LEARNING AND PHYSIOLOGICAL STRESS:

OUTCOMES ON EXPRESSION OF RELATED GENES

IN HONEY BEES (APIS MELLIFERA)

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LEARNING AND PHYSIOLOGICAL STRESS: OUTCOMES ON EXPRESSION OF

RELATED GENES IN HONEY BEES (*APIS MELLIFERA*)

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Abstract: Stress is defined as any deviation form an organism's baseline physiological levels. As such, introduction of new stimuli and information, such as in learning, can be operationally defined stressors. A large body of research exists examining the role that stress plays in learning, but virtually none addresses whether or not learning itself is a measurable cause of stress. The current work seeks to explore stress in conjunction with learning to determine whether expression of three genes of interest are affected in similar or different fashions by both learning and stress. The current work employs three studies, including aversive conditioning, appetitive conditioning, and naturalistic observation to explore how expression of the candidate genes is altered under a variety of experimental contexts. Gene expression was quantified using reverse-transcriptase quantitative polymerase chain reaction. Results were analyzed using both traditional parametric statistics, and novel non-parametric statistics in Observational Oriented Modeling. Results indicate that stress and learning appear to affect all genes of interest in separate fashions, and do not appear to cause a physiological stress response as a result of learning.

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CHAPTER I

INTRODUCTION

The purpose of this work is to examine the effects of a variety of different learning paradigms on expression of stress and learning associated genes present within the honey bee brain. These learning paradigms include a shuttle box passive avoidance learning, free-flight foraging choice, and an appetitive Y-maze task. This work seeks to clarify whether learning or external physiological stress predicts alterations in expression of three genes of interest. While some data exists to suggest learning may cause a physiological stress response (Black et al., 2021), more data is required to elucidate the direct relationship between learning and expression of these genes.

The Acute Stress Response

In order to adapt to environmental threats, an organism must first be able to make a physiological response to react to environmental stimuli. This response often consists of hormonal changes (Romero, 2004; Wada, 2008; Denver, 2009; Wingfield, 2013), cellular changes (Irwin, 1994; Kültz, 2005), and activation of physiological systems present within an organism (Irwin, 1994; Gabella, 2001). In general, this suite of responses is referred to as the general adaptation syndrome, as posited by Hans Selye (1950).

In his original characterization of the general adaptation syndrome, Selye (1950)

posited that the reaction to potentially threatening stimuli occurred in a series of three stages. These stages consist of alarm reaction, resistance and finally exhaustion. Selye (1950) suggested that during the alarm reaction stage, an organism activates physiological systems in order to flee or defend itself from a threatening stimulus. Subsequently, during the resistance stage, the organism is experiencing the physiological cost of maintaining the activated systems. Perhaps the most contentious of these stages is the exhaustion stage, wherein Selye (1950) suggested that continued physiological exertion to maintain stress responses becomes deleterious, and as such may result in stress pathology. Researchers such as Boonstra et al. (1998) have argued that while sustained exposure to stress may have short term detrimental effects on many physiological measures, they do not themselves result in pathology in the long run, and may in fact be adaptive to present environmental pressures.

While still subject to some debate regarding the true nature of the three phases of stress response, the general adaptation syndrome, and more importantly, acute responses to stress, remain a highly researched physiological mechanism, particularly in the case of endocrine responses (Romero, 2004; Wada, 2008; Denver, 2009; Wingfield, 2013). For most vertebrate systems, the primary endocrine response as a result of stressors is the secretion of corticosteroids. In vertebrates, release of corticosteroids occurs as a result of stimulation of a system known as the hypothalamic-pituitary-adrenal axis (HPA axis). This system, by virtue of its conserved nature across vertebrates (Romero et al., 1998; Boonstra, 2004; Romero, & Wikelski, 2006; Hawlena & Schmitz, 2010; Harris et al., 2012), and the high degree of detectability of glucocorticoids, mineralocorticoids, and their subsequent metabolites (Breuner et al., 2013; Desantis et al., 2013), has become the primary measure of physiological stress for a number of study organisms.

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Endocrine secretion across the HPA axis begins with detection of a stressor by the organism. Initial detection by sensory systems results in secretion of norepinephrine from dedicated neurosecretory cells. The elevation of norepinephrine is detected by the hypothalamus, which in turn begins to secrete corticotrophin-releasing hormone through the infundibulum to stimulate the pituitary gland. The pituitary gland in turn releases adrenocorticotropic hormone from the anterior pituitary, and vasopressin from the posterior pituitary. Secretion of these endocrine messengers into the bloodstream results in stimulation if the adrenal medulla and cortex. Subsequently, the adrenal glands release glucocorticoids and mineralocorticoids.

While corticosteroids have become a ubiquitous measure of stress across a wide variety of vertebrate models, there exists some debate about whether or not glucocorticoids serves as an adequate measure. Desantis et al. (2013) argue that while glucocorticoids can affect up to 10 % of a vertebrate's genomic expression due to regulatory effects, the majority of these steroids exist primarily in a bound compound with corticosteroid binding globulins, and are therefore inert. Likewise, Crespi et al. (2013) note that glucocorticoids are subject to change depending on both the organism, and their corresponding life stage, suggesting that interpretation of glucocorticoid measures between populations or species must be made with care.

Despite widespread focus of the stress literature on glucocorticoids and their expression as primary measures of physiological stress, it is important to note that other systems are activated as a result of encountering threatening stimuli. In most organisms, systems controlled by the autonomic nervous system, such as heart, lungs, and gastrointestinal functioning have their baseline levels of functioning altered in order to

address the organismal needs presented by the stressor (Hawlena & Schmitz, 2010). Likewise, in more localized cases, organisms may experience cellular responses, such as the release of cytokines (Irwin, 1994; Kültz, 2005), edema (Schulz et al., 1999), or alterations in protein expression (Hranitz et al., 2010; Kültz, 2005) resulting from detection of stimuli in the local environment. This vast suite of responses suggests that changes in both the organismal, and individual cellular environment may be interpreted by physiological systems as stressors, and should be investigated as such.

Invertebrate Stress Response

A major distinction between the stress responses of vertebrate systems and invertebrates, particularly insects, is the lack of a corticosteroid system. Insects in particular lack an HPA axis, possessing instead an analogous system in the corpora cardiac hormonal cascade (Ivanovic, 2018). Activation of this system begins as a result of accumulation of biogenic amines, such as dopamine and octopamine in the corpora cardiac. The corpora cardiac a brain region consisting of a high density of neurosecretory cells analogous to the hypothalamus (Perić-Mataruga et al., 2006; Evan et al., 2012; Ivanovic, 2018). In the insect brain, dopamine and octopamine serve as trophic neurohormones. Binding of these amines to receptors throughout the insect results in the release of the metabolic hormones allostatin and vasopressin into the vascular system (Perić-Mataruga et al., 2006; Evan et al., 2012; Ivanovic, 2018). As with vertebrate glucocorticoids, these factors promote increased rates of cellular metabolism and result in the breakdown of fatty substances within the insect's fat body.

It is important to note that while invertebrate systems do not possess glucocorticoids, they do possess a system of similar function. In insects, vasopressin is theorized to bind to

with a corticotrophin releasing factor in order to perform functions such as binding to nucleic steroid receptors, which are expected within the well understood vertebrate systems (Evan et al., 2012).

Environmental Stress

While stress is often conceptualized as an immediate threat (such as predation) to an organism, there are many factors present in an environment that may cause an organism to mount a stress response. Factors such as food availability (Boonstra et al., 1998), population density (Schultner et al., 2013), weather (Wingfield, 2013) and predation risk are all environmental stressors that often cause stress responsive systems to activate. Further, these causes often exist in both predictable and unpredictable forms (Love et al., 2013). Love et al. (2013) suggest that in the case of unpredictable stressors, the organism must engage an acute stress response in order to survive. In juxtaposition, predictable stressors allow the organism to adjust their homeostatic baseline through repeated exposure. This shift results in a change in the amount of physiological resources necessary to maintain standard function.

Allostatic theory suggests that the constant shift in physiological resources required to return an organism to homeostasis is what results in deleterious effects occurring as a result of the acute stress response (Ganzel et al., 2010). However, in addition to Selye's (1950) general adaptation model, the allostatic theory suggests that organisms may alter physiological systems in an anticipatory fashion. This may include experiencing early stress responses, or in cases of sustained environmental pressure (See decline phases in hare populations in Boonstra et al., 1998), alterations of systems to encourage new homeostatic levels that are more adaptive to the present environment.

Adaptation of the stress response is a well-known phenomenon, best exemplified in the adaptive calibration model of stress responsivity (Del Giudice et al., 2011). Traditionally researched in mice and rats, the adaptive calibration model has shown that parental care within some species can be interpreted by offspring as a signal of a stressor, allowing the offspring to express corticosteroid receptors at an up-regulated, or down-regulated rate depending on the care (Walker et al., 1986; Del Giudice et al., 2011). It is believed that this mechanism allows for the offspring to experience physiological stressors early in life in a safe context, such that their later interaction with environmental stressors may be better matched in order to reduce allostatic load and metabolic resources.

Collectively these factors suggest that either through generational calibration (Walker et al., 1986; Boonstra et al., 1998; Del Giudice et al., 2011) or habituation to continued acute environmental pressures (Ganzel et al., 2010), organisms of similar populations may have vastly different stress responses based on their geographic and environmental conditions. To this end, traditional laboratory experiments using maintained colonies of the same breeding stock may be insufficient to truly examine the effects of stress on an organism's ability to learn in an environmental context.

Learning in Conjunction with Stress

Traditional examinations of learning and stress explore how physiological stress affects learning outcomes. When examining the relationship in this manner, results are consistent across species suggesting an inverted-U pattern of performance dependent on the individual's stress level (Joëls et al., 2006; Oitzl et al., 2001). Individuals under low or high amounts of physiological stress display impaired learning and recall, while those under moderate amounts often show improved performance (Kim & Diamond, 2002; Joëls et al.,

2006). Results of this nature, while consistent, appear to be context dependent. Repeated studies have shown that the effect of stress on learning and memory performance varies slightly depending on timing of the stressor in relation to the task, as well as the type of task performed (Sandi & Gauza, 2006; Smeets et al., 2009).

Sandi and Gauza (2006) in particular, noted increased recall performance in rats which experienced simulated stress prior to a memory task. Rats which were injected with a small dosage of cortisol to simulate physiological stress showed more rapid learning acquisition and improved recall time when exposed to a Morris water-maze. These results suggest that physiological stress actively promotes learning in response to moderate stressors.

In extreme cases of stress, such as impaired glucocorticoid systems, or instances of chronic stress, laboratory animals have been shown to exhibit significantly decreased performance on learning outcomes (Oitzl et al., 2001; Song et al., 2006). Oitzl et al. (2001) made use of genetic knockout mice with decreased glucocorticoid receptor expression to display the effects of impaired stress responses on learning. Knockout mice performed more poorly in spatial learning tasks on learning acquisition and recall when compared to wild type mice.

In a contrasting study, Song et al. (2006) made use of chronic stressors to simulate instances of high physiological stress in rats. Chronic stress, in the form of unpredictable enclosure movement, induced a physiological state consistent with the exhaustion phase of the stress response (Selye, 1950; Boonstra et al., 1998). Animals subjected to this chronic stress showed decreased performance in spatial learning tasks, when compared to individuals in non-stressed groups.

While results from the above mentioned studies may be consistent, researchers tend to focus on a one-directional flow of cause and effect between physiological stress and learning outcomes: namely that stress affects learning. In many cases, however, learning outcomes have been shown to result in significant changes to neural pathways, including changes in protein production, receptor density, cellular projections, synaptic density, and synaptic activity (Kami et al., 1995; Kelly & Garavan, 2005; Lisberger, 1988; Houweling et al., 2008). All of the above require a large degree of metabolic and energetic resources, suggesting that these neurological changes may cause significant oxidative and organismal stress within neural tissue.

Lisberger (1988) in particular noted substantial changes in firing rates of neurons associated with eye movement in non-human primates. Results indicated that signaling rates increased across the neurons involved following repeated activation through training in an eye-movement task. While these results are not surprising, they do show that physiological change occurs as a result of learning at a cellular level. These changes in signaling rate in conjunction with observed changes in receptor density (Sapolsky & Meaney, 1986), suggest widespread alterations in individual cell metabolism following learning related restructuring.

Findings similar to Lisberger (1988) have been corroborated in human beings using magnetic resonance imaging and electroencephalograph measures (Kami et al., 1995; Kelly & Garavan, 2005; Houweling et al., 2008). Kami et al. (1995) in particular noted synaptic changes present in adult humans following a motor reproduction task. Similarly Kelly and Garavan (2005) noticed improved connectivity between brain regions as a result of performing repeated tasks. It is worth noting that in their review of the extant literature, Kelly and Garavan (2005) illustrated that neuroplastic changes resulting from experience do not

always result in improved neural communication. In fact, many of the changes noted were a result of reorganization, as well as generation and degeneration of synaptic connections (Kelly & Garavan, 2005).

Collectively, these findings suggest that active changes within the brain are a consistent process across taxa. The high metabolic cost of generating cellular projections, corresponding proteins, and subsequent cost of repeated cellular activation, indicate that neuroplasticity is capable of generating a significant amount of cellular stress. Moreover, a gap exists in the literature regarding whether or not the cost of physical reorganization in neural pathways is capable of generating a physiological stress response.

A previous inquiry by Black et al. (2021) examined expression of a suite of genes related to both learning and physiological stress following exposure of honey bees to a passive avoidance task developed by Dinges et al. (2013). Results indicated that of the genes examined, a serotonin receptor and dopamine receptor both exhibited altered expression as a result of learning and exposure to a physiological stressor, in the form of shock. Down regulation occurred in the serotonin receptor following both application of shock, and learning. Conversely, the dopamine receptor was affected by both learning and physiological stress in distinct ways. Black et al. (2021) noted upregulation of the gene as a result of learning, while physiological stress appeared to downregulate it. These results lend credence to the idea that learning may induce a response similar to physiological stress at the molecular level in some genes, while other genes may be differentially affected.

Honey Bees as Learning Models

While the majority of research regarding stress and learning is conducted in vertebrate models, honey bees offer a set of unique opportunities when examining the two

phenomena together. Honey bees are a model organism for a wide variety of behavioral learning paradigms in invertebrates. These paradigms include appetitive conditioning (Abramson,1986; et al.,), aversive conditioning (Dinges et al., 2013; Giannoni-Guzmán et al., 2014; Black et al., 2018), geospatial learning (Amaya-Márquez et al., 2014), avoidance behaviors (Agarwal et al., 2011; Giannoni-Guzmán et al., 2014; Black et al., 2018), and free-flight foraging behaviors (Menzel, & Erber, 1978; Hill et al., 1997; Menzel, 1999; Amaya-Márquez, et al., 2014). This robust suite of learning paradigms ensures that Behavioral and physiological outcomes may be examined in both laboratory and naturalistic contexts.

In addition to a wide variety of available learning paradigms, honey bees have been shown to rapidly incorporate new information in both laboratory and free flight studies. An experiment designed to determine the effect of previous color associations with appetitive stimuli by Black et al. (2018) showed that while a preference for the associated color is present initially, after a few pairings with shock, honey bees readily learn to avoid the color. Likewise, in free flying experiments, honey bees have been shown to alter foraging behavior to maximize reward when a higher concentration of sucrose is paired with a given stimulus (Wells et al., 1981). The ability to alter existing schema and discriminate between stimuli make honey bees an excellent candidate for research involving learning.

Honey bees have also become a popular model organism for explorations of genetic expression among invertebrates. While most genetic research in invertebrates is conducted in fruit flies, *Drosophila melanogaster*, studies have shown that honey bees have a high degree of genomic similarity to this well-known study species (Walldorf et al., 1989). Walldorf et al., (1989) suggest that comparison of homeobox regions within the genomes of both honey bees and *D. melanogaster* show a 90% similarity between the two**.** This genetic similarity,

coupled with recent expansive genome sequencing efforts within honey bees (Honeybee Genome Sequencing Consortium, 2006) place honey bees at a unique juncture. Genes previously examined in *D. melanogaster* may be interpreted in a second organism, while novel genes may be identified within honey bees themselves.

Further, honey bees possess one of the most well mapped stress response systems of any invertebrate. Efforts from Evan et al., (2012) have expanded on the general stress response posited for most invertebrates (Perić-Mataruga et al., 2006; Ivanovic, 2018). The honey bee stress response incorporates endocrine signaling found in other insects, such as allostatin, dopamine, and vasopressin (Ivanovic, 2018). Evan et al. (2012) have noted species specific pathways, which allow honey bees to access food stuffs stored in their foraging crop to serve as a secondary reservoir for metabolic resources when subject to stress. The extensive mapping of the honey bee genome and stress response make them an ideal candidate species for examination of stress related phenomena, as they allow for easy extrapolation to both other insect systems, and analogous systems in vertebrates.

Collectively, the wide variety of learning paradigms and extensively mapped stress response make honey bees a perfect candidate for examining the effects of learning in conjunction with stress at the molecular level. Experiments may be designed to allow for extensive experimental control regarding the application of physiological stressors, or naturalistic experiments, which allow subjects to experience stress and exhibit learning in an environmental context.

Genes of Interest

In order to assess physiological changes present within an organism, a wide variety of biomarkers are used. Most commonly when examining stress and related factors, these

systems focus on quantification of endocrine products, such as glucocorticoids (Crespi et al., 2013; Desantis et al., 2013). Due to the wide nature of physiological responses to stress, other measures, such as quantification of protein (Oitzl et al., 2001; Hranitz et al., 2010) or transcriptional RNA fragments (Gregory et al., 2012; Black et al., 2021) are often more appropriate.

Transcriptional RNA fragments are of particular interest when examining the combined effects of learning physiological stress. Past research has shown that physiological stress is capable of altering expression of genes through direct promotion (Hranitz et al., 2010; Mano et al., 2018) and suppression (Gregory, 2005), as well as epigenetic and generational pathways (Walker et al., 1986; Del Giudice et al., 2011). Moreover, genes related to cytoskeletal scaffolding (Mauri et al., 2014), and transmission (Humphries et al., 2003) have all been shown to display altered expression as a result of changes in neuroplasticity.

Three individual genes of interest were identified as candidates for investigation to determine whether or not neurological changes as a result of learning can generate a response similar to physiological stress. These genes consist of a serotonin receptor (5HT2A), a dopamine receptor (DOP2), and a fragment of non-coding microRNA (miR932). Of these genes, 5HT2A and DOP2 have been examined previously, and were selected for their differential patterns of expression relating to stress and learning respectively (Black et al., 2021). The third gene of interest, miR932, was identified based on its link to neuroplastic cellular reorganization (Lin et al., 2011).

Dopamine Receptor – DOP2

In the initial inquiry by Black et al. (2021) identified significant differences in DOP2 expression between experimental groups in a shuttle box aversive conditioning task (Dinges et al., 2017). Individuals in this task which were exposed to an inescapable displayed down regulation as a result of stress when compared to unstressed controls. Further, individuals which were capable of learning to avoid the stressor also displayed decreased expression, though not as severe as that seen in the inescapable shock condition. The results of this particular study suggest that physiological stress and learning affect expression of DOP2 in competing manners, with stress suppressing expression, and learning promoting expression (Black et al., 2021).

Past research regarding dopamine, and other biogenic amines, in honey bees supports this interpretation of DOP2's differential expression. Humphries et al. (2003) has illustrated that DOP2 is implicated in neural plasticity and aging processes. Results indicated that young individuals and drones (reproductive males) showed significantly lower expression of DOP2 in neural circuits when compared to foragers. In addition, behavioral results comparing the drones and foragers has shown that drones are significantly less capable of adapting behavior as a result of experiencing a noxious stimulus (Dinges et al., 2013).

When quantifying biogenic amines, Agarwal et al. (2011) showed that higher levels of dopamine corresponded with increased performance on passive avoidance tasks. Further studies have shown that general locomotor performance is impaired when dopamine receptor antagonists are present within honey bee vascular systems (Mustard et al., 2010), indicating that DOP2 may play a role in the maintenance of general locomotor behavior. Collectively these results show that DOP2 is a largely important genetic component to learning performance and locomotion in honey bees.

Serotonin Receptor 5HT2A

Examination of the 5HT2A gene is relatively new in honey bees. A study by Thamm et al. (2013) highlighted the distribution of serotonin receptors throughout the honey bee brain, and noted that two distinct subtypes were present. Studies in *D. melanogaster* have shown that 5HT2A is linked to both circadian rhythmicity, as well as anticipatory behaviors (Nichols, 2007). Further results show that receptor proteins coded for by the 5HT2A gene are necessary in *D. melanogaster* for spatial learning (Sitaraman et al., 2008) and olfactory learning (Johnson et al., 2011).

Examination of 5HT2A gene showed that expression was downregulated as a result of physiological stress, as well as learning in honey bees (Black et al., 2021). This result suggests that for 5HT2A, learning and stress act in tandem, and are potentially markers of the same system. This effect is corroborated by those seen in the vertebrate literature, as physiological stress has been shown to reduce serotonergic signaling in rats (McKittrick et al., 2000).

Additional results in rats suggest that artificial suppression of serotonergic systems can affect learning and memory (Izquierdo et al., 2012). Following pharmaceutical suppression of serotonin synthesis, rats displayed increased persistence in effortful tasks, but also showed decreased sensitivity to reward. Further, Majlessi et al. (2003) noted that artificial suppression of serotonergic systems was capable of reversing learning impairment caused by nitrous oxide neurotoxicity. Such results suggest that downregulation of serotonin is capable of promoting learning and memory.

Taken together, the decrease in serotonin signaling produced by physiological stress may promote the learning and recall enhancement see in vertebrates subject to moderate

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levels of stress (Joëls et al., 2006; Oitzl et al., 2001). Of particular interest, is whether the same expression patterns is observed in invertebrates. Expression results observed for 5HT2A from Black et al., (2021) would indicate that this system is conserved across taxa.

MicroRNA Fragment 932 – miR932

In addition to genes coding for receptor proteins, a new class of genes which produce small, non-coding segments of RNA has become of interest, particularly regarding honey bees and learning (Behura & Whitfield, 2010). These segments of RNA are known as microRNA, and consist of short RNA sequences that do not code for proteomic products (Ghildiyal & Zamore, 2009). Instead, these microRNA segments regulate posttranscriptional effects of other genes, often inactivating or destabilizing alternative mRNA strands (Bushati & Cohen, 2007). These products are often enzymatically lysed into smaller segments following transcription, and have been implicated in learning through promotion of neural plasticity, resulting in increased learning and memory in both vertebrate model organisms such as mice (Edbauer et al., 2010; Gao et al., 2010) and invertebrate systems including *D. melanogaster* (Ashraf et al., 2006) and *C.elegans* (Schratt et al., 2009).

The microRNA segment encoded for by the miR932 gene has been shown to occur both in honey bees and *D. melanogaster* (Biswas et al., 2008; Qian et al., 2016). The miR932 fragment itself has been linked to regulation of actin and its related proteins in honey bees (Lin et al., 2011; Cristino et al., 2014). The actin family of proteins consists of a variety of polymorphic proteins all linked to production of cytoskeletal scaffolding in the honey bee brain (Mullins & Pollard, 1999; Li et al., 2008). While highly conserved across eukaryotic species (Mullins, 2013), actin and its related proteins have been identified as key factors in

synaptic changes involved in memory consolidation (Cristino et al, 2014; Lamprecht et al., 2016).

In both honey bees and *D. melanogaster*, it has been shown that miR932 is responsible for regulation of the actin related Act5C gene (Lin et al., 2011; Fink et al., 2003). Christino et al., (2014) further noted that upregulation of the miR932 fragment assisted in promotion of actin and related proteins within honey bees. This suggests that expression of miR932 plays a strong role in the neuroplastic processes related to learning. As both learning and physiological stress result in neural restructuring, miR932 is likely an adequate measure of both in neural tissue (See Appendix A, Table 1).

The Present Work

The current work seeks to examine the effects of learning and physiological stress on expression of three genes of interest in honey bees. In addition, this work aims to assess whether or not the effects of learning are consistent across experimental contexts, including laboratory and naturalistic settings. In order to examine expression of learning independent from physiological stress, three experiments were designed. Each experiment was intended to explore learning in a different behavioral and environmental context in order to capture the scope of learning, and best exemplify expression of the genes of interest.

Experiment 1

Experiment 1 consisted of a shuttle box passive avoidance task developed by Dinges et al., (2013). While originally designed to explore behaviors such as learned helplessness within honey bees (Dinges et al., 2017), the shuttle box task offers a series of inherent experimental controls, ideal for examinations of learning and physiological stress. Baseline groups experience neither a physiological stressor nor learning, and serve as a control for

standard gene expression. Additional groups consist of experimental individuals, which are capable of learning to avoid a noxious stimulus (electric shock), and a yoked individual, which experiences inescapable shock of the same duration and intensity. Yoked individuals therefore provide an ideal example of stress based alteration in gene expression, while experimental individuals provide illustration of expression affected by both learning and stress.

In addition to the degrees of experimental control offered by the shuttle box task (Dinges et al., 2013), previous examinations of learning in conjunction with physiological stress have made use of the same paradigm (Black et al., 2021). As such, this experiment serves as a replication for the expression results of 5HT2A and DOP2, while providing comparable data on expression of miR932.

Experiment 2

Experiment 2 consisted of a flower patch foraging task, similar to that of Wells et al. (1981). Flower patch experiments allow for free-flight foraging choice in order to assess learning in a natural environment with minimal experimenter interference. Past research has shown two distinct behavioral syndromes in foraging within honey bees: generalist foragers, and specialist foragers. Generalists forage on all available food sources present in their patch, while specialists adapt their foraging preference based on the energy cost and reward associated with specific food sources within a patch (Hill et al., 1997). Specialist foragers, as a result of adapting their behavior, may be said to learn whereas the pattern of unchanged behavior in generalists is inconsistent with learning.

Naturalistic experiments also offer a more inclusive view of learning, as organisms are generally subject to predictable stressors present within their environment (Love et al.,

2013). As predictable stressors elicit consistent, and often small stress responses, naturalistic observation offers a necessary viewpoint when assessing expression of 5HT2A, DOP2 and miR932. In addition, comparison of expression seen in a naturalistic environment may be made to results seen in controlled laboratory experiments to examine the role of environmental contexts on expression.

Experiment 3

Experiment 3 consisted of a free choice appetitive conditioning task in a Y-maze. The Y-maze paradigm has been used in many study species, including honey bees (Nouvian & Galizia, 2019), fruit flies (Simonnet et al., 2014), as well as vertebrates, such as rats and mice (Aggleton, 1985; Kraeuter et al., 2019). Use of the Y-maze was chosen to assess effects of appetitive conditioning, on expression of the genes of interest. As the freedom of individual movement reduces handling stress that may be present in other appetitive conditioning paradigms, such as the proboscis extension response (Abramson & Boyd, 2001) this method allowed for exploration of gene expression with as few stressors as possible. As such, expression of 5HT2A, DOP2 and miR932 may be examined solely based on subject learning performance. Expression results based on learning may be compared to those of Experiment 3 in order to assess the role of environmental context on gene expression clearly.

Hypotheses

For experiment 1, it is hypothesized that the act of learning will alter expression of 5HT2A and MiR-932 in a fashion similar to physiological stressors. As such, it is expected that bees learning passive avoidance will express lower levels of 5HT2A and higher levels of miR932 than bees experiencing shock only, or those experiencing no shock. Expression of DOP2 is hypothesized to be similar to results of Black et al. (2021), such that bees

experiencing shock only will express lowest DOP2, bees experiencing no shock will express highest, and those experiencing both shock and learning will fall between (Appendix A, Table 2).

For the second experiment, it is hypothesized that bees will perform one of two foraging strategies: generalist foragers which do not alter their behavior, and specialist foragers which alter their behavior as a result of increased reward at select feeding locations. As the specialist foragers learn and adapt, they are expected to display expression of the genes of interest consistent with learning. It is expected that specialist bees will display lower 5HT2A, and higher DOP2 and miR932 expression (Appendix A, Table 2).

For the third experiment it is hypothesized that bees with higher performance on the Y-maze task will express higher levels of DOP2 and miR932, and lower levels of 5HT2A when compared to poor performing bees (Appendix A, Table 2).

CHAPTER II

METHODOLOGY

Subjects

All subjects consisted of honey bees (*Apis mellifera* L.) collected from research

hives maintained by the Laboratory of Comparative Psychology and Behavioral Biology

at Oklahoma State University. Hives are located in a rural area north-east of Stillwater,

Oklahoma. Subjects for all experiments were first trained to a feeder containing a 10%

sucrose by volume solution placed roughly 50m from all active hives. Bees collected

from such feeders are known to be foragers, and as such ensured to be in the same life

stage (Seeley, 1995).

For experimental procedures conducted in a laboratory setting, subjects were

collected from the feeder individually in 15 mL Falcon tubes, and transferred to a

communal wire mesh cage with internal dimensions of 35 cm x 30 cm x 40 cm. Each

cage contained a petri dish with a sucrose and honey mixture to ensure bees had

sufficient food between time of collection and initiation of behavioral protocol. All

subjects captured in this way were subject to behavioral protocols within 24 hours.

In the case of experimental procedures conducted in naturalistic settings, subjects

were collected one at a time in individual cardboard match boxes. Subjects collected in

were transferred to a secondary feeder containing a 1.5M sucrose solution. Boxes were opened slowly in order to allow the subject to make antennal contact with the sucrose solution at the feeder, but not exit the matchbox. Upon proboscis extension, and initiation of drinking behaviors, the box was slowly opened to allow the subject to exit onto the feeder. Subjects transported in this manner were allowed to drink from the feeder to satiation, and return to the hive. Individuals who freely returned to the feeder were marked using Testors™ enamel paints for identification, and subsequently recorded for behavioral protocols.

Experiment 1: Shuttle box

Experiment 1 consisted of a passive avoidance task based on the learned helplessness design of Dinges et al. (2017). This protocol was selected in order to identify the effects of learning through aversive conditioning and uncontrollable physiological stress on the expression of miR932, as well as provide additional data on expression of 5HT2A and DOP2 in order to replicate the findings of Black et al. (2021). As the previous study made use of a tropical subspecies of honey bee (*Apis mellifera mellifera/scutellata* hybrid; Avalos et al., 2017), data were collected on all genes of interest to avoid extrapolation of results across populations.

Honey bees have been shown to vary between subspecies on both behavioral and physiological responses to similar protocols, rendering conclusions across population's questionable (Abramson et al., 1997; Chicas-Mosier et al., 2017; Chicas-Mosier et al., Under Review).

Apparatus

The shuttle box apparatus consists of two shuttle chambers made from 3D-printed acrylonitryle butadiene styrene with internal dimensions of 135 mm x 20 mm x 5 mm. Each chamber was affixed to a shock grid containing 2.5 mm stainless steel pins set at 2.5 mm intervals, so that when shock was applied to the grid and subjects were in contact with two consecutive pins, they completed the circuit, resulting in administration of shock. A Plexiglas cover was affixed to the top of each chamber in order to ensure subjects were in contact with the grid at all times. Color stimuli consisting of a yellow and blue paint swatch were placed underneath the shock grid to allow subjects to distinguish the two halves of the chamber. These colors were selected due to past research showing that the two are easily distinguishable by honey bees (Hill et al., 1997; Dinges et al., 2013; See Appendix B, Figure 1).

At the center of each chamber were two pairs of infrared LEDs, and corresponding detectors. All components were wired to a Propeller Experiment Microcontroller (Varnon & Abramson, 2013; 2018). The controller recorded instances of subjects crossing between an infrared LED and its detector, and tracked number of interactions and position of subjects within the chamber. Location information was used to control the onset and offset of shock to the grid. Shock was supplied using an external DC power supply (BK Precision 9110, BK Precision Corporation, Yorba Linda, CA) set to administer 6.0V at 0.05A.

Behavioral Protocol

Individual subjects were collected from the communal cage using 15 mL Falcon tubes. Subjects were captured by placing the open end of the tube over the individual and

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closing the tube with the corresponding lid once the bee began to move freely within the tube. Each subject was randomly assigned to one of three experimental conditions: experimental bees (n=26), yoked controls (n=26), and baseline bees (n=17). Bees were introduced to the apparatus in pairs, one per chamber. In the case of experimental bees, the second bee was a yoked control.

For experimental and yoked pairs, one of the two colors was designated to correspond with administration of shock for the experimental bee. During the conditioning phase, when the experimental bee entered the side designated as shock, an electrical stimulus was applied to the entire grid, shocking both the experimental bee, and the yoked control, regardless of the latter's position. Shock was terminated once the experimental bee exited the designated side of the apparatus. In this design, experimental bees experienced shock as a paired stimulus with color, while the yoked controls experienced both in an unpaired fashion. The color associated with shock was counterbalanced between experimental bees. Baseline bees experienced no shock during their time in the apparatus in order to serve as transcriptional controls.

Prior to introduction of subjects to the apparatus, both chambers and the shock grid were cleaned with a 70% ethanol solution. This cleaning ensured any detritus or pheromones from previous subjects were removed between sessions. Subjects were placed in their individual chambers, and the session began. Upon detection by the apparatus, subjects experienced a 3-minute adaptation period, wherein no shock was administered. Following the adaptation period, and subsequent detection of both subjects by the apparatus, a 5-minute experimental phase began. During the experimental phase, unpaired controls were allowed to move freely between the two colors, and experienced

no shock. Experimental bees experienced shock paired with one of the colors, and yoked control bees experienced both shock and color in an unpaired fashion as previously described. A 5-minute experimental session was selected, as past studies have found that longer experimental sessions can lead to fatigue or habituation to shock on the part of experimental and yoked bees (Black et al., 2017).

Following the experimental session, bees were removed from the apparatus and placed in a 15 mL Falcon tube labeled with their respective sample number. Subjects were let sit for 45 minutes following the experimental protocol to allow for expression of genes of interest. The 45-minute period was selected based on a study by Alaux and Robinson (2007), which suggests that in honey bees, immediate early genes reach peak expression 30 minutes after an eliciting stimulus. As the genes of interest in this study are not immediate early genes, but do possess rapid transcription, 45-minutes was selected to allow for transcription. This method was also employed by Black et al. (2021), and likewise selected for consistency between the two experiments. Following this 45-minute period, subjects were submerged in liquid nitrogen to halt all metabolic activity, and transferred to labeled sterile 1.5 mL microcentrifuge tubes for storage and bioassay. All samples were stored at -80°C until brain dissection and qPCR analysis.

Experiment 2: Flower Patch

Experiment 2 made use of the flower patch foraging study design (Wells et al., 1981). This design was selected in order to assess expression of the genes of interest associated with learning in an environmental context. Learning in this context was assessed based on behavioral changes in foraging strategy observed as a result of changing reward density within the flower patch over time.

While methodologically similar to classical flower patch experiments such as Wells et al., (1981), and Hill et al., (1991) substantial changes were made to both methodology and apparatus. Experiment 2 employed a significantly smaller apparatus with reduced density of artificial flowers. Results from studies utilizing a similar reduction have shown that behavioral patterns are similar between the two forms of flower patch (Chicas-Mosier et al., 2020), while reducing risk of non-subject individuals interacting with the patch.

Similarly, where past studies have paired reward with color (Hill et al., 1991; Amaya- Márquez et al., 2014), reward was instead based on location within the flower patch. Basing high versus low reward on location rather than color was selected as past research has suggested that colors encountered in past foraging trips may affect subsequent interactions with stimuli of that color (Black et al., 2018). Honey bees have been shown to respond to conditioning based on location (Amaya-Márquez et a., 2014), suggesting this was a suitable alternative.

Apparatus

The flower patch apparatus consisted of four artificial flowers, each consisting of a 85 mm x 85 mm piece of colored Plexiglas with a small well placed at the center. Each well consisted of the lid of a 1 mL microcentrifuge tube. The flowers consisted of two colored yellow, and two colored blue (See Appendix B, Figure 2). As with experiment 1, these colors were selected as they have been shown to be easily distinguishable by honey bees (Hill et al., 1997; Dinges et al., 2013).

Artificial flowers were placed side by side in a 2x2 grid on a white background. Wells were filled with 40 µL of a sucrose solution between individual subject visits to the

flower patch. Sucrose solutions consisted of a 1.5 M solution during the first and third phase of the experiment, and a 2M phase for half of the flowers in the second phase of the experiment. The location of the colors was counterbalanced between research sessions in order to remove potential positional bias.

Behavioral Protocol

Subjects were first trained to a secondary feeder and allowed to return freely following their release from the match box. Individuals that returned to the secondary feeder were marked using Testors™ enamel paints for individual identification. Individuals were allowed to leave and return to the secondary feeder at will. At the fifth return to the feeder following marking, bees were considered adequately trained to the location. After leaving the feeder on their fifth visit, the feeder was removed and replaced with the flower patch apparatus.

The first experimental phase began once the subject returned to the flower patch, and successfully drank from one of the flower wells. Following the initiation of feeding from a well, a 45-minute session began. Feeding was defined by extension of the proboscis into the well and making contact with the sucrose solution. During experimental phases, time, location, and duration of feeding behaviors, as well as number of individual visits to the flower patch was recorded for all marked subjects. Individuals which did not return within a 15-minute span were removed from the sample pool.

At the end of the first experimental phase, two adjacent flowers were randomly assigned to become high reward conditions. During the second phase, the wells from these flowers were removed, and replaced with wells containing 40 µL of a 2M sucrose solution. This design was selected to allow honey bees to express foraging behavior

based on either a generalist or specialist strategy (Burns, & Dyer, 2008; Hill et al., 1997).

The second phase consisted of a similar 45-minute session. During the second phase, time, location, and duration of feeding behaviors, as well as number of individual visits to the flower patch were recorded for all marked subjects. As with the previous phase, individuals which did not return within a 15-minute span were removed from the sample pool.

Following the end of the second experimental phase, the high reward wells were removed and replaced with low reward wells, such that all flowers once again contained 1.5 M solutions. This was done to examine persistence of learned behavior in honey bees which elected to pursue high reward food locations. A third 45-minute session was conducted; as with prior sessions, data were collected and bees which do not return in a 15-minute time frame were removed from the subject pool.

Upon completion of the third phase, subjects were captured in a 15 mL Falcon tube, and a 45-minute genetic expression phase similar to that of experiment 1 begins. During the expression phase, subjects were transported to the laboratory maintained at Oklahoma State University's Stillwater OK campus. At the end of the expression phase, subjects were submerged in liquid nitrogen to halt metabolic activity. Subjects were then transferred to a labeled sterile 1 mL microcentrifuge tube, and stored at -80°C for subsequent brain dissection, RNA extraction, and RT-qPCR analysis.

Experiment 3: Y-Maze

For experiment 3, a Y-maze protocol was employed. Y-mazes are often used in invertebrate models, such as honey bees and *D. melanogaster* (Nouvian, & Galizia, 2019; Simonnet et al., 2014) in order to assess spatial learning and conditioning. This method

was selected in order to explore the expression of genes of interest in conjunction with appetitive conditioning in a controlled laboratory environment. While past appetitive conditioning paradigms in honey bees have made use of proboscis extension (Smith et al., 1991; Abramson & Boyd, 2001), this method requires restraining the subjects. The Y-maze allows for free conditioning within subjects without the risk of inducing a physiological stress response due to restraint.

Apparatus

The Y-maze apparatus consisted of a three armed maze 3D-printed using white acrylonitryle butadiene styrene (Hitesh & Abramson, 2020). Each arm possessed internal dimensions measuring 50 mm x 50 m x 100 mm, positioned at an equidistant angle from the opposing two arms. Slots were positioned at the end of two arms to allow insertion of color stimuli, which consisted of 53 mm x 55 mm x 2 mm 3D-printed acrylonitryle butadiene styrene plate painted with Testors™ blue or yellow enamel paint. Placed at the bottom of each color stimulus within the Y-maze was a well containing either a 1.5 M sucrose solution or a 1.5 M NaCl solution. Each solution was paired with a corresponding color for the duration of the experiment, such that sucrose solutions acted as a reward for visiting a color stimulus, while the NaCl solution served as punishment. NaCl was selected, as past research has shown it is an effective, non-lethal punishment for a variety of insect models (Selcho et al., 2009; Unoki et al., 2005). Pairing of solutions to color was counterbalanced between experimental subjects.

Located at the end of the third arm was a docking slot, to allow for introduction of subjects from containment chambers. Each containment chamber consisted of a 3D-printed acrylonitryle butadiene styrene chamber with internal dimensions of 4 cm x 5 cm

x 4 cm. On the one wall of the gamber, a slot was present to allow for the opening and closing of a 45 mm x 45 mm x 2 mm acrylonitryle butadiene styrene gate. This allowed the release of subjects from the containment chamber into the docking arm of the Y-maze (See Appendix B, Figure 3).

Behavioral Protocol

Subjects were individually collected from the communal wire mesh cage using the containment chamber of the Y-maze apparatus. The open portion of the chamber was placed around the subject, and the gate was closed once the subject moved into the chamber. Prior to the introduction of a subject to the apparatus, and between experimental sessions, the Y-maze was cleaned with a 70% ethanol solution. This was done in order to remove pheromone trails and detritus from the apparatus. Subjects remained in the containment chamber for 5-minutes prior to the onset of the conditioning sessions. This was done to allow adaptation to the containment chamber as well as induce hunger in subjects.

Wells were filled with 40 µL of either a 1.5 M sucrose or 1.5 M NaCl solution, with each solution being paired with a color stimulus. These stimuli were counterbalanced, such that half of subjects (n=20) experience blue paired with sucrose, and half experience yellow paired with sucrose. Location of blue or yellow were randomized at the onset of each experimental session. Between experimental sessions, location of yellow and blue stimuli and their corresponding solutions, were switched, to ensure that honey bees were associating color, and not position, with reward and punishment.

Conditioning sessions consisted of six 5-minute sessions, wherein the subject was released into the Y-maze from the containment chamber. During each 5-minute session, researchers recorded any instance of drinking from one of the two wells. Drinking behavior was defined as extension of the proboscis into the well, making contact with either the solution or bottom of the well. Time of the drinking behavior, location, and duration were all recorded for each 5-minute session. Following the 5-minute conditioning session, the subject was returned to the containment chamber, and subject to a 5-minute rest period. Subjects which did not interact with a well for three consecutive sessions were discarded from the subject pool.

Test trials began following successful completion of the six conditioning sessions. For each test trial, clean wells were placed with each color stimulus. No solution was added to either well. Subjects were allowed 5-minutes to select either well. Upon successful interaction with either well, subjects were returned to the containment chamber for the remainder of the 5-minute session. Following this, individuals were subject to the same 5-minute rest session between trials. Subjects which did not interact with a well for three consecutive sessions were discarded from the subject pool.

Individuals which successfully completed the six test trials were removed from the Y-maze apparatus and placed in individual 15 mL Falcon tubes. These subjects remained in isolation for 45-minutes following the behavioral protocol to allow for expression of the genes of interest. Following the expression phase, subjects were submerged in liquid nitrogen in order to halt metabolic activity. Subjects were then transferred to labeled sterile 1 mL microcentrifuge tubes, and stored at -80°C for subsequent brain dissection, RNA extraction, and RT-qPCR analysis.

Bioassay

Tissue Sample Preparation

Samples for bioassay were subject to brain dissection. Heads were removed from frozen samples, and placed on a bed of solid CO_2 under a dissection microscope. Using a fine point 11-blade scalpel, hair and cuticle were removed from the surface of the head. Any remaining hypopharangeal gland is also removed. Following removal of hypopharangeal tissue, ocelli and compound eyes were removed, leaving the brain attached to remaining cuticle tissue below the olfactory bulbs. Brain tissue was removed from remaining cuticle by placing the scalpel tip underneath the olfactory bulb and gently prying the tissue loose.

Individual brain samples were collected whole, including optic lobes, mushroom bodies, olfactory bulbs and antennal lobes. Samples were placed in sterile 1 mL microcentrifuge tubes with 500 µL of TRIzol reagent. Brains were then be mechanically homogenized using a sterile 0.5-10 µL pipette tip.

RNA Extraction

RNA extraction was performed using a prepared extraction reaction (Zymo Research Direct-zol RNA MiniPrep Kit, Zymo Research, Irvine, CA, Cat. No. R2050). 500 µL of 95% ethanol was added to each 500 µL TRIzol and tissue sample. Preparations were then agitated to become homogenous. Samples were transferred to a binding column and collection tube assembly at 14,000 rpm for 60 seconds. To ensure binding, flow-through underwent two additional centrifugations.

Following binding, 400 µL of RNA wash buffer was added to the column assembly, and centrifuged for 30 seconds. Following the wash, 5 µL of a DNase solution,

and 35 µL of a DNA digestion buffer was added to the column. Columns prepared for DNA digestion were allowed to incubate at room temperature for 15 minutes before final wash and RNA elution.

Following DNA digestion, 400 µL of a RNA prewash was added to the column and centrifuged. Flow-through was discarded. A subsequent wash using 700 µL of a RNA wash buffer was then added, and centrifuged for 2 minutes. Following this step, the collection tube was to be removed, and the column transferred to a sterile 1.5 mL microcentrifuge tube. Elution was conducted by placing 50 µL of Nuclease free water in the column and centrifuging for 60 seconds. All samples were subject to nanodrop analysis to assess mass of RNA present. Samples were then diluted with Nuclease free water to a constant 20ng/µL to ensure consistent interaction with RT-qPCR master mixes. Samples were stored at -80°C until RT-qPCR analysis.

RT-qPCR Analysis

To quantify relative genetic expression, reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was conducted on the RNA samples, using a BioRad iTaq Universal qPCR kit (Bio-Rad Laboratories, Hercules, CA). For each gene of interest, a master mix was created containing 750 µL SYBR Green, 15 µL reverse transcriptase, 4.5 µL reconstituted forward primers, 4.5 µL reconstituted reverse primers (See Appendix A, Table 3), and 426 µL nuclease free water. Master Mixes were then distributed throughout plates in 8 µL aliquots, with 2 µL aliquots of the respective diluted sample. Each sample was replicