Median CI (Upper)	Sample Size
Pre-Test	Training	0.58	0.56	0.62	75
	Yoked	0.56	0.54	0.59	146
	Blank	0.53	0.47	0.57	73
Test 1	Training	0.42	0.37	0.48	75
	Yoked	0.51	0.49	0.54	146
	Blank	0.49	0.46	0.51	73
Test 2	Training	0.15	0.06	0.24	75
	Yoked	0.45	0.41	0.50	146
	Blank	0.47	0.44	0.50	73

The training flies' activity level is $15.0\% \pm 9.0\%$, whereas the yoked and the blank groups' activity level are: $45.0\% \pm 4.5\%$ for the yoked group and $47.0\% \pm 3.0\%$ for the blank control group. The significant difference between the training flies and the control flies further reinforces that flies in the training group have learned to act anti-instinctively with recurring movement-triggered heat stress, while flies in the control groups fail to do so.

In addition to the behavioral effect, we kept track of all flies' life span after experiments. The Kaplan-Meier estimation of the survival probability after receiving recurring heat stress shows no significant difference among the three groups of flies, indicating that heat stress endured by the flies in the training group and the yoked group did not induce major physiological damages in the flies (Figure 3.9). Taken together, wild type flies are able to learn to inhibit walking after receiving recurring movement-triggered heat stress. Such learning persists after the two Train sessions finish. The result shows that flies are capable of anti-instinctive learning.

3.3.4 Yoked Flies Show A Moderate Decrease in Activity Level

In addition to the training flies' learning effect, we also observed that the yoked flies' activity level showed a moderate decrease as they receive more recurring mild heat stress (Figure 3.8). As the yoked flies receive randomly occurring heat stress, it is interesting that

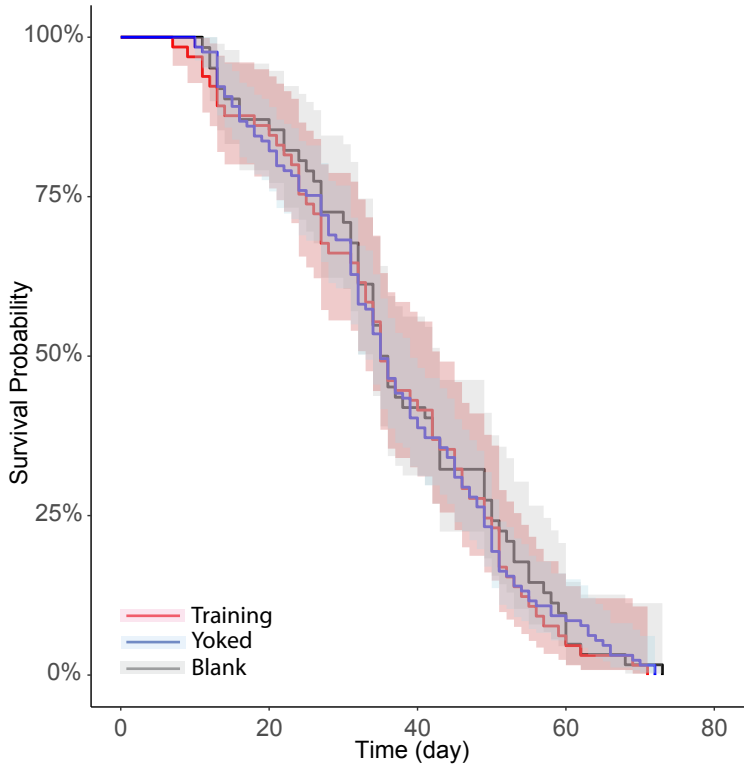


Figure 3.9: Kaplan-Meier estimation of survival probability after experiment

their activity level decrease more than the blank flies' activity level (Figure 3.4, 3.5). Two possible factors may underlie this phenomenon: the effect of prolonged heat stress or the effect of learning.

Prolonged Heat Stress' Effect on Activity Level

Due to the operant nature of the experiment, different training flies require different amounts of total laser exposure to complete both Training sessions. As a result, yoked flies inevitably experience laser stimulation of varying durations. Although the heat stress intensity is mild ($26-27°C$), it is still possible that a subset of flies experience longer exposure to heat stress and become more exhausted as a result.

To find out if this is the case, we investigated the correlation between the total duration of laser exposure during the two Train sessions (Duration (sec)) and the change in activity levels between Pre-test and Test 2 sessions (Activity Difference) (Figure 3.10). If such an correlation exists, yoked flies having been exposed to longer heat stress will show

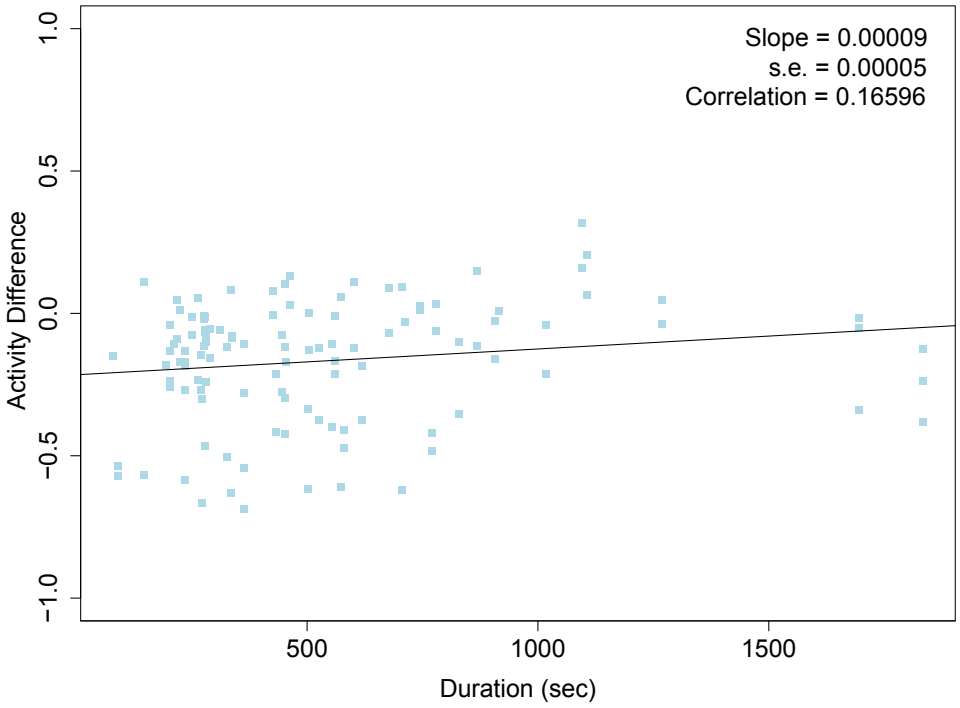


Figure 3.10: The relationship between total laser exposure duration and yoked flies' changes in activity level before and after training

more negative activity changes. The result, however, shows that the correlation between the amount of heat stress received and the decrease in activity level is negligible, indicating that the decrease in the yoked flies' activity level is likely not due to prolonged heat stress-induced exhaustion.

Learning

The second possible cause for the yoked flies' decrease in activity level after experiencing heat stress is learning. Although both the timing and amount of heat stress yoked flies experience are determined by the training flies and are, in general, unrelated to the yoked flies' own activity, a subset of yoked flies may have received, by chance, more heat stress when they are walking than when they were in pause. To understand if that's the case, we measured the randomness of heat stress received by a given fly. The randomness of heat stress can be quantified by measuring the difference between the likelihood of receiving heat

stress during walking and the likelihood of receiving heat stress during pause (Equation 3.1). This measurement will be referred to as the exposure differential, or ED. For example, if a yoked fly experiences heat stress 50% of the time during walking and 50% of the time during the pause, its' ED will be 0. If another yoked fly experiences heat stress 80% of the time during walking, and 30% of the time during the pause, its ED would be 0.5. If learning exist, we would expect yoked flies to show more negative activity difference (a negative Activity Difference) when they receive more heat stress during walking (with a more positive ED), or less negative activity change (a positive Activity Difference) when they receive less heat stress during walking (with a more negative ED).

$$ED = P(heated|walking) - P(heated|pause) \tag{3.1}$$

Our result shows that, during Train 1 session, the correlation between the yoked flies' ED and their change in activity level between Pre-Test and Test 1 showed a moderate but significant negative relationship (Figure 3.11). This indicates that a small amount of flies did change their behavior based on not-so-randomly occurring heat stress. During Train 2 session, the yoked flies' ED is still negatively correlated with their activity changes between Test 1 and Test 2, though the correlation coefficient is less negative than that of the Train 1 session (Figure 3.12). The reduced negative correlation seen in the Train 2 session ED and the activity difference between Test 1 and Test 2 is not surprising. This is likely due to that many training flies have become completely stationary during Train 2 and do not trigger any heat stress, resulting in the yoked flies receiving no heat stress during walking or during a pause. These flies' ED, together with the yoked flies receiving truly random heat stress, would both be 0. Therefore, the correlation between ED and activity difference is affected by the two different groups of yoked flies for the Train 2 session. Our results show that some yoked flies indeed seemed to have a higher likelihood of experiencing heat stress during walking than during the pause, especially during the two Train sessions. These flies, in turn, show a greater activity level decrease. For these flies, a significant negative correlation exists

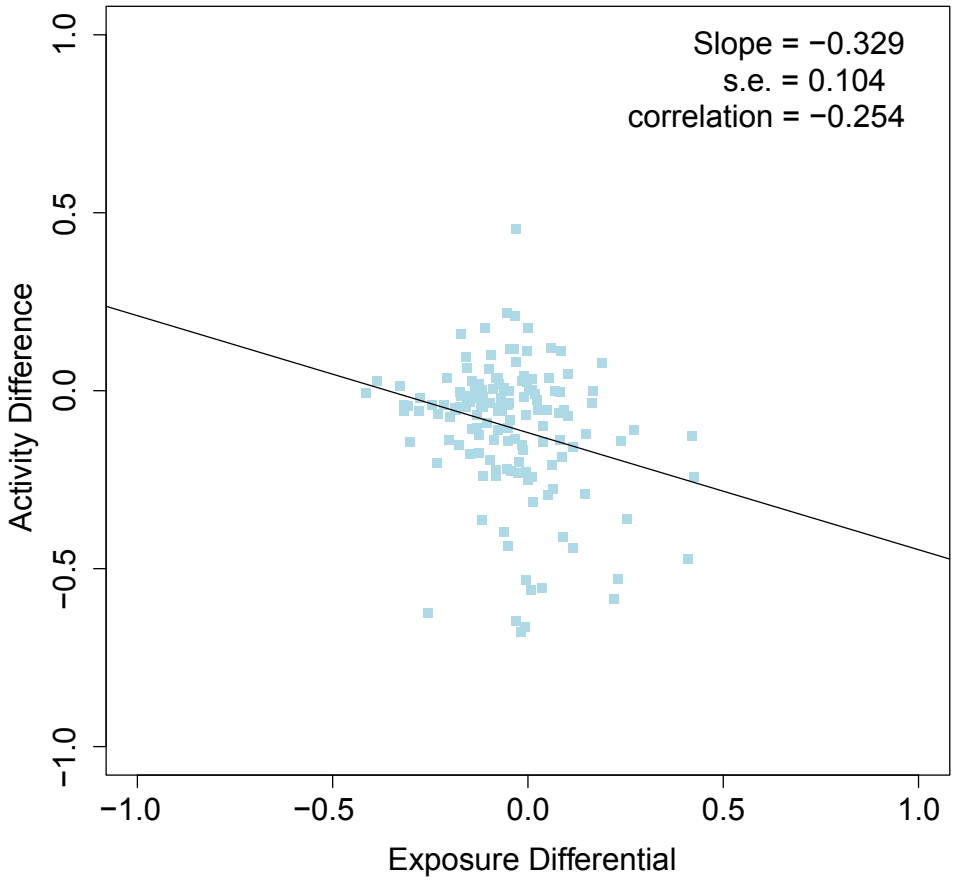


Figure 3.11: The relationship between ED and activity difference of yoked flies during Train 1 session

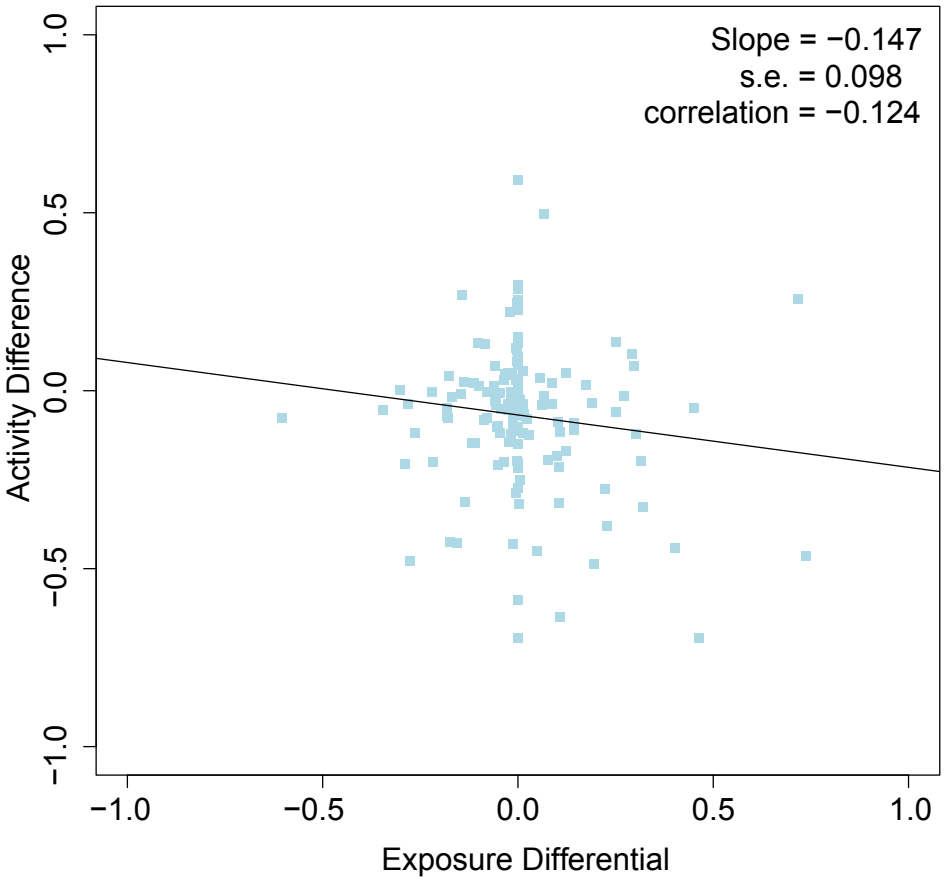


Figure 3.12: The relationship between ED and activity difference of yoked flies during Train 2 session

between the change in activity level and ED. Thus, the observed activity level decrease in some yoked flies' may be due to their experiencing, by chance, more heat stress when they were walking than when they were in pause, indicating that anti-instinctive learning in the *LaserSync* can occur when the ED is positive. This result, together with previous results, further validates the flies' ability to perform anti-instinctive learning when they experience more heat stress during walking than during the pause.

3.4 Acknowledgment

Chapter 3 includes materials from the following article, currently being prepared for submission for publication. This dissertation author is the first author of the paper.

Sun, Ruichen; Delly, Joseph; Sereno, Emily; Wong, Sean; Huang, Yan; Chen, Xinyu; Wang, Yuxuan; Greenspan, Ralph. "Anti-Instinctive Learning Revealed by Locomotion-Triggered Heat Stress in *Drosophila*".

3.5 Appendix: Data analysis R code

See below for customized functions written in R for analyzing data. For complete scripts written for this study, see `https://github.com/Ruichensun/fly_source`.

```
# Computing a fly's transient speed in a recording
get_fly_moving_speed = function(x, framerate) {
  data_start = 31
  fly_pos = x[data_start:min(600 * framerate, length(x))]
  experiment_time = length(fly_pos) / framerate
  tot_moving_dist = sum(abs(diff(fly_pos)))
  return(tot_moving_dist * (47 / 768) / experiment_time)
}

# Computing a fly's initial pause duration of a recording
get_fly_initial_pause = function(x, framerate){
  data_start = 31
  fly_pos = x[data_start:min(600 * framerate, length(x))]
  experiment_time = length(fly_pos) / framerate
  fly_speed = diff(c(x[data_start - 1], fly_pos))
  pause = sum(fly_speed == 0) / framerate
  return(pause / experiment_time)
}

# Find all of the pauses a fly makes in a recording and obtain their time points and
    durations
get_pause_df = function(fly_pos, fly_speed){
  label_for_pause = rep(0, length(fly_pos))
  for (i in 2:length(label_for_pause)) {
    if ((fly_speed[i] == 0) & (fly_speed[i - 1] > 0)) {label_for_pause[i] = 1}
    else if ((fly_speed[i] > 0) & (fly_speed[i - 1] == 0)) {label_for_pause[i] = 2}
```

```
  else if ((fly_speed[i] < 0) & (fly_speed[i - 1] == 0)) {label_for_pause[i] = 3}
  else if ((fly_speed[i] == 0) & (fly_speed[i - 1] < 0)) {label_for_pause[i] = 4}
}

# Getting the index for the pause start and ends #
starts = c()
ends = c()
is_start = 1
if (fly_speed[1] == 0){
  starts = c(starts, 1)
  is_start = 0
}

for (i in 1:length(label_for_pause)) {
  if (label_for_pause[i] != 0) {
    if (is_start == 1) {
      if ((label_for_pause[i] == 1) | (label_for_pause[i] == 4)) {
        starts = c(starts, i)
        is_start = 0
      }
    }
    else{
      ends = c(ends, i)
      is_start = 1
    }
  }
}

if (length(starts) < 1) {
  starts = 1
}
pause_df = data.frame()

if (length(ends) < 1) {
  start_type = label_for_pause[starts]
  start_position = fly_pos[starts - 1]
  ends = length(fly_pos)
  end_position = fly_pos[ends]
  end_type = label_for_pause[ends]
  pause_df = data.frame(
    starts[1:length(ends)] - 1,
    start_position[1:length(ends)],
    start_type[1:length(ends)],
    ends,
    end_position,
    end_type,
    ends - starts[1:length(ends)]
  )
} else{
  if (length(starts) - length(ends) == 1){
    ends = c(ends, length(fly_pos))
  }
  end_type = label_for_pause[ends]
  start_type = label_for_pause[starts]
  if (starts[1] == 1){
    start_position = fly_pos[starts]
    startindex = starts[1:length(ends)]
  }else{
    start_position = fly_pos[starts - 1]
    startindex = starts[1:length(ends)] - 1
  }
  end_position = fly_pos[ends]
  pause_df = data.frame(
    startindex,
    start_position[1:length(ends)],
    start_type[1:length(ends)],
    ends - 1,
    end_position,
    end_type,
    ends - starts[1:length(ends)]
  )
}
colnames(pause_df) = c(
  "Start_Index",
  "Start_Position",
  "Start_Type",
```

```
    "End_Index",
    "End_Position",
    "End_Type",
    "Pause_Duration"
  )
  return(pause_df)
}

# Quantifications of a fly's walking behaviors in a recording
one_fly_statistics = function(input_file,
                              framerate = 50,
                              speed_max_thres = 30,
                              speed_zero_thres = 1e-2,
                              pause_frame_thres = 25,
                              chamber_end_thres = 60,
                              chamber_left = 21,
                              chamber_right = 752
                              ){
  #  pause_frame_thres - Least of number of frames in a pause
  #  speed_zero_thres - How small the speed is to be treated as not moving (i.e. zero)
  #  speed_max_thres - Maximum speed allowed (The setting for this threshold is as follows:
  #  The distance of centers of adjacent pixels is 63.5um
  #  A fly needs 5-6 seconds to travel from one end of the tube to the other end without
     pause (in a 50fps setting, 250 - 300 frames ==> 5-6sec)
  #  The tube length is 48.7mm.
  #  So the average walking speed is 48.7mm/5sec = 9.74mm/s or 48.7mm/6sec = 8.11mm/s
  #  Between two adjacent frames (which are 0.02s apart in a 50fps setting), the possible
     transient speed is 8.11mm/s x 0.02s = 0.1622mm

     or 9.74mm/s x 0.02s = 0.1948mm
  #  This transient displacement translates to 0.1622(mm)/63.5um = 2.55px, or 0.1948mm/63.5
     um = 3.06px
  #  Therefore, the fly typically moves 2-3px per 0.02sec.
  #  Therefore, I set the maximum speed threshold in 0.02s duration to be 30px (10 times the
      usual speed)
  #  chamber_end_thres - How close (in ) a position to one end of the chamber to be treated
     as part of the end

  # Get File Info

  file_path = unlist(strsplit(input_file, "/"))
  Experimenter = file_path[2]
  file_name = unlist(strsplit(file_path[4], "_"))
  file_session = file_name[3]
  genotype = unlist(strsplit(file_name[4],".csv"))[1]
  fly_num = as.integer(unlist((strsplit(file_name[2], "Fly")))[2])

  # Load data

  tryCatch({
    x = read.table(input_file, header = T, sep = ",", stringsAsFactors = F)
  }, error = function(e) {
    stop(paste0("Input file is empty!:\n","  Input files is: ", input_file, "\n\n"))
  })
  if (nrow(x) < 10) {stop(paste0("Input file is empty!:\n", "  Input files is: ", input_file
      , "\n\n"))}
  x = as.numeric(x[[1]])
  data_start = 31
  fly_pos = x[data_start:min(600 * framerate, length(x))]
  set_time = length(fly_pos)
  experiment_time = length(fly_pos)

  # Remove system noise using speed threshold
  fly_speed = diff(c(x[data_start - 1], fly_pos))
  for (i in 1:length(fly_pos)) {
    if (abs(fly_speed[i]) >= speed_max_thres) {
      fly_speed[i] = 0
    }
  }

  # Activity
  pause_df = get_pause_df(fly_pos, fly_speed)

  # Real pause is the pauses with duration longer than pause_frame_thres
  real_pause_df = subset(pause_df, Pause_Duration >= pause_frame_thres)

  # Pauses not at the end
```

```
mid_pause_df = subset(real_pause_df, (Start_Position >= chamber_end_thres) & (
    Start_Position <= 767 - chamber_end_thres))
pause_middle_dur = mid_pause_df$Pause_Duration

num_pause = length(real_pause_df$Start_Index)
num_mid_pause = length(mid_pause_df$Start_Index)

md_pause_dur = median(real_pause_df$Pause_Duration) / framerate
md_pause_middle_dur = (median(pause_middle_dur)) / framerate
avg_pause_dur = mean(real_pause_df$Pause_Duration) / framerate
avg_pause_middle_dur = (mean(pause_middle_dur)) / framerate

frac_pause = sum(real_pause_df$Pause_Duration) / experiment_time
frac_pause_middle = (sum(pause_middle_dur)) / experiment_time

max_pause = max(real_pause_df$Pause_Duration) / framerate
max_pause_middle = (max(pause_middle_dur)) / framerate

# First_pause_duration: first real pause or first real pause not at the end
first_pause = real_pause_df$Pause_Duration[1] / framerate
first_pause_middle = (pause_middle_dur[1]) / framerate

# Turns

# Step 0 - smoothing
ma = function(x, bin_size){filter(x, rep(1/bin_size, bin_size), sides=2)}
bin_size = 150
fly_pos_sm = ma(fly_pos, bin_size)
fly_speed_sm = diff(fly_pos_sm)

# Step 1 - get the moving direction (speed sign)
bin_positive_frac = NULL
t = bin_size
while (t < experiment_time) {
  bin_fly_speed = fly_speed_sm[t - 1:bin_size + 1]
  frac = sum(bin_fly_speed > 0, na.rm = T) / sum(bin_fly_speed != 0, na.rm = T)
  bin_positive_frac = c(bin_positive_frac, frac)
  t = t + bin_size
}

# Step 2 - get the turns
turns = find_intersect_points(bin_positive_frac, rep(0.5, length(bin_positive_frac)))
turns = ceiling(turns * bin_size - bin_size / 2)
position_turns = (fly_pos[turns] + fly_pos[turns - 1]) / 2 # Use original fly_pos value as
     the actual position
mid_turns = turns[position_turns > chamber_end_thres & position_turns < 767 -
    chamber_end_thres]
position_mid_turns = position_turns[position_turns > chamber_end_thres & position_turns <
    767 - chamber_end_thres]

# Step 3 - get turn numbers
num_turns = length(turns)
num_mid_turns = length(mid_turns)

if (num_turns == 0){
  frac_mid_turns = 0
} else{
  frac_mid_turns = num_mid_turns / num_turns
}

#Return output

ret = data.frame(cbind(
  Experimenter,
  genotype,
  fly_num,
  file_session,
  num_pause,
  num_mid_pause,
  1 - frac_pause, #unit: percentage
  1 - frac_pause_middle,
  md_pause_dur,
  md_pause_middle_dur,
  max_pause,
  max_pause_middle,
  first_pause,
  first_pause_middle,
  num_turns,
```

```
    num_mid_turns,
    frac_mid_turns,
    avg_pause_dur,
    avg_pause_middle_dur
  ), stringsAsFactors=FALSE)

  colnames(ret) = c(
    "experimenter", #1
    "genotype", #2
    "flynum", #3
    "session", #4
    "num of pause", #5
    "num of middle pause", #6
    "percentage time active", #7
    "percentage time active - pause not at the end", #8
    "median pause duration",#9
    "median middle mause duration", #10
    "max pause duration", #11
    "max middle pause duration", #12
    "first pause duration", #13
    "first middle pause duration", #14
    "number of turns",#15
    "number of middle turns",#16
    "fraction of middle turns out of total turns",#17
    "average pause duration", #18
    "average middle pause duration" #19
  )
  return(ret)
}

# Find intersect points in order to define a turn
find_intersect_points = function(x1, x2){
  ##Adapted from code by nograpes
  ##http://stackoverflow.com/questions/20519431/finding-point-of-intersection-in-r
  ## Find points where x1 is above x2.
  above = x1 > x2
  ## Points always intersect when above=TRUE, then FALSE or reverse
  intersect.points = which(diff(above) != 0)
  ## Find the slopes for each line segment.
  x1.slopes = x1[intersect.points + 1] - x1[intersect.points]
  x2.slopes = x2[intersect.points + 1] - x2[intersect.points]
  ## Find the intersection for each segment.
  x.points = intersect.points + ((x2[intersect.points] - x1[intersect.points]) / (x1.slopes
      - x2.slopes))
  return(x.points)
}

shuffle_is_pause = function(is_pause) {
  if (max(is_pause) == 0) {
    return(is_pause)
  }
  r = rle(is_pause)
  ## Prepare sequence for shuffling
  folded_is_pause = NULL
  for (i in 1:length(r$values)) {
    if (r$values[i] == 1) {
      ## Pause
      ## Fold
      folded_is_pause = c(folded_is_pause, i)
    } else{
      ## Move
      ## Do not fold
      folded_is_pause = c(folded_is_pause, rep(0, r$lengths[i]))
    }
  }
}

# Find the yoked flies for every training fly
Use_T_find_R = function(fly.info, Tindex){
  if (fly.info[Tindex, ]$Category != "T"){
    return(c())
  }else{
    Rlst = c()
    setup_T = fly.info[Tindex, ]$Setup
    for (i in ((Tindex - setup_T + 1):(Tindex + 4 - setup_T))){
      if ((i < 1) | (i > nrow(fly.info))){
        next
      }
```

```
      if (fly.info[i, ]$Category == "R" &
          fly.info[i, ]$Genotype == fly.info[Tindex, ]$Genotype &
          fly.info[i, ]$Exp.date == fly.info[Tindex, ]$Exp.date &
          fly.info[i, ]$Experimenter == fly.info[Tindex, ]$Experimenter &
          fly.info[i, ]$Setup != fly.info[Tindex, ]$Setup){
        Rlst = c(Rlst, i)
      }
    }
    return (Rlst)
  }
}

# Find the blank flies for every training fly
Use_T_find_N = function(fly.info, Tindex){
  if (fly.info[Tindex, ]$category != "T"){
    return(c())
  }else{
    Nlst = c()
    setup_T = fly.info[Tindex, ]$setup
    for (i in ((Tindex - setup_T + 1):(Tindex + 4 - setup_T))){
      if ((i < 1) | (i > nrow(fly.info))){
        next
      }
      if (fly.info[i, ]$category == "N" &
          fly.info[i, ]$genotype == fly.info[Tindex, ]$genotype &
          fly.info[i, ]$exp_date == fly.info[Tindex, ]$exp_date &
          fly.info[i, ]$experimenter == fly.info[Tindex, ]$experimenter &
          fly.info[i, ]$setup != fly.info[Tindex, ]$setup){
        Nlst = c(Nlst, i)
      }
    }
    return (Nlst)
  }
}

# Computing the laser exposure details of a fly during Train sessions
get_laser_df = function(fly_laser, framerate){
  laser_ON  = rle(fly_laser)$length[rle(fly_laser)$values == 1]
  laser_OFF = rle(fly_laser)$length[rle(fly_laser)$values == 0]
  label_for_laser = rep(0, length(fly_laser))
  for (i in 1:(length(label_for_laser) - 1)) {
    if ((fly_laser[i] == 0) & (fly_laser[i + 1] > 0)) {
      label_for_laser[i + 1] = 1
    }
    if ((fly_laser[i] > 0) & (fly_laser[i + 1] == 0)) {
      label_for_laser[i + 1] = 2
    }
  }
  laser_df = data.frame()
  if (laser_OFF[1] == length(fly_laser)) {
    laser_df = data.frame(0,
                          0,
                          0,
                          (laser_OFF[1]) / framerate,
                          (laser_OFF[1] - 0) / framerate,
                          TRUE)
  } else{
    laser_df = data.frame (which(label_for_laser == 1),
                           which(label_for_laser == 2),
                           laser_ON / framerate,
                           (laser_OFF[2:length(laser_OFF)]) / framerate,
                           (laser_OFF[2:length(laser_OFF)] - laser_ON) / framerate,
                           laser_OFF[2:length(laser_OFF)] >= 8 * 60 * framerate
    )
  }
  colnames(laser_df) = c(
    "Laser_On",
    "Laser_Off",
    "ON_duration",
    "OFF_duration",
    "Difference",
    "eight_min_OFF"
  )
  return(laser_df)
}
```

```
# Computing the amount of laser exposure a fly receives during the Train sessions based on
    the get_laser_df function
one_fly_laser_statistics = function(input_file, framerate){
  file_path = unlist(strsplit(input_file, "/"))
  Experimenter = file_path[2]
  file_name = unlist(strsplit(file_path[4], "_"))
  file_session = file_name[3]
  genotype = unlist(strsplit(file_name[4],".csv"))[1]
  fly_num = as.integer(unlist((strsplit(file_name[2], "Fly")))[2])

  fly.file = read.csv(input_file, header = T, stringsAsFactors = F)
  fly.position.raw = as.numeric(fly.file[[1]])
  fly.laser.raw = as.numeric(fly.file[[2]])
  if (is.na(fly.laser.raw[1]) == T) {
    number_of_laser_clicks = -1
    total_laser_ON = -1
    ret = data.frame(cbind(
      Experimenter,
      genotype,
      fly_num,
      file_session,
      number_of_laser_clicks,
      total_laser_ON #in seconds
    ))
    colnames(ret) = c(
      "experimenter",
      "genotype",
      "flynum",
      "session",
      "laser_count",
      "laser_exposure"
    )
    return(ret)
  }else{
    data_start = 31
    fly_pos = fly.position.raw[data_start:length(fly.position.raw)]
    fly_laser = fly.laser.raw[data_start:length(fly.laser.raw)]
    if (fly_laser[length(fly_laser)] > 0) {
      fly_laser[length(fly_laser)] = 0
    }
    if (fly_laser[1] > 0) {
      fly_laser[1] = 0
    }
    for (i in 1:length(fly_laser)) {
      if (fly_laser[i] > 0) {
        fly_laser[i] = 1
      }
    }
    laser_df = get_laser_df(fly_laser, framerate)
    number_of_laser_clicks = length(laser_df$Laser_On)
    total_laser_ON = sum(laser_df$ON_duration)
    if (number_of_laser_clicks == 1) {
      if (laser_df$ON_duration == 0) {
        number_of_laser_clicks = 0
      }
    }
    ret = data.frame(cbind(
      Experimenter,
      genotype,
      fly_num,
      file_session,
      number_of_laser_clicks,
      total_laser_ON
    ))
    colnames(ret) = c(
      "experimenter",
      "genotype",
      "flynum",
      "session",
      "laser_count",
      "laser_exposure")
    return(ret)
  }
}

#Quantify percentage of time being hit by laser both during walking and during pause
```

```
chance_of_being_hit_by_laser = function(input_file){

  fly.file = read.csv(input_file, header = T, stringsAsFactors = F)
  fly.position.raw = fly.file$fly_pos.framerate.50
  fly.laser.raw = fly.file$laser_status
  # If laser status not recorded, return NA
  if (is.na(fly.laser.raw[1]) == T) {
    return(c(NA,NA,NA))
  }else{
    fly.moving.status.raw = fly_pos_to_moving_status(fly.position.raw)
    starting_point = 21
    fly.position = fly.position.raw[starting_point:length(fly.position.raw)]
    fly.laser.status = fly.laser.raw[starting_point:length(fly.laser.raw)]
    fly.moving.status = fly.moving.status.raw[(starting_point-1):length(fly.moving.status.
        raw)]
    if (sum(fly.position.raw)==0){
      fly.moving.status = rep(0, length(fly.moving.status))

    }else{
      for (i in 1:length(fly.moving.status)){
        if (fly.position[i] < 60){
          fly.moving.status[i] = 1
        }
        if (fly.position[i] > 708){
          fly.moving.status[i] = 1
        }
      }
    }
    moving_status_summary = rle(fly.moving.status)

    total_frame_moving = sum(moving_status_summary$lengths[moving_status_summary$values!=0])
    total_frame_pause = sum(moving_status_summary$lengths[moving_status_summary$values==0])

    moving_laser_status.df = data.frame(fly.moving.status,fly.laser.status)
    moving_status_during_laser_ON =
      rle(moving_laser_status.df$fly.moving.status[moving_laser_status.df$fly.laser.status
          !=0])

    moving_laser_ON = sum(moving_status_during_laser_ON$lengths[
        moving_status_during_laser_ON$values==1])
    pause_laser_ON = sum(moving_status_during_laser_ON$lengths[
        moving_status_during_laser_ON$values==0])

    if (total_frame_moving == 0){
      chance_of_being_hit_by_laser_during_moving = 0
      chance_of_being_hit_by_laser_during_pause = pause_laser_ON/total_frame_pause
      laser_on_percentage =
        length(moving_laser_status.df$fly.moving.status[moving_laser_status.df$fly.laser.
            status!=0])/
        length(fly.moving.status)
    }else{
      chance_of_being_hit_by_laser_during_moving = moving_laser_ON/total_frame_moving
      chance_of_being_hit_by_laser_during_pause = pause_laser_ON/total_frame_pause
      laser_on_percentage =
        length(moving_laser_status.df$fly.moving.status[moving_laser_status.df$fly.laser.
            status!=0])/
        length(fly.moving.status)
    }
    return(c(chance_of_being_hit_by_laser_during_moving,
             chance_of_being_hit_by_laser_during_pause,
             laser_on_percentage))
  }
}

# Computing all the flies' chance of being hit by types (T/R)
Hit_by_laser = function(file_name_filter, fly.info.movement) {
  laser_chance = data.frame()
  for (ind in 1:nrow(fly.info.movement)) {
    if(fly.info.movement$genotype[ind]=="WT"){
      input.file = list.files(
        path = paste0("data/",fly.info.movement$experimenter[ind],"/CS/"),
        pattern = paste0("ProcessedData_Fly", fly.info.movement$fly[ind], "_",
            file_name_filter, "_WT", ".csv"),
        full.names = T)
      if(length(input.file)==0){next()}
```

```
    }else if(fly.info.movement$genotype[ind]=="CS"){
      input.file = list.files(
        path = paste0("data/", fly.info.movement$experimenter[ind], "/mutants/"),
        pattern = paste0("ProcessedData_Fly", fly.info.movement$fly[ind], "_",
            file_name_filter, "_CS", ".csv"),
        full.names = T)
      if(length(input.file)==0){next()}
    }else{
      input.file = list.files(
        path = paste0("data/", fly.info.movement$experimenter[ind], "/mutants/"),
        pattern = paste0("ProcessedData_Fly", fly.info.movement$fly[ind], "_",
            file_name_filter,"_", fly.info.movement[ind,]$genotype, ".csv"),
        full.names = T)
      if(length(input.file)==0){next()}
      print(input.file)
    }
    laser_profile = chance_of_being_hit_by_laser(input.file)
    print(laser_profile)
    temp = cbind(fly.info.movement[ind, ], laser_profile[1], laser_profile[2], laser_profile
        [3])
    laser_chance = rbind(laser_chance, temp)
  }
  names(laser_chance) = c(names(fly.info.movement[ind, ]), "Hit_W", "Hit_P", "Hit_All")
  return(laser_chance)
}

#Calculating all the flies' chance of being hit by types (T/R)
fly_pos_to_moving_status = function(fly_pos){
  # This is determined by quantile(abs(fly_moving_status),c(0.97, 0.975, 0.98)), and the
      97.5%
  # corresponds to 28.6
  speed_threshold = 30
  fly_moving_status = diff(fly_pos)
  # Finding out the fly's moving status by two criteria: velocity = 0 or velocity much
      larger than
  # a speed threshold
  fly_moving_status_discretized = replace(fly_moving_status, fly_moving_status >
                                            speed_threshold, 0)
  fly_moving_status_discretized = replace(fly_moving_status_discretized,
                                          fly_moving_status_discretized != 0,
                                          1)
  return(fly_moving_status_discretized)
}
# Labelling each frame from a recording of a fly's walking behavior as walking or pause
moving_status = function(input_file) {
  a = read.csv(input_file, header = T, stringsAsFactors = F)
  if(is.na(a$laser_status[1])){
    return(NA)
  }else{
    fly_pos = a$fly_pos.framerate.50
    fly_moving_status = fly_pos_to_moving_status(fly_pos)
    starting_point = 31
    fly_moving_status = fly_moving_status[(starting_point-1):length(fly_moving_status)]
    return(cumsum(fly_moving_status))
  }
}

# Get all CS flies' cumulated moving status grouped by types
get_sequence_length = function(file_name) {
  if (sum(is.na(moving_status(file_name)))==0){
    return (length(moving_status(file_name)))
  }else{return(NA)}
}

# Computing the cumulative activity duration of a group of flies
get_cumsums_total = function(file_name_filter, fly.info.movement) {
  file_names = c()
  for (ind in 1:nrow(fly.info.movement)) {
    if (fly.info.movement$Genotype[ind] == "WT"){
      input.file = list.files(
        path = paste0("data/", fly.info.movement$Experimenter[ind], "/CS/"),
        pattern = paste0("ProcessedData_Fly", fly.info.movement$Fly[ind], "_",
                         file_name_filter, "_", fly.info.movement$Genotype[ind], ".csv"),
        full.names = T)
    } else if (fly.info.movement$Genotype[ind] == "CS"){
```

```
      input.file = list.files(
        path = paste0("data/", fly.info.movement$Experimenter[ind], "/mutants/"),
        pattern = paste0("ProcessedData_Fly", fly.info.movement$Fly[ind], "_",
                         file_name_filter, "_", fly.info.movement$Genotype[ind], ".csv"),
        full.names = T)
    }

    if(length(input.file)==0){
      print( paste0("ProcessedData_Fly", fly.info.movement$Fly[ind], "_",
                    file_name_filter, "_", fly.info.movement$Genotype[ind], ".csv"))
      next()
    }
    if (!is.na(get_sequence_length(input.file))){
      # print(input.file)
      file_names = c(file_names, input.file)
    }else{
      next()
    }
  }
  # Get min sequence length
  sequence_lengths = unlist(lapply(file_names, get_sequence_length))
  min_sequence_length = min(sequence_lengths)
  # Concat cumsums to matirx
  cumsums = matrix(nrow = min_sequence_length, ncol = 0)
  for (file_name in file_names) {
    cumsums = cbind(cumsums, moving_status(file_name)[1:min_sequence_length])
  }
  return(cumsums)
}

# Bootstrapping the 95% confidence interval from raw data
get_Wald_CI = function(data){
  Mboot = boot(data,
               function(x,i) median(x[i]),
               R=10000)

  CI = boot.ci(Mboot,
               conf = 0.95,
               type = c("norm")
  )
  return(c(CI$t0, CI$normal[2], CI$normal[3]))
}
```

Chapter 4

The Mechanisms of Anti-Instinctive Learning in Fruit Flies

4.1 Introduction

Over the past several decades, many genetic and molecular tools have been developed for *Drosophila melanogaster* [135, 76, 108, 42, 34, 80, 62, 6]. These tools allow researchers to interrogate the cellular and molecular mechanisms underlying a biological phenomenon. As an example, our understanding of molecular and cellular processes underlying the olfactory learning and memory behavior of fruit flies were built upon these powerful tools [119, 143, 149, 62, 7, 6]. And using the spatially targeted manipulation of specific genes, different genes and neurons have been identified in the learning and memory process [149, 103, 164, 163, 6]). Using these molecular and genetic tools, in this chapter, we investigate what the neural circuits and molecules are involved in fruit fly's anti-instinctive learning behavior. Particularly, this exploratory investigation focuses on a neurotransmitter, dopamine, and two central neuropils in the fly brain, ellipsoid body and mushroom bodies, as they have been reported to be involved in various learning paradigms.

4.1.1 Dopamine

Dopamine is an evolutionarily-conserved neurotransmitter capable of modifying motor behaviors [66, 13]. In higher organisms, dopamine is also involved in behaviors such as reward-seeking, executive control, mood regulation, and learning [113, 10, 154, 96]. Previous studies have found that the dopamine signaling pathway is highly conserved between the fruit fly's brain and mammals, and that fruit flies also employ dopamine for a variety of behaviors, including learning [161, 148, 121, 18, 150].

Four types dopamine receptors are found in the fly's brain: dopamine 1-like receptor 1 (Dop1R1), dopamine 1-like receptor 2 (Dop1R2), dopamine 2-like receptor (Dop2R), and dopamine/ecdysteroid receptor (DopEcR) [49]. All 4 types of receptors are expressed in the mushroom bodies[31]. Previous studies showed that Dop1R1 is involved in aversive and appetitive olfactory learning, arousal level regulation, and temperature preference behaviors [70, 46, 11]. Dop1R2 plays a role in olfactory memory formation and courtship drive [18, 166]. Dop2R has been reported to be important in memory formation and olfactory learning [36, 112, 102]. Lastly, DopEcR modulates memories of courtship conditioning and sensitization to ethanol [57, 100, 54]. Given the ample evidence of the importance of dopamine receptors in learning, it is worth exploring the roles of dopamine receptors in the anti-instinctive learning process.

4.1.2 Ring Neurons in the Ellipsoid Body

The ellipsoid body (EB), located in the central complex of the fly brain, is a donut-shaped structure that has been found involved in visual and spatial orientation memory, maintaining heading direction, and flight control [74, 114, 56, 156, 165]. The EB can be further divided into rings with 4 ring-like layers: R1, R2, R3, R4 [47], or into 16 wedges that tile the entire EB. As anti-instinctive learning behavior involves changes in locomotion, whether the EB is involved in the locomotion changes seen in the training flies will be important to our understanding of the neural mechanisms underlying anti-instinctive learning.

4.1.3 Mushroom Bodies γ Lobes

The mushroom bodies (MB), known for their crucial role in associative memory in fruit flies, are a pair of mushroom-shaped neuropils located at the center of the fly's brain [51, 30, 86, 7]. Each of the two MBs is comprised of Kenyon cells whose axons form three distinct lobes within MB: α/β, α'/β', and γ lobes [58, 29, 7]. All three lobes have been reported to be involved in associative memory. Particularly, the neurons in the γ lobes have been found to mediate aversive associative olfactory learning [103]. As the anti-instinctive learning paradigm (described in Chapter 3) involves recurring aversive stimuli (mild heat stress), it would be interesting to see whether neurons in γ lobes are involved in the process of anti-instinctive learning. In γ lobes of MB, two layers exist: main layer and dorsal (d) layer [7]. The γ main layer processes olfactory signals that are relayed from the antennal lobes' projection neurons (which receive information from olfactory sensory neurons in the antennae), while inputs to the γ d layer have been largely elusive [7]. If γ lobes are required for flies to perform anti-instinctive learning, it is then necessary to investigate which part of the γ lobes are responsive.

4.2 Materials and Methods

4.2.1 Fly Strains

For details about fly rearing, see the methods section 3.2.1. A complete list of transgenic flies and their origins are listed in Table 4.1. In the 4.1, BDSC stands for Bloomington *Drosophila* Stock Center and JFRC stands for Janelia Farm Research Campus.

4.2.2 Immunohistochemistry and Confocal Imaging

Adult fly brains are dissected following a previously described protocol [158]. The dissected brains are subsequently stained according to the Janelia Farm Research Campus' FlyLight IHC-Anti-GFP protocol (`https://www.janelia.org/project-team/flylight/`

Table 4.1: Fly strains

Figure	Genotype	Source
4.1	$D2R^{f06521}$/;+/+; $DopR1^{attP}$,$DopEcR^{c02142}$/ $DopR1^{attP}$,$DopEcR^{c02142}$	Michael Crickmore lab
4.2	$Dop1R1^{attP}$/$Dop1R1^{attP}$	Michael Crickmore lab
4.3	nSyb-Gal4/UAS-Dop2R-RNAi	BDSC (51635, 78804)
4.4	nSyb-Gal4/10XUAS-IVS-myr::GFP	BDSC (51635, 32197)
4.5	$Dop1R2^{attP}$/$Dop1R2^{attP}$	Michael Crickmore lab
4.6, 4.7	R60D05-Gal4/10XUAS-IVS-myr::GFP	BDSC (39247, 32197)
4.8	R60D05-Gal4/UAS-Kir2.1	BDSC(39247), Greenspan lab
4.9	189Y-Gal4/10XUAS-IVS-myr::GFP	Greenspan lab, BDSC (32197)
4.10	189Y-Gal4/UAS-Kir2.1	Greenspan lab
4.11	MB009B-Gal4/10XUAS-IVS-myr::GFP	JFRC, BDSC (32197)
4.12	MB009B-Gal4/UAS-Kir2.1	JFRC, Greenspan lab
4.13	MB009B-Gal4/+	JFRC, Greenspan lab
4.14	+/UAS-Kir2.1	Greenspan lab
4.15	MB131B-Gal4/10XUAS-IVS-myr::GFP	JFRC, BDSC (32197)
4.16	MB131B-Gal4/UAS-Kir2.1	JFRC, Greenspan lab
4.17	MB131B-Gal4/+	JFRC, Greenspan lab
4.18	MB419B-Gal4/10XUAS-IVS-myr::GFP	JFRC, BDSC (32197)
4.19	MB419B-Gal4/UAS-Kir2.1	JFRC, Greenspan lab
4.20	MB419B-Gal4/+	JFRC, Greenspan lab
4.21	MB607B-Gal4/10XUAS-IVS-myr::GFP	JFRC, BDSC (32197)
4.22	MB607B-Gal4/UAS-Kir2.1	JFRC, Greenspan lab
4.23	MB607B-Gal4/+	JFRC, Greenspan lab

protocols). Brains were imaged immediately following staining with dual color (488 nm and 561 nm) using ZEISS LSM 800 with Airyscan system. Maximum intensity projection is used for all confocal images.

4.2.3 Statistical Analysis

The Kruskal–Wallis test followed by pairwise Wilcoxon rank sum tests (with Benjamini Hochberg correction) is used for all statistical comparisons. Confidence intervals are calculated using permutation tests with 10,000 permutations of the raw data. Interpretation of p values are shown in Table 3.1.

4.3 Results

4.3.1 Lack of Dopamine Receptors Alters Flies' Anti-Instinctive Learning

Dopamine receptors have been reported to be important in fruit fly olfactory learning [70, 18, 112, 57]. It is highly likely that dopamine receptors are also involved in anti-instinctive learning behaviors. To test this hypothesis, we tested the anti-instinctive learning behaviors in homozygous mutants of the Dop1R1, Dop2R, and DopEcR dopamine receptors. The triple mutants' walking ability was not affected by the disruption of the three dopamine receptor genes, as shown in the blank flies activity level during the Pre-Test and the Test 2 session (Figure 4.1). Interestingly, after 2 Train sessions, training flies with defective Dop1R1, Dop2R, and DopEcR receptors showed a higher activity level compared to yoked flies of the same genotype (Figure 4.1). As wild type training flies showed a more pronounced decrease in activity in Test 2 than their yoked counterparts, the reverse trend seen in the Dop1R1, Dop2R, and DopEcR triple mutants indicates that the abnormal learning behavior may be due to the lack of any of (or any combinations of) the three dopamine receptors.

Therefore, we set out to test the individual dopamine receptor's involvement in the

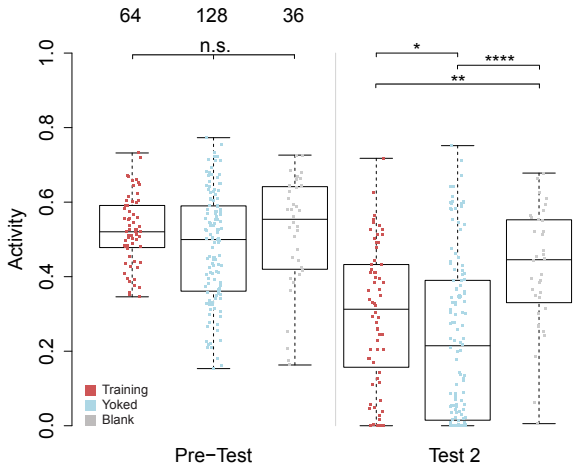


Figure 4.1: Dop1R1, Dop2R, DopEcR triple mutants' showed no significant learning

Table 4.2: P values from Dop1R1, Dop2R, DopEcR Triple Mutants Kruskal-Wallis Test, Followed by Mann-Whitney U Test with Benjamini-Hochberg correction

Test Groups	Pre-Test P Value	Test 2 P Value
Krusal-Wallis Test	0.08	7.754e-06
Training vs Yoked, Mann-Whitney U Test	N/A	0.0248
Training vs Blank, Mann-Whitney U Test	N/A	0.0017
Yoked vs Blank, Mann-Whitney U Test	N/A	1.8e-05

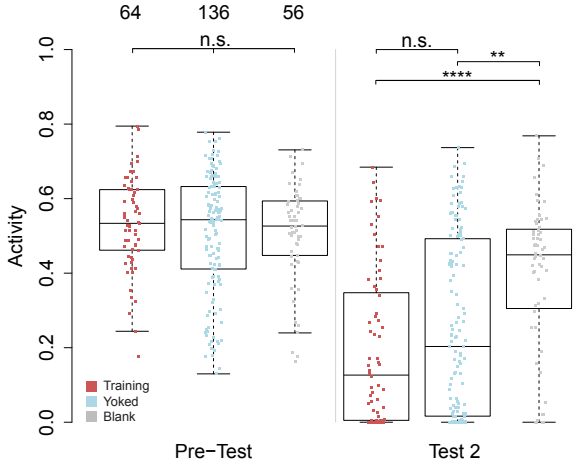


Figure 4.2: Dop1R1 mutants showed no significant learning

Table 4.3: P Values from Dop1R1 Single Mutants Kruskal-Wallis Test, Followed by Mann-Whitney U Test with Benjamini-Hochberg correction

Test Groups	Pre-Test P Values	Test 2 P Values
Krusal-Wallis Test	0.7427	3.607e-05
Training vs Yoked, Mann-Whitney U Test	N/A	0.1432
Training vs Blank, Mann-Whitney U Test	N/A	7.7e-06
Yoked vs Blank, Mann-Whitney U Test	N/A	0.0012

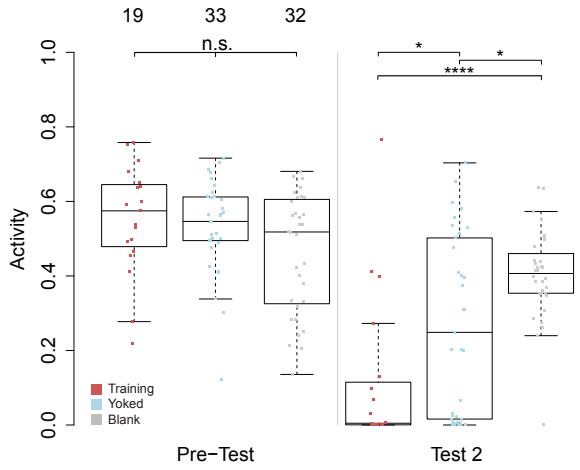


Figure 4.3: nSyb-Gal4/UAS-Dop2R-RNAi flies showed significant learning

Table 4.4: P Values from nSyb-Gal4/UAS-Dop2R-RNAi Flies Kruskal-Wallis Test, Followed by Mann-Whitney U Test with Benjamini-Hochberg correction

Test Groups	Pre-Test P Values	Test 2 P Values
Krusal-Wallis Test	0.1511	0.0001158
Training vs Yoked, Mann-Whitney U Test	N/A	0.028
Training vs Blank, Mann-Whitney U Test	N/A	4.4e-05
Yoked vs Blank, Mann-Whitney U Test	N/A	0.046

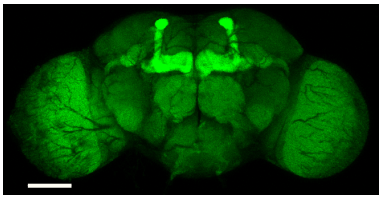


Figure 4.4: nSyb-Gal4 expression pattern. Green areas represent the green fluorescent protein (GFP) expressed in the targeted neurons. Scale bar: 100μm

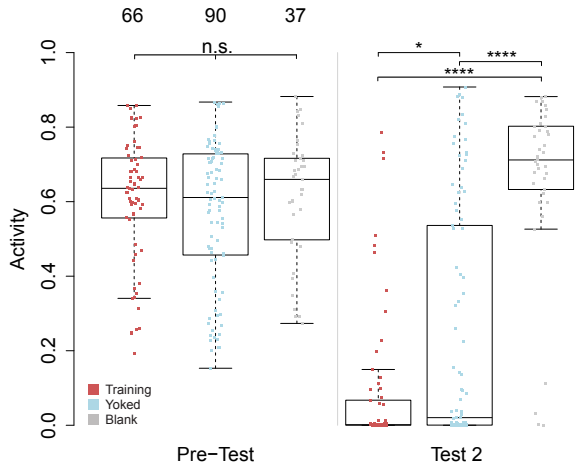


Figure 4.5: Dop1R2 mutants' normal anti-instinctive learning behavior

Table 4.5: P Values from Dop1R2 Single Mutants Kruskal-Wallis Test, Followed by Mann-Whitney U Test with Benjamini-Hochberg correction

Test Groups	Pre-Test P Values	Test 2 P Values
Krusal-Wallis Test	0.5273	3.607e-05
Training vs Yoked, Mann-Whitney U Test	N/A	0.014
Training vs Blank, Mann-Whitney U Test	N/A	8.8e-13
Yoked vs Blank, Mann-Whitney U Test	N/A	9.1e-09

anti-instinctive learning process. We first assayed the Dop1R1 homozygous mutants (Figure 4.2, previously described in [68]). The Dop1R1 training flies' activity level decrease after 2 Train sessions, similar to the pattern seen in the wild type fruit flies. Yet, Dop1R1 mutants' activity levels do not differ significantly with the Dop1R1 yoked flies' activity level either in the Pre-Test session and the Test 2 session. This indicates that disrupting the Dop1R1 gene may reduce learning phenotype seen in wild type flies.

We then used nSyb-Gal4/UAS-Dop2R-RNAi flies to test if using RNA interference technology to knock down the expression of Dop2R receptor will affect flies' anti-instinctive learning behavior (Figure 4.3, [36]). The nSyb-Gal4 targets all neurons in the fly brain, allowing the whole brain Dop2R expression knockdown when crossed with UAS-Dop2R-RNAi strains (Figure 4.4). Our result showed that the whole brain knockdown of Dop2R preferentially reduced the training flies' activity level, indicating that general knocking down Dop2R receptor in the fly brain does not affect anti-instinctive learning behavior (Figure 4.3).

As we do not have the DopEcR specific mutants in a wild type genetic background, it would be interesting to investigate the role of DopEcR in the anti-instinctive learning process.

In addition to the three dopamine receptors mentioned earlier, we also tested the Dop1R2 mutant' anti-instinctive learning behaviors. Dop1R2 mutant training flies' activity level is low after 2 Train sessions, and its yoked counterpart also shows low activity in Test 2 (Figure 4.5, Dop1R2 mutant previously described in [68]). In Test 2, activity levels of training Dop1R2 mutant flies and yoked Dop1R2 flies show a significant difference, similar to that of the wild type flies, indicating the lack of Dop1R2 receptors do not affect learning. It is interesting to note that the degree with of Dop1R2 reduce their activity level after experiencing 2 Train sessions are the largest among all dopamine receptor mutants tested. Thus, it would be interesting to see if the lack of Dop1R2 receptors affects renders the flies more sensitive to stress in general.

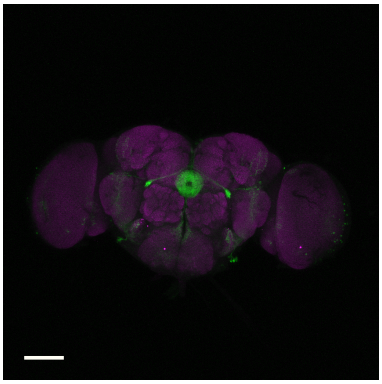


Figure 4.6: R60D05-Gal4 expression pattern (dorsal view). Magenta areas represent contour of the fly brain while green areas represent the green fluorescent protein (GFP) expressed in the targeted neurons. Scale bar: 100μm

4.3.2 Columnar Wedge Neurons and R3 Neurons in Ellipsoid Body Are Not Required for Anti-Instinctive Learning Behavior

Next, we investigated the role of the EB in anti-instinctive learning behavior by selectively expressing Kir2.1, an inward-rectifying potassium channel inhibiting normal functions of the neurons expressing it, in different regions of the EB using the R60D05-Gal4 and 189Y-Gal4 lines [20] (Figure 4.6, 4.7, 4.9). The R60D05-Gal4 targets the columnar wedge neurons whose dendrites tile all wedges in the EB [115, 62]. The 189Y-Gal4 targets the R3 neurons in EB [64]. The reason for choosing these two Gal4 lines is that most of the other EB-targeting Gal4 lines produce few or no viable progeny when crossed with UAS-Kir2.1 lines, presumably due to the importance of EB in development.

Both R60D05-Gal4/UAS-Kir2.1 and 189Y-Gal4/UAS-Kir2.1 flies show walking behavior in Pre-Test, and, in Test 2, the training flies' activity level is significantly lower than that of their yoked counterparts for both genotypes (Figure 4.8, 4.10). This results indicate that anti-instinctive learning behavior does not require participation from either the columnar wedge neurons or the R3 neurons in the EB.

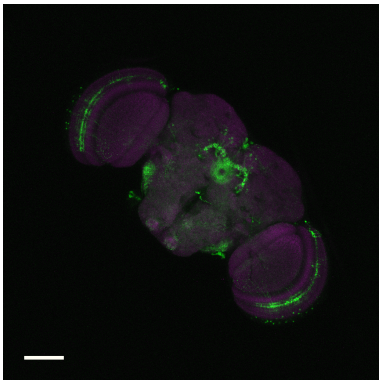


Figure 4.7: R60D05-Gal4 expression pattern (ventral view). Magenta areas represent contour of the fly brain while green areas represent the green fluorescent protein (GFP) expressed in the targeted neurons. Scale bar: 100μm

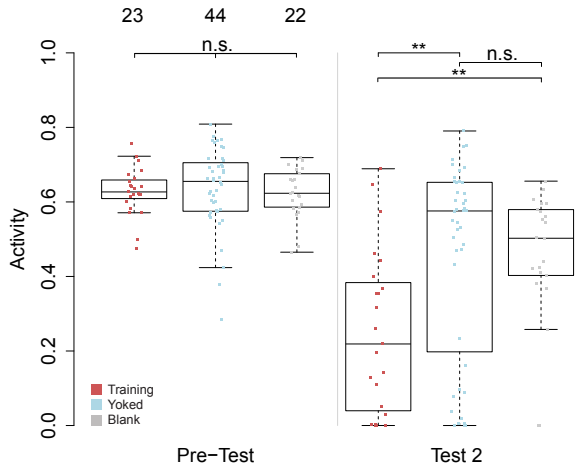


Figure 4.8: R60D05-Gal4/UAS-Kir2.1 flies showed significant learning

Table 4.6: P Values from R60D05-Gal4/UAS-Kir2.1 Flies Kruskal-Wallis Test, Followed by Mann-Whitney U Test with Benjamini-Hochberg correction

Test Groups	Pre-Test P Values	Test 2 P Values
Krusal-Wallis Test	0.6051	0.001556
Training vs Yoked, Mann-Whitney U Test	N/A	0.0031
Training vs Blank, Mann-Whitney U Test	N/A	0.0031
Yoked vs Blank, Mann-Whitney U Test	N/A	0.2254

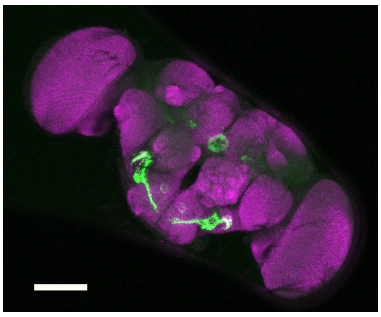


Figure 4.9: 189Y-Gal4 expression pattern. Magenta areas represent contour of the fly brain while green areas represent the green fluorescent protein (GFP) expressed in the targeted neurons. Scale bar: 100μm

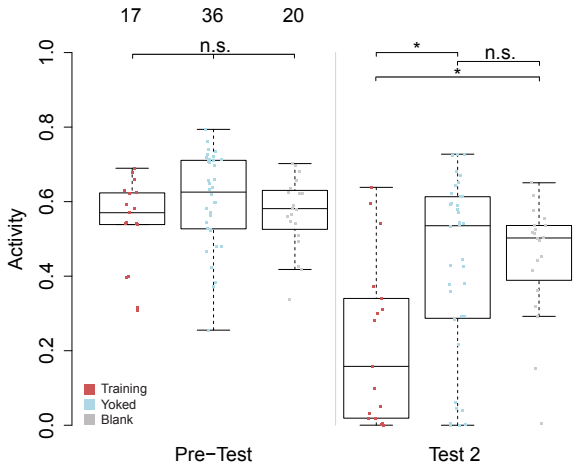


Figure 4.10: 189Y-Gal4/UAS-Kir2.1 flies showed significant learning

Table 4.7: P Values from 189Y-Gal4/UAS-Kir2.1 Flies Kruskal-Wallis Test, Followed by Mann-Whitney U Test with Benjamini-Hochberg correction

Test Groups	Pre-Test P Values	Test 2 P Values
Krusal-Wallis Test	0.113	0.0098
Training vs Yoked, Mann-Whitney U Test	N/A	0.01
Training vs Blank, Mann-Whitney U Test	N/A	0.01
Yoked vs Blank, Mann-Whitney U Test	N/A	0.60

4.3.3 Mushroom Bodies γ lobes' Role in Anti-Instinctive Learning Behavior is Unclear

To investigate whether MB γ lobes are involved in anti-instinctive learning behavior, we again employed the Gal4/UAS system and used four split Gal4 lines: MB009B-Gal4, MB131B-Gal4, MB419B-Gal4, and MB607-Gal4 from the Fly Light Split-Gal4 Driver Collection to selectively express Kir2.1, in the MB γ lobes (Table 4.1) [20, 7, 6]. Most of the previously described MB-targeting Gal4 lines were either not specific enough or their trans-heterozygous progenies with UAS-Kir2.1 were unhealthy or lethal, leaving split Gal4 lines as our only alternative in this project. Also, the anti-instinctive learning assay uses heat as a recurring stressor, thus methods requiring temperature control for disrupting neuronal function disruption (such as expressing *shibire* or dTrpA protein in targeted neurons), are not suitable for this study [44, 91]. Each of these split Gal4 transgenic fly lines targets a specific areas in the MB γ lobes in the fly brain, with MB009B-Gal4 and MB131B-Gal4 target the entire γ lobe (both the main and d layers), whereas MB419B-Gal4 and MB607B-Gal4 target only the main layer in the γ lobe [7] (Figure 4.11, 4.15, 4.18. 4.21). The specificity of these γ lobe neuron-targeting Gal4 lines enables local functional disruption in these neurons while leaving other brain areas intact. When the MB009B-Gal4/UAS-Kir2.1 trans-heterozygous files were tested in the *LaserSync* system, the training flies' activity is lower than that of the yoked flies in Test 2 (Figure 4.12). However, the two groups of flies do not differ significantly after receiving two Train sessions, indicating MB009B-Gal4/UAS-Kir2.1 flies' anti-instinctive learning ability is reduced compared to that of the wild type flies, whose training flies show significantly lower activity levels after training than their yoked counterparts. The functional disruption of neurons in the γ lobes is done via MB γ lobe-specific expression Gal4 proteins which leads to expression of Kir2.1 proteins in these neurons. Therefore, the reduction in learning may be due to the specific functional disruption mediated by Gal4 activated Kir2.1 expression in the entire γ lobes, or it could be due to expressing exogenous Gal4 proteins in the γ lobes alone. In order to find out which one is

Figure 4.11: MB009B-Gal4 expression pattern. Magenta areas represent contour of the fly brain while green areas represent the green fluorescent protein (GFP) expressed in the targeted neurons. Scale bar: 100μm

the case, we then assay the anti-instinctive learning behavior in MB009B-Gal4/+ heterozygous flies and +/UAS-Kir2.1 heterozygous flies (Figure 4.13, 4.14). The MB009B-Gal4/+ flies express Gal4 proteins in MB γ lobes without incurring functional disruption due to the lack of UAS-Kir2.1. The +/UAS-Kir2.1 flies do not express either Gal4 proteins or Kir2.1 proteins as it does not has the Gal4 transgene, and that the Kir2.1 expression can only be activated by Gal4 protein [20].

MB009B-Gal4/+ heterozygous flies showed no learning phenotype, while +/UAS-Kir2.1 flies show normal learning behavior (Figure 4.13, 4.14). This result indicates that expression of Gal4 transgene in the MB γ lobe neurons has a dominant effect, i.e. affecting the normal function of the MB γ lobe neurons, while the UAS-Kir2.1 transgene does not affect the phenotype of interest. Therefore, our results indicate that the heterozygous MB009B-Gal4/+ is not a suitable control.

We then tested the anti-instinctive learning behavior of MB131B-Gal4/UAS-Kir2.1, MB419B-Gal4/UAS-Kir2.1, and MB607B-Gal4/UAS-Kir2.1 flies, along with their respective controls: MB131B-Gal4/+, MB419B-Gal4/+, and MB607B-Gal4/+ flies. In all 6 strains of flies, no anti-instinctive learning behaviors was found (Figure 4.16, 4.17, 4.19, 4.20, 4.22, 4.23). These results, taken together, indicates that the role of MB γ lobes in anti-instinctive learning behavior requires further investigation.

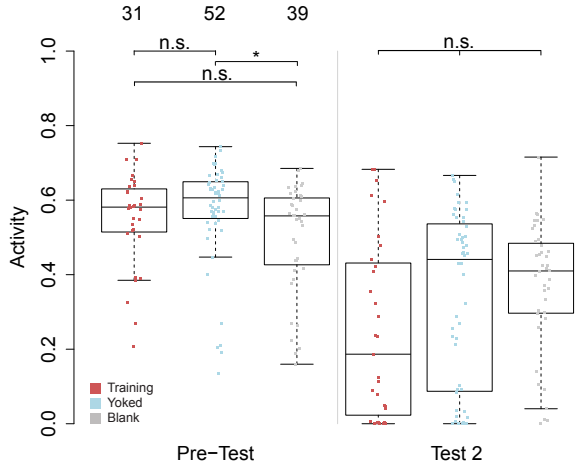


Figure 4.12: MB009B-Gal4/UAS-Kir2.1 flies showed no learning

Table 4.8: P Values from MB009B-Gal4/UAS-Kir2.1 Flies Kruskal-Wallis Test, Followed by Mann-Whitney U Test with Benjamini-Hochberg correction

Test Groups	Pre-Test P Values	Test 2 P Values
Krusal-Wallis Test	0.02411	0.09253
Training vs Yoked, Mann-Whitney U Test	0.269	N/A
Training vs Blank, Mann-Whitney U Test	0.269	N/A
Yoked vs Blank, Mann-Whitney U Test	0.018	N/A

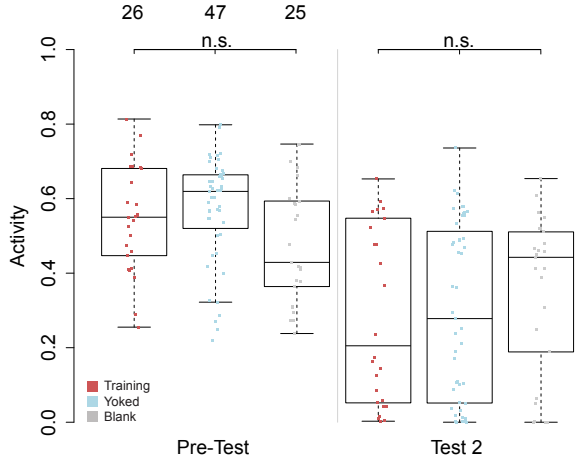


Figure 4.13: MB009B-Gal4/+ flies showed no learning

Table 4.9: P Values from MB009B-Gal4/+ Flies Kruskal-Wallis Test, Followed by Mann-Whitney U Test with Benjamini-Hochberg correction

Test Groups	Pre-Test P Values	Test 2 P Values
Krusal-Wallis Test	0.0534	0.91
Training vs Yoked, Mann-Whitney U Test	N/A	N/A
Training vs Blank, Mann-Whitney U Test	N/A	N/A
Yoked vs Blank, Mann-Whitney U Test	N/A	N/A

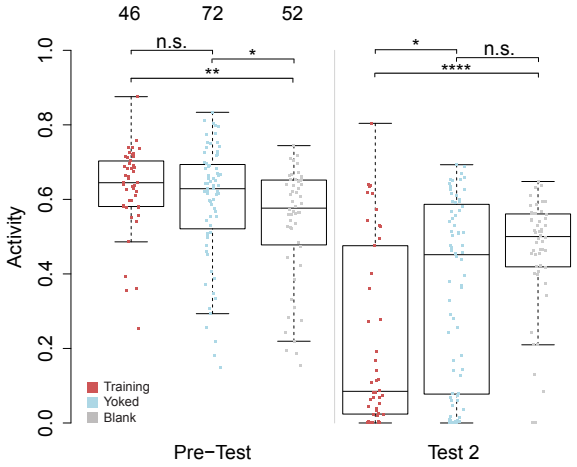


Figure 4.14: +/UAS-Kir2.1 showed significant learning

Table 4.10: P Values from +/UAS-Kir2.1 Flies Kruskal-Wallis Test, Followed by Mann-Whitney U Test with Benjamini-Hochberg correction

Test Groups	Pre-Test P Values	Test 2 P Values
Krusal-Wallis Test	0.00428	0.0002009
Training vs Yoked, Mann-Whitney U Test	0.3582	0.012
Training vs Blank, Mann-Whitney U Test	0.0024	5.7e-05
Yoked vs Blank, Mann-Whitney U Test	0.0334	0.147

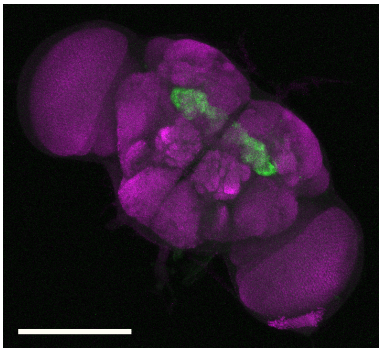


Figure 4.15: MB131B-Gal4 expression pattern. Magenta areas represent contour of the fly brain while green areas represent the green fluorescent protein (GFP) expressed in the targeted neurons. Scale bar: 100μm

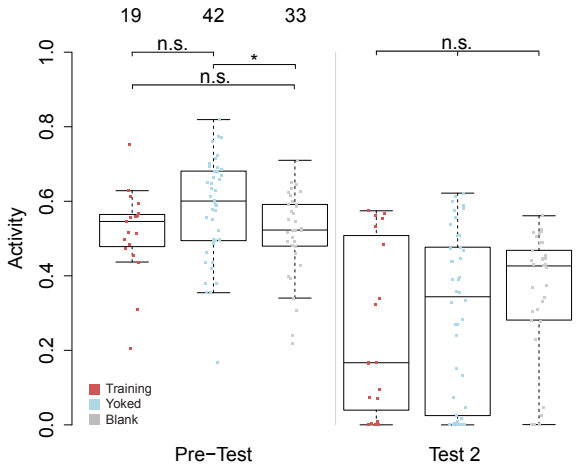


Figure 4.16: MB131B-Gal4/UAS-Kir2.1 flies showed no learning

Table 4.11: P Values from MB131B-Gal4/UAS-Kir2.1 Flies Kruskal-Wallis Test, Followed by Mann-Whitney U Test with Benjamini-Hochberg correction

Test Groups	Pre-Test P Values	Test 2 P Values
Krusal-Wallis Test	0.02686	0.5881
Training vs Yoked, Mann-Whitney U Test	0.067	N/A
Training vs Blank, Mann-Whitney U Test	0.895	N/A
Yoked vs Blank, Mann-Whitney U Test	0.049	N/A

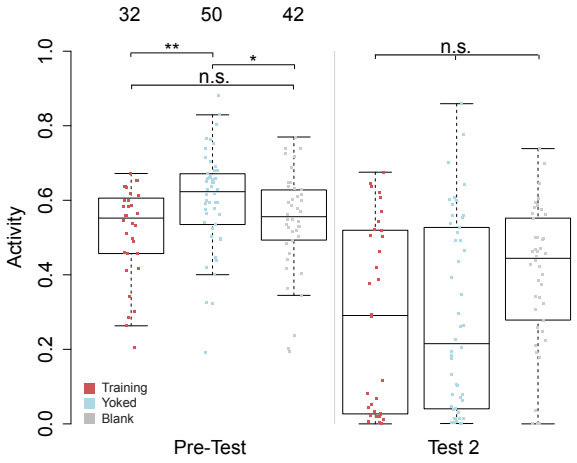


Figure 4.17: MB131B-Gal4/+ flies showed no significant learning

Table 4.12: P Values from MB131B-Gal4/+ Flies Kruskal-Wallis Test, Followed by Mann-Whitney U Test with Benjamini-Hochberg correction

Test Groups	Pre-Test P Values	Test 2 P Values
Krusal-Wallis Test	0.006132	0.1187
Training vs Yoked, Mann-Whitney U Test	0.0076	N/A
Training vs Blank, Mann-Whitney U Test	0.4441	N/A
Yoked vs Blank, Mann-Whitney U Test	0.0371	N/A

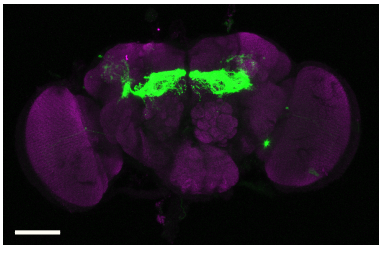


Figure 4.18: MB419B-Gal4 expression pattern. Magenta areas represent contour of the fly brain while green areas represent the green fluorescent protein (GFP) expressed in the targeted neurons. Scale bar: 100μm

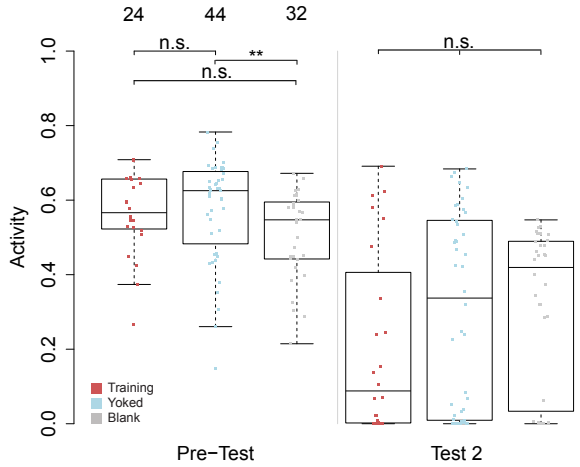


Figure 4.19: MB419B-Gal4/UAS-Kir2.1 flies showed no significant learning

Table 4.13: P Values from MB419B-Gal4/UAS-Kir2.1 Flies Kruskal-Wallis Test, Followed by Mann-Whitney U Test with Benjamini-Hochberg correction

Test Groups	Pre-Test P Values	Test 2 P Values
Krusal-Wallis Test	0.0139	0.4048
Training vs Yoked, Mann-Whitney U Test	0.4556	N/A
Training vs Blank, Mann-Whitney U Test	0.1347	N/A
Yoked vs Blank, Mann-Whitney U Test	0.0094	N/A

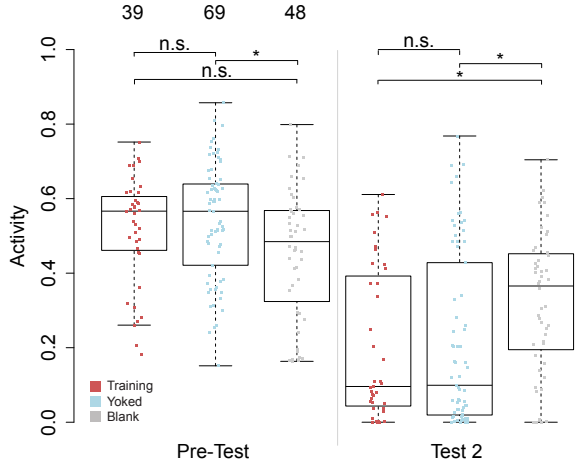


Figure 4.20: MB419B-Gal4/+ flies showed no significant learning

Table 4.14: P Values from MB419B-Gal4/+ Flies Kruskal-Wallis Test, Followed by Mann-Whitney U Test with Benjamini-Hochberg correction

Test Groups	Pre-Test P Values	Test 2 P Values
Krusal-Wallis Test	0.01742	0.006578
Training vs Yoked, Mann-Whitney U Test	0.364	0.93
Training vs Blank, Mann-Whitney U Test	0.061	0.01
Yoked vs Blank, Mann-Whitney U Test	0.025	0.01

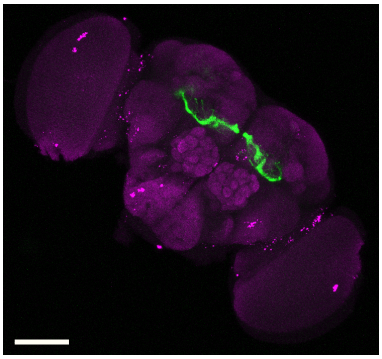


Figure 4.21: MB607B-Gal4 expression pattern. Magenta areas represent contour of the fly brain while green areas represent the green fluorescent protein (GFP) expressed in the targeted neurons. Scale bar: 100μm

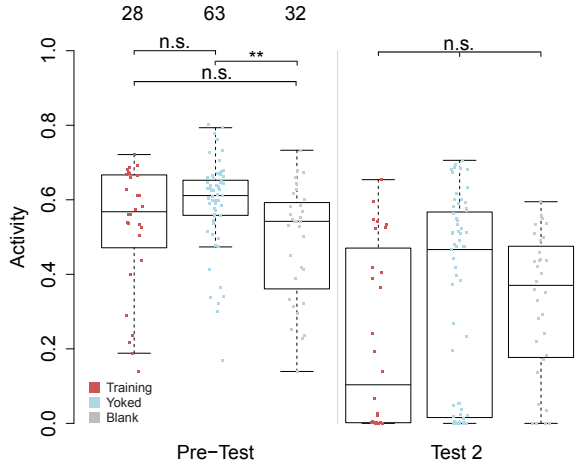


Figure 4.22: MB607B-Gal4/UAS-Kir2.1 flies showed no significant learning

Table 4.15: P Values from MB607B-Gal4/UAS-Kir2.1 Flies Kruskal-Wallis Test, Followed by Mann-Whitney U Test with Benjamini-Hochberg correction

Test Groups	Pre-Test P Values	Test 2 P Values
Krusal-Wallis Test	0.004041	0.1726
Training vs Yoked, Mann-Whitney U Test	0.2122	N/A
Training vs Blank, Mann-Whitney U Test	0.2122	N/A
Yoked vs Blank, Mann-Whitney U Test	0.0019	N/A

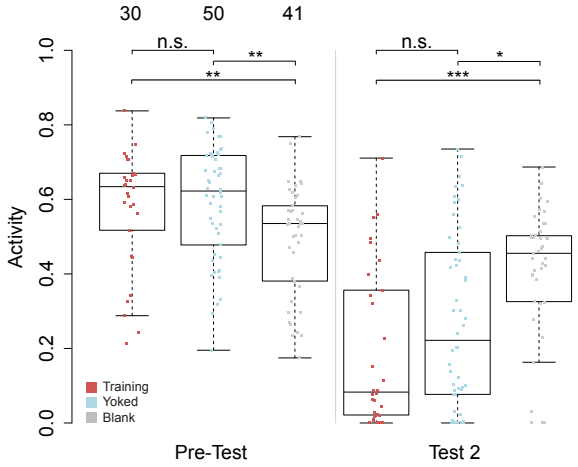


Figure 4.23: MB607B-Gal4/+ flies showed no significant learning

Table 4.16: P Values from MB607B-Gal4/+ Flies Kruskal-Wallis Test, Followed by Mann-Whitney U Test with Benjamini-Hochberg correction

Test Groups	Pre-Test P Values	Test 2 P Values
Krusal-Wallis Test	0.001376	0.0007024
Training vs Yoked, Mann-Whitney U Test	0.7317	0.0970
Training vs Blank, Mann-Whitney U Test	0.0040	0.0009
Yoked vs Blank, Mann-Whitney U Test	0.0036	0.0157

4.4 Discussion

The results presented in this chapter provide an exploration of the mechanisms underlying anti-instinctive learning behavior described in 3. Specifically, the role of the previously reported learning-relevant dopamine receptors, ellipsoid body and the mushroom body γ lobes in anti-instinctive learning behavior were investigated in this study. Our results show that Dop1R1, one of the 4 types of dopamine receptors exist in the fruit fly brain, is involved in the anti-instinctive learning process, while Dop1R2 and Dop2R do not seem to be involved in this process. DopEcR's role in learning is unclear. Our results also show that the learning effect does not require EB, a brain region previously reported involved in motor control and visual memory, as flies with their EB functionally disrupted show normal learning. In addition, MB γ lobes' role in the anti-instinctive learning process is currently unclear, as both flies with the MB γ lobes functionally disrupted and flies expressing exogenous Gal4 proteins in MB γ lobes show comparable activity levels between the training and yoked flies after receiving 2 Train sessions. It is still likely that MB γ lobes are still relevant to the anti-instinctive learning behavior, as both flies with the MB γ lobes functionally disrupted and flies expressing exogenous Gal4 proteins in MB γ lobes have their MB γ lobes affected at the cellular level.

Future works may build upon our existing results in the following areas. First, what is the role of DopEcR in the anti-instinctive learning process? Second, what are the roles of neurons in other MB lobes (α/β and α'/β' lobes) are in the anti-instinctive learning process? Lastly, as the *LaserSync* system uses heat as stressor when training flies, which making temporal control of neuronal function disruption unfeasible, future technology using non-heat based temporal control of gene expression, such as optogenetics, may elucidate the details of how learning happens in action [81, 101].

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Bibliography

[1] Susan L Ackerman and Richard W Siegel. Chemically reinforced conditioned courtship in Drosophila: responses of wild-type and the dunce, amnesiac and don giovanni mutants. *Journal of neurogenetics*, 3(2):111–123, 1986.

[2] U Alon, M G Surette, N Barkai, and S Leibler. Robustness in bacterial chemotaxis. *Nature*, 397(6715):168–171, jan 1999.

[3] John Robert Anderson. *Learning and memory: An integrated approach*. John Wiley & Sons Inc, 2000.

[4] Adam R Aron, Trevor W Robbins, and Russell A Poldrack. Inhibition and the right inferior frontal cortex. *Trends in Cognitive Sciences*, 8(4):170–177, 2004.

[5] Kenta Asahina. Neuromodulation and Strategic Action Choice in Drosophila Aggression. *Annual review of neuroscience*, mar 2017.

[6] Yoshinori Aso, Daisuke Hattori, Yang Yu, Rebecca M Johnston, Nirmala A Iyer, Teri T B Ngo, Heather Dionne, L F Abbott, Richard Axel, Hiromu Tanimoto, and Gerald M Rubin. The neuronal architecture of the mushroom body provides a logic for associative learning. *eLife*, 3:e04577, 2014.

[7] Yoshinori Aso, Daisuke Hattori, Yang Yu, Rebecca M. Johnston, Nirmala A. Iyer, Teri T.B. Ngo, Heather Dionne, L. F. Abbott, Richard Axel, Hiromu Tanimoto, and Gerald M. Rubin. The neuronal architecture of the mushroom body provides a logic for associative learning. *eLife*, 3:e04577, 2014.

[8] Vincent Baggett, Aditi Mishra, Abigail L Kehrer, Abbey O Robinson, Paul Shaw, and Troy Zars. Place learning overrides innate behaviors in Drosophila. *Learning & Memory*, 25(3):122–128, mar 2018.

[9] Vincent Baggett, Aditi Mishra, Abigail L Kehrer, Abbey O Robinson, Paul Shaw, and Troy Zars. Place learning overrides innate behaviors in Drosophila. *Learning & Memory*, 25(3):122–128, mar 2018.

[10] Bernard W Balleine, Nathaniel D Daw, and John P O Doherty. Multiple Forms of Value Learning and the Function of Dopamine BT - Neuroeconomics: Decision Making and the Brain. *Neuroeconomics Decision Making and the Brain*, page 538, 2009.

[11] Sunhoe Bang, Seogang Hyun, Sung Tae Hong, Jongkyun Kang, Kyunghwa Jeong, Joong Jean Park, Joonho Choe, and Jongkyeong Chung. Dopamine signalling in mushroom bodies regulates temperature-preference behaviour in Drosophila. *PLoS Genetics*, 7(3), 2011.

[12] Belinda Barbagallo and Paul A Garrity. Temperature sensation in Drosophila. *Current Opinion in Neurobiology*, 34:8–13, 2015.

[13] Andrew B. Barron, Eirik Søvik, and Jennifer L. Cornish. The Roles of Dopamine and Related Compounds in Reward-Seeking Behavior Across Animal Phyla. *Frontiers in Behavioral Neuroscience*, 4(October):1–9, 2010.

[14] Daniel E Bath, John R Stowers, Dorothea Hörmann, Andreas Poehlmann, Barry J Dickson, and Andrew D Straw. FlyMAD: rapid thermogenetic control of neuronal activity in freely walking Drosophila. *Nature Methods*, 11:756, may 2014.

[15] Sophie Batsching, Reinhard Wolf, and Martin Heisenberg. Inescapable stress changes walking behavior in flies - Learned helplessness revisited. *PLoS ONE*, 11(11):1–16, 2016.

[16] S Benzer. Behavioral mutants of Drosophila isolated by countercurrent distribution. *Proceedings of the National Academy of Sciences of the United States of America*, 58(3):1112–1119, sep 1967.

[17] Howard C. Berg. The rotary motor of bacterial flagella. *Annual review of biochemistry*, 72(1):19–54, 2003.

[18] Jacob A Berry, Isaac Cervantes-Sandoval, Eric P Nicholas, and Ronald L Davis. Dopamine Is Required for Learning and Forgetting in Drosophila. *Neuron*, 74(3):530–542, 2012.

[19] Daniel Bovet, Filomena Bovet-Nitti, and Alberto Oliverio. Genetic aspects of learning and memory in mice., 1969.

[20] A H Brand and Norbert Perrimon. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*, 118(5):289–295, 1993.

[21] Keller Breland and Marian Breland. The misbehavior of organisms. *American Psychologist*, 16(11):681–684, 1961.

[22] Björn Brembs. Operant conditioning in invertebrates. *Current Opinion in Neurobiology*, 13(6):710–717, 2003.

[23] T J Carew, E T Walters, and E R Kandel. Classical conditioning in a simple withdrawal reflex in Aplysia californica. *Journal of Neuroscience*, 1(12):1426–1437, 1981.

[24] V Castellucci and E R Kandel. Presynaptic facilitation as a mechanism for behavioral sensitization in Aplysia. *Science*, 194(4270):1176–1178, 1976.

[25] Vincent F Castellucci and Eric R Kandel. A Quantal Analysis of the Synaptic Depression Underlying Habituation of the Gill-Withdrawal Reflex in Aplysia. *Proceedings of the National Academy of Sciences*, 71(12):5004–5008, 1974.

[26] M Chandler, R E Bird, and L Caro. The replication time of the Escherichia coli K12 chromosome as a function of cell doubling time. *Journal of Molecular Biology*, 94(1):127–132, 1975.

[27] Ann-Shyn Chiang, Chih-Yung Lin, Chao-Chun Chuang, Hsiu-Ming Chang, Chang-Huain Hsieh, Chang-Wei Yeh, Chi-Tin Shih, Jian-Jheng Wu, Guo-Tzau Wang, Yung-Chang Chen, Cheng-Chi Wu, Guan-Yu Chen, Yu-Tai Ching, Ping-Chang Lee, Chih-Yang Lin, Hui-Hao Lin, Chia-Chou Wu, Hao-Wei Hsu, Yun-Ann Huang, Jing-Yi Chen, Hsin-Jung Chiang, Chun-Fang Lu, Ru-Fen Ni, Chao-Yuan Yeh, and Jenn-Kang Hwang. Three-dimensional reconstruction of brain-wide wiring networks in Drosophila at single-cell resolution. *Current biology : CB*, 21(1):1–11, jan 2011.

[28] FRANCIS CRICK. Central Dogma of Molecular Biology. *Nature*, 227(5258):561–563, aug 1970.

[29] J R Crittenden, E M Skoulakis, K A Han, D Kalderon, and R L Davis. Tripartite mushroom body architecture revealed by antigenic markers. *Learning & memory (Cold Spring Harbor, N.Y.)*, 5(1-2):38–51.

[30] Steven de Belle and Martin Heisenberg. Associative Odor Learning in Drosophila Abolished by Chemical Ablation of Mushroom Bodies. 263(February):692–695, 1994.

[31] Bowen Deng, Qi Li, Xinxing Liu, Yue Cao, Bingfeng Li, Yongjun Qian, Rui Xu, Renbo Mao, Enxing Zhou, Wenxia Zhang, Juan Huang, and Yi Rao. Chemoconnectomics: Mapping Chemical Transmission in Drosophila. *Neuron*, pages 876–893, 2019.

[32] Michael H Dickinson. How Animals Move: An Integrative View. *Science*, 288(5463):100–106, 2000.

[33] Soeren Diegelmann, Melissa Zars, and Troy Zars. Genetic dissociation of acquisition and memory strength in the heat-box spatial learning paradigm in Drosophila. *Learning and Memory*, 13(1):72–83, 2006.

[34] Georg Dietzl, Doris Chen, Frank Schnorrer, Kuan-Chung Su, Yulia Barinova, Michaela Fellner, Beate Gasser, Kaolin Kinsey, Silvia Oppel, Susanne Scheiblauer, Africa Couto, Vincent Marra, Krystyna Keleman, and Barry J Dickson. A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature*, 448:151, jul 2007.

[35] Michael E. Dillon, George Wang, Paul a. Garrity, and Raymond B. Huey. Thermal preference in Drosophila. *Journal of Thermal Biology*, 34(3):109–119, 2009.

[36] Isabelle Draper, T. Peri Kurshan, Edward McBride, F. Rob Jackson, and S. Alan Kopin. Locomotor Activity Is Regulated by D2-Like Receptors in Drosophila: An Anatomic and Functional Analysis Isabelle. *Developmental Neurobiology*, 67(3):378–393, 2007.

[37] Josh Dubnau. *Behavioral Genetics of the Fly (Drosophila melanogaster)*. Cambridge University Press, 2014.

[38] Y Dudai, Y N Jan, D Byers, W G Quinn, and S Benzer. dunce, a mutant of Drosophila deficient in learning. *Proceedings of the National Academy of Sciences*, 73(5):1684–1688, 1976.

[39] Charles B Ferster and Burrhus Frederic Skinner. Schedules of reinforcement. 1957.

[40] Marco Gallio, Tyler A Ofstad, Lindsey J Macpherson, Jing W Wang, and Charles S Zuker. The Coding of Temperature in the Drosophila Brain. *Cell*, 144(4):614–624, 2011.

[41] Abhrajyoti Ghosh and Sonja-Verena Albers. Assembly and function of the archaeal flagellum. *Biochemical Society Transactions*, 39(1):64–69, 2011.

[42] Ralph J Greenspan. Fly pushing: the theory and practice of Drosophila genetics. 2004.

[43] Ralph J Greenspan and J F Ferveur. Courtship in Drosophila. *Annual review of genetics*, 34:205–232, 2000.

[44] Thomas A Grigliatti, Linda Hall, Raja Rosenbluth, and David T Suzuki. Temperature-sensitive mutations in Drosophila melanogaster. *Molecular and General Genetics MGG*, 120(2):107–114, jun 1973.

[45] Fumika N Hamada, Mark Rosenzweig, Kyeongjin Kang, Stefan R Pulver, Alfredo Ghezzi, Timothy J Jegla, and Paul A Garrity. An internal thermal sensor controlling temperature preference in Drosophila. *Nature*, 454:217, jun 2008.

[46] K.-A. Han, R. Andretic, R. J. Greenspan, F. S. Jones, and Y.-C. Kim. Drosophila D1 dopamine receptor mediates caffeine-induced arousal. *Proceedings of the National Academy of Sciences*, 105(51):20392–20397, 2008.

[47] U. Hanesch, K. F. Fischbach, and M. Heisenberg. Neuronal architecture of the central complex in Drosophila melanogaster. *Cell and Tissue Research*, 257(2):343–366, 1989.

[48] Rasika M Harshey. Bacterial Motility on a Surface: Many Ways to a Common Goal. *Annual Review of Microbiology*, 57(1):249–273, oct 2003.

[49] Frank Hauser, Giuseppe Cazzamali, Michael Williamson, Wolfgang Blenau, and Cornelis J.P. Grimmelikhuijzen. A review of neurohormone GPCRs present in the fruitfly Drosophila melanogaster and the honey bee Apis mellifera. *Progress in Neurobiology*, 80(1):1–19, 2006.

[50] R D Hawkins, T W Abrams, T J Carew, and E R Kandel. A cellular mechanism of classical conditioning in Aplysia: activity-dependent amplification of presynaptic facilitation. *Science*, 219(4583):400–405, 1983.

[51] Martin Heisenberg, Alexander Borst, Sibylle Wagner, and Duncan Byers. drosophila mushroom body mutants are deficient in olfactory learning: Research papers. *Journal of Neurogenetics*, 2(1):1–30, 1985.

[52] Suzana Herculano-Houzel. The human brain in numbers: a linearly scaled-up primate brain. *Frontiers in Human Neuroscience*, 3:31, 2009.

[53] Bastian Herzog and Reinhard Wirth. Swimming behavior of selected species of Archaea. *Applied and environmental microbiology*, 78(6):1670–1674, mar 2012.

[54] Samantha J. Hinojos, Paul R. Sabandal, Kyung-An Han, Peter D. Evans, and Gissel P. Aranda. Behavioral Sensitization to the Disinhibition Effect of Ethanol Requires the Dopamine/Ecdysone Receptor in Drosophila. *Frontiers in Systems Neuroscience*, 11(August):1–10, 2017.

[55] A. Horowitz, C. B. Menice, R. Laporte, and K. G. Morgan. Mechanisms of smooth muscle contraction. *Physiological Reviews*, 76(4):967–1003, 1996.

[56] M. Ilius, R. Wolf, and M. Heisenberg. The central complex of Drosophila melanogaster is involved in flight control: Studies on mutants and mosaics of the gene Ellipsoid body open. *Journal of Neurogenetics*, 21(4):321–338, 2007.

[57] Hiroshi Ishimoto, Zhe Wang, Yi Rao, Chun Fang Wu, and Toshihiro Kitamoto. A Novel Role for Ecdysone in Drosophila Conditioned Behavior: Linking GPCR-Mediated Noncanonical Steroid Action to cAMP Signaling in the Adult Brain. *PLoS Genetics*, 9(10), 2013.

[58] K Ito, W Awano, K Suzuki, Y Hiromi, and D Yamamoto. The Drosophila mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. *Development (Cambridge, England)*, 124(4):761–71, 1997.

[59] M. E. Jacobs. Influence of Light on Mating of Drosophila Melanogaster. 41(1):182–188, 1960.

[60] Ken F Jarrell and Mark J McBride. The surprisingly diverse ways that prokaryotes move. *Nat Rev Micro*, 6(6):466–476, jun 2008.

[61] Gáspár Jékely. Origin and early evolution of neural circuits for the control of ciliary locomotion. *Proceedings of the Royal Society B: Biological Sciences*, 278(1707):914 LP – 922, feb 2011.

[62] Arnim Jenett, Gerald M. Rubin, Teri T.B. Ngo, David Shepherd, Christine Murphy, Heather Dionne, Barret D. Pfeiffer, Amanda Cavallaro, Donald Hall, Jennifer Jeter, Nirmala Iyer, Dona Fetter, Joanna H. Hausenfluck, Hanchuan Peng, Eric T. Trautman, Robert R. Svirskas, Eugene W. Myers, Zbigniew R. Iwinski, Yoshinori Aso, Gina M. DePasquale, Adrianne Enos, Phuson Hulamm, Shing Chun Benny Lam, Hsing Hsi Li, Todd R. Laverty, Fuhui Long, Lei Qu, Sean D. Murphy, Konrad Rokicki, Todd

Safford, Kshiti Shaw, Julie H. Simpson, Allison Sowell, Susana Tae, Yang Yu, and Christopher T. Zugates. A GAL4-Driver Line Resource for Drosophila Neurobiology. *Cell Reports*, 2(4):991–1001, 2012.

[63] Jian Jing and Rhanor Gillette. Central Pattern Generator for Escape Swimming in the Notaspid Sea Slug Pleurobranchaea californica. *Journal of Neurophysiology*, 81(2):654 LP – 667, feb 1999.

[64] Kim Kaiser, J. Douglas Armstrong, Zongsheng Wang, Susan C. P. Renn, Paul H. Taghert, Mingyao Yang, and Xin An. Genetic analysis of the Drosophila ellipsoid body neuropil: Organization and development of the central complex. *Journal of Neurobiology*, 41(2):189–207, 2002.

[65] Eric R Kandel, James H Schwartz, Thomas M Jessell, Department of Biochemistry, Molecular Biophysics Thomas Jessell, Steven Siegelbaum, and A J Hudspeth. *Principles of neural science*, volume 4. McGraw-hill New York, 2000.

[66] G. Kass-Simon and Paola Pierobon. Cnidarian chemical neurotransmission, an updated overview. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, 146(1):9–25, 2007.

[67] Almut Kelber and Miriam J Henze. Colour Vision: Parallel Pathways Intersect in Drosophila. *Current Biology*, 23(23):R1043 – R1045, 2013.

[68] Krystyna Keleman, Eleftheria Vrontou, Sebastian Kruttner, Jai Y Yu, Amina Kurtovic-Kozaric, and Barry J Dickson. Dopamine neurons modulate pheromone responses in Drosophila courtship learning. *Nature*, 489(7414):145–149, 2012.

[69] H Keshishian, K Broadie, A Chiba, and M Bate. The Drosophila Neuromuscular Junction: A Model System for Studying Synaptic Development and Function. *Annual Review of Neuroscience*, 19(1):545–575, mar 1996.

[70] Y.-C. Kim, H.-G. Lee, and K.-A. Han. D1 Dopamine Receptor dDA1 Is Required in the Mushroom Body Neurons for Aversive and Appetitive Learning in Drosophila. *Journal of Neuroscience*, 27(29):7640–7647, 2007.

[71] Ronald J Konopka and Seymour Benzer. Clock Mutants of Drosophila melanogaster. *Proceedings of the National Academy of Sciences*, 68(9):2112–2116, 1971.

[72] K G Kozminski, K A Johnson, P Forscher, and J L Rosenbaum. A motility in the eukaryotic flagellum unrelated to flagellar beating. *Proceedings of the National Academy of Sciences*, 90(12):5519–5523, jun 1993.

[73] C Kung and Y Saimi. The Physiological Basis of Taxes in Paramecium. *Annual Review of Physiology*, 44(1):519–534, oct 1982.

[74] Sara Kuntz, Burkhard Poeck, Marla B. Sokolowski, and Roland Strauss. The visual orientation memory of Drosophila requires Foraging (PKG) upstream of Ignorant (RSK2) in ring neurons of the central complex. *Learning and Memory*, 19(8):337–340, 2012.

[75] K A Lawrence and W H Watson. Swimming Behavior of the Nudibranch Melibe leonina. *The Biological Bulletin*, 203(2):144–151, oct 2002.

[76] Tzumin Lee and Liqun Luo. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron*, 22(3):451–461, 1999.

[77] Daniel A Levitis, William Z Lidicker, and Glenn Freund. Behavioural biologists don't agree on what constitutes behaviour. *Animal behaviour*, 78(1):103–110, jul 2009.

[78] Konrad Lorenz. *Here am I - where are you? The behavior of the graylag goose*. R. Piper GmbH & Co., München, 1988.

[79] Konrad Lorenz. *The foundations of ethology*. Springer Science & Business Media, 2013.

[80] Liqun Luo, Edward M Callaway, and Karel Svoboda. Genetic dissection of neural circuits. *Neuron*, 57(5):634–660, 2008.

[81] Kristen W Lynch, Laura B Motta-Mena, Anna Reade, Orion D Weiner, Michael J Mallory, Spencer Glantz, and Kevin H Gardner. An optogenetic gene expression system with rapid activation and deactivation kinetics. *Nature Chemical Biology*, 10(3):196–202, 2014.

[82] Robert M Macnab. Genetics and Biogenesis of Bacterial Flagella. *Annual Review of Genetics*, 26(1):131–158, dec 1992.

[83] Martin E.P. Seligman. On the generality of the laws of learning. *Psychol. Rev.*, (77):406–18, 1970.

[84] Michael L May. Insect thermoregulation. *Annual review of entomology*, 24(1):313–349, 1979.

[85] Sean E McGuire, Mitch Deshazer, and Ronald L Davis. Thirty years of olfactory learning and memory research in Drosophila melanogaster. *Progress in Neurobiology*, 76(5):328–347, aug 2005.

[86] Sean E McGuire, Phuong T Le, and Ronald L Davis. The Role of Drosophila Mushroom Body Signaling in Olfactory Memory. *Science*, 293(5533):1330 LP —- 1333, 2001.

[87] A Joiner Mei-ling and Leslie C Griffith. CaM kinase II and visual input modulate memory formation in the neuronal circuit controlling courtship conditioning. *Journal of Neuroscience*, 17(23):9384–9391, 1997.

[88] W Mischel, Y Shoda, and M I Rodriguez. Delay of gratification in children. *Science*, 244(4907):933–938, 1989.

[89] Walter Mischel, Ebbe B. Ebbesen, and Antonette Raskof Zeiss. Cognitive and attentional mechanisms in delay of gratification. *Journal of personality and social psychology*, 21(2):204–218, 1972.

[90] David R Mitchell. The evolution of eukaryotic cilia and flagella as motile and sensory organelles. *Eukaryotic Membranes and Cytoskeleton*, pages 130–140, 2007.

[91] Craig Montell. Drosophila TRP channels. *Pflügers Archiv*, 451(1):19–28, oct 2005.

[92] James M Newcomb and Paul S Katz. Homologues of serotonergic central pattern generator neurons in related nudibranch molluscs with divergent behaviors. *Journal of Comparative Physiology A*, 193(4):425–443, 2007.

[93] S Y M Ng, B Chaban, and K F Jarrell. Archaeal Flagella, Bacterial Flagella and Type IV Pili: A Comparison of Genes and Posttranslational Modifications. *Journal of Molecular Microbiology and Biotechnology*, 11(3-5):167–191, 2006.

[94] Steven P Nilsen, Yick-Bun Chan, Robert Huber, and Edward A Kravitz. Gender-selective patterns of aggressive behavior in Drosophila melanogaster. *Proceedings of the National Academy of Sciences of the United States of America*, 101(33):12342–12347, 2004.

[95] C K Omoto, I R Gibbons, R Kamiya, C Shingyoji, K Takahashi, and G B Witman. Rotation of the central pair microtubules in eukaryotic flagella. *Molecular biology of the cell*, 10(1):1–4, jan 1999.

[96] Mark G. Packard and Barbara J. Knowlton. Learning and Memory Functions of the Basal Ganglia. *Annual Review of Neuroscience*, 25(1):563–593, 2002.

[97] Ivan Pavlov. Conditioned Reflexed: an investigation of the physiological activity of the cerebral cortex. *Annals of Neurosciences*, 17(3):136–140, 2010.

[98] Ivan Petrovitch Pavlov and W Gantt. Lectures on conditioned reflexes: Twenty-five years of objective study of the higher nervous activity (behaviour) of animals. 1928.

[99] Bastianella Perazzona, Guillaume Isabel, Thomas Preat, and Ronald L Davis. The role of cAMP response element-binding protein in Drosophila long-term memory. *Journal of Neuroscience*, 24(40):8823–8828, 2004.

[100] E. Petruccelli, Q. Li, Y. Rao, and T. Kitamoto. The Unique Dopamine/Ecdysteroid Receptor Modulates Ethanol-Induced Sedation in Drosophila. *Journal of Neuroscience*, 36(16):4647–4657, 2016.

[101] Lauren R. Polstein and Charles A. Gersbach. A light-inducible CRISPR-Cas9 system for control of endogenous gene activation. *Nature Chemical Biology*, 11(3):198–200, 2015.

[102] Cheng Qi and Daewoo Lee. Pre- and Postsynaptic Role of Dopamine D2 Receptor DD2R in Drosophila Olfactory Associative Learning. *Biology*, 3(4):831–845, 2014.

[103] Hongtao Qin, Michael Cressy, Wanhe Li, Jonathan S Coravos, Stephanie A Izzi, and Joshua Dubnau. Gamma Neurons Mediate Dopaminergic Input during Aversive Olfactory Memory Formation in Drosophila. *Current Biology*, 22(7):608–614, 2012.

[104] Catherine H. Rankin, Christine D.O. Beck, and Catherine M. Chiba. Caenorhabditis elegans: A new model system for the study of learning and memory. *Behavioural Brain Research*, 37(1):89–92, 1990.

[105] Robert A Rescorla. Behavioral studies of Pavlovian conditioning. *Annual review of neuroscience*, 11(1):329–352, 1988.

[106] Robert A Rescorla, Allan R Wagner, and Others. A theory of Pavlovian conditioning: Variations in the effectiveness of reinforcement and nonreinforcement. *Classical conditioning II: Current research and theory*, 2:64–99, 1972.

[107] Alice A Robie, Jonathan Hirokawa, Austin W Edwards, Lowell A Umayam, Allen Lee, Mary L Phillips, Gwyneth M Card, Wyatt Korff, Gerald M Rubin, Julie H Simpson, Michael B Reiser, and Kristin Branson. Mapping the Neural Substrates of Behavior. *Cell*, 170(2):393–406, 2017.

[108] Yikang S Rong and Kent G Golic. Gene targeting by homologous recombination in Drosophila. *science*, 288(5473):2013–2018, 2000.

[109] Yoshiro Saimi and Ching Kung. Behavioral genetics of paramecium. (24):47–65, 1987.

[110] O Sayeed and S Benzer. Behavioral genetics of thermosensation and hygrosensation in Drosophila. *Proceedings of the National Academy of Sciences*, 93(12):6079–6084, 1996.

[111] Birgit E Scharf, Karen A Fahrner, Linda Turner, and Howard C Berg. Control of direction of flagellar rotation in bacterial chemotaxis. *Proceedings of the National Academy of Sciences*, 95(1):201–206, 1998.

[112] Sabrina Scholz-Kornehl and Martin Schwärzel. Circuit Analysis of a Drosophila Dopamine Type 2 Receptor That Supports Anesthesia-Resistant Memory . *The Journal of Neuroscience*, 36(30):7936–7945, 2016.

[113] W. Schultz. Reward signaling by dopamine neurons. *Neuroscientist*, 7(4):293–302, 2001.

[114] Johannes D. Seelig and Vivek Jayaraman. Feature detection and orientation tuning in the Drosophila central complex. *Nature*, 503(7475):262–266, 2013.

[115] Johannes D. Seelig and Vivek Jayaraman. Neural dynamics for landmark orientation and angular path integration. *Nature*, 521(7551):186–191, 2015.

[116] Katja Seipel and Volker Schmid. Evolution of striated muscle: Jellyfish and the origin of triploblasty. *Developmental Biology*, 282(1):14–26, jun 2005.

[117] Piali Sengupta. The belly rules the nose: feeding state-dependent modulation of peripheral chemosensory responses. *Current Opinion in Neurobiology*, 23(1):68–75, feb 2013.

[118] R K Shultzaberger, S J Johnson, J Wagner, K Ha, T A Markow, and R J Greenspan. Conservation of the behavioral and transcriptional response to social experience among Drosophilids. *Genes, Brain and Behavior*, 2018.

[119] Richard W Siegel and Jeffrey C Hall. Conditioned responses in courtship behavior of normal and mutant Drosophila. 76(7):3430–3434, 1979.

[120] Alcino J Silva, Jeffrey H Kogan, Paul W Frankland, and Satoshi Kida. CREB AND MEMORY. *Annual Review of Neuroscience*, 21(1):127–148, mar 1998.

[121] Divya Sitaraman, Yoshinori Aso, Gerald M. Rubin, and Michael N. Nitabach. Corrigendum: Control of Sleep by Dopaminergic Inputs to the Drosophila Mushroom Body. *Frontiers in Neural Circuits*, 9(November):1–8, 2015.

[122] Divya Sitaraman, Elizabeth F. Kramer, Lily Kahsai, Daniela Ostrowski, and Troy Zars. Discrete Serotonin Systems Mediate Memory Enhancement and Escape Latencies after Unpredicted Aversive Experience in Drosophila Place Memory. *Frontiers in Systems Neuroscience*, 11:92, 2017.

[123] Divya Sitaraman, Melissa Zars, Holly LaFerriere, Yin-Chieh Chen, Alex Sable-Smith, Toshihiro Kitamoto, George E Rottinghaus, and Troy Zars. Serotonin is necessary for place memory in Drosophila. *Proceedings of the National Academy of Sciences*, 105(14):5579–5584, 2008.

[124] Burrhus F Skinner. Operant behavior. *American psychologist*, 18(8):503, 1963.

[125] Carolyn L Smith, Natalia Pivovarova, and Thomas S Reese. Coordinated Feeding Behavior in Trichoplax, an Animal without Synapses. *PLOS ONE*, 10(9):e0136098, sep 2015.

[126] Benjamin Soibam, Monica Mann, Lingzhi Liu, Jessica Tran, Milena Lobaina, Yuan Yuan Kang, Gemunu H Gunaratne, Scott Pletcher, and Gregg Roman. Open-field arena boundary is a primary object of exploration for Drosophila. *Brain and Behavior*, 2(2):97–108.

[127] M B Sokolowski. Drosophila: genetics meets behaviour. *Nature reviews. Genetics*, 2(11):879–890, 2001.

[128] Mansi Srivastava, Emina Begovic, Jarrod Chapman, Nicholas H Putnam, Uffe Hellsten, Takeshi Kawashima, Alan Kuo, Therese Mitros, Asaf Salamov, Meredith L Carpenter, Ana Y Signorovitch, Maria A Moreno, Kai Kamm, Jane Grimwood, Jeremy Schmutz, Harris Shapiro, Igor V Grigoriev, Leo W Buss, Bernd Schierwater, Stephen L Dellaporta, and Daniel S Rokhsar. The Trichoplax genome and the nature of placozoans. *Nature*, 454(7207):955–960, aug 2008.

[129] Patrick R H Steinmetz, Johanna E M Kraus, Claire Larroux, Jorg U Hammel, Annette Amon-Hassenzahl, Evelyn Houliston, Gert Worheide, Michael Nickel, Bernard M Degnan, and Ulrich Technau. Independent evolution of striated muscles in cnidarians and bilaterians. *Nature*, 487(7406):231–234, jul 2012.

[130] Roland Strauss. The central complex and the genetic dissection of locomotor behaviour. *Current opinion in neurobiology*, 12(6):633–638, 2002.

[131] Chih-Ying Su and Jing W Wang. Modulation of neural circuits: how stimulus context shapes innate behavior in Drosophila. *Current Opinion in Neurobiology*, 29:9–16, 2014.

[132] Greg S B Suh, Allan M Wong, Anne C Hergarden, Jing W Wang, Anne F Simon, Seymour Benzer, Richard Axel, and David J Anderson. A single population of olfactory sensory neurons mediates an innate avoidance behaviour in Drosophila. *Nature*, 431:854, sep 2004.

[133] Paul H. Taghert and Michael N. Nitabach. Peptide Neuromodulation in Invertebrate Model Systems. *Neuron*, 76(1):82–97, oct 2012.

[134] LEANNE TAMM, VINOD MENON, and ALLAN L REISS. Maturation of Brain Function Associated With Response Inhibition. *Journal of the American Academy of Child & Adolescent Psychiatry*, 41(10):1231–1238, 2002.

[135] Stephen T Thibault, Matthew A Singer, Wesley Y Miyazaki, Brett Milash, Nicholas A Dompe, Carol M Singh, Ross Buchholz, Madelyn Demsky, Robert Fawcett, Helen L Francis-Lang, and Others. A complementary transposon tool kit for Drosophila melanogaster using P and piggyBac. *Nature genetics*, 36(3):283, 2004.

[136] Stuart Thompson and Winsor H Watson. Central pattern generator for swimming in Melibe. *The Journal of Experimental Biology*, 208(7):1347 LP – 1361, mar 2005.

[137] N Tinbergen. On aims and methods of Ethology. *Zeitschrift f??r Tierpsychologie*, 20(4):410–433, 1963.

[138] Nikolaas Tinbergen. *The Study of Instinct*, volume 228. Clarendon Press, Oxford, 1951.

[139] Laurie Tompkins and Jeffrey C Hall. The different effects on courtship of volatile compounds from mated and virgin Drosophila females. *Journal of Insect Physiology*, 27(1):17–21, 1981.

[140] Laurie Tompkins, Richard W Siegel, Donald A Gailey, and Jeffrey C Hall. Conditioned courtship inDrosophila and its mediation by association of chemical cues. *Behavior Genetics*, 13(6):565–578, 1983.

[141] W.Daniel Tracey, Rachel I Wilson, Gilles Laurent, and Seymour Benzer. painless, a Drosophila Gene Essential for Nociception. *Cell*, 113(2):261–273, 2003.

[142] Thomas C Tubon, Jiabin Zhang, Eugenia L Friedman, Haining Jin, Erin D Gonzales, Hong Zhou, Diana Drier, Jason R Gerstner, Emily A Paulson, Robin Fropf, and Others. dCREB2-mediated enhancement of memory formation. *Journal of Neuroscience*, 33(17):7475–7487, 2013.

[143] Tim Tully and William G Quinn. Classical conditioning and retention in normal and mutantDrosophila melanogaster. *Journal of Comparative Physiology A*, 157(2):263–277, 1985.

[144] Stephanie Lynn Turner and Anandasankar Ray. Modification of CO2 avoidance behaviour in Drosophila by inhibitory odorants. *Nature*, 461:277, aug 2009.

[145] Atsushi Ueda and Yoshiaki Kidokoro. Aggressive behaviours of female Drosophila melanogaster are influenced by their social experience and food resources. *Physiological Entomology*, 27(1):21–28, 2002.

[146] Tetsuo Ueda, Susumu Koya, and Yoshihiko K Maruyama. Dynamic patterns in the locomotion and feeding behaviors by the placozoan Trichoplax adhaerence. *Biosystems*, 54(1–2):65–70, dec 1999.

[147] Judith van Houten. Kinetic Analysis of Chemokinesis of Paramecium. *The Journal of Protozoology*, 29(2):226–230, 1982.

[148] Bruno Van Swinderen and Rozi Andretic. Dopamine in Drosophila: Setting arousal thresholds in a miniature brain. *Proceedings of the Royal Society B: Biological Sciences*, 278(1707):906–913, 2011.

[149] Katrin Vogt, Christopher Schnaitmann, Kristina V. Dylla, Stephan Knapek, Yoshinori Aso, Gerald M. Rubin, and Hiromu Tanimoto. Shared mushroom body circuits underlie visual and olfactory memories in Drosophila. *eLife*, 3:e02395, 2014.

[150] Scott Waddell. Reinforcement signalling in Drosophila; dopamine does it all after all. *Current Opinion in Neurobiology*, 23(3):324–329, 2013.

[151] Jan Walker. *Psychology: The Science of Mind and Behaviour (4th edition)*, volume 22. 2005.

[152] E T Walters, T J Carew, and E R Kandel. Associative Learning in Aplysia: evidence for conditioned fear in an invertebrate. *Science*, 211(4481):504–506, 1981.

[153] J D WATSON and F H CRICK. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature*, 171(4356):737–738, apr 1953.

[154] Paul Willner. Dopamine and depression: A review of recent evidence. I. Empirical studies. *Brain Research Reviews*, 6(3):211–224, 1983.

[155] Reinhard Wolf and Martin Heisenberg. Basic organization of operant behavior as revealed in Drosophila flight orientation. pages 699–705, 1991.

[156] Tanya Wolff, Nirmala A. Iyer, and Gerald M. Rubin. Neuroarchitecture and neuroanatomy of the Drosophila central complex: A GAL4-based dissection of protocerebral bridge neurons and circuits. *Journal of Comparative Neurology*, 523(7):997–1037, 2015.

[157] Jacqueline N Wood and Jordan Grafman. Human prefrontal cortex: processing and representational perspectives. *Nature Reviews Neuroscience*, 4(2):139–147, 2003.

[158] Joy S Wu and Liqun Luo. A protocol for dissecting Drosophila melanogaster brains for live imaging or immunostaining. *Nature Protocols*, 1(4):2110–2115, 2006.

[159] Ming-Chin Wu, Li-An Chu, Po-Yen Hsiao, Yen-Yin Lin, Chen-Chieh Chi, Tsung-Ho Liu, Chien-Chung Fu, and Ann-Shyn Chiang. Optogenetic control of selective neural activity in multiple freely moving Drosophila adults. *Proceedings of the National Academy of Sciences*, 2014.

[160] G Wustmann, K Rein, R Wolf, and M Heisenberg. A new paradigm for operant conditioning of Drosophila melanogaster. *Journal of Comparative Physiology A*, 179(3):429–436, sep 1996.

[161] Shinya Yamamoto and Elaine S. Seto. Dopamine Dynamics and Signaling in Drosophila: An Overview of Genes, Drugs and Behavioral Paradigms. *Experimental Animals*, 63(2):107–119, 2014.

[162] Zhenghong Yang, Franco Bertolucci, Reinhard Wolf, and Martin Heisenberg. Flies Cope with Uncontrollable Stress by Learned Helplessness. *Current Biology*, 23(9):799–803, 2013.

[163] J C P Yin, M Del Vecchio, H Zhou, and T Tully. CREB as a Memory Modulator: induced expression of a dCREB2 activator isoform enhances long-term memory in drosophila. *Cell*, 81(1):107–115, 1995.

[164] J C P Yin, J S Wallach, M Del Vecchio, E L Wilder, H Zhou, W G Quinn, and T Tully. Induction of a dominant negative CREB transgene specifically blocks long-term memory in Drosophila. *Cell*, 79(1):49–58, 1994.

[165] J. M. Young and J. D. Armstrong. Structure of the adult central complex in Drosophila: Organization of distinct neuronal subsets. *Journal of Comparative Neurology*, 518(9):1500–1524, 2010.

[166] Stephen X Zhang, Dragana Rogulja, and Michael A Crickmore. Dopaminergic Circuitry Underlying Mating Drive. *Neuron*, 91(1):168–181, 2016.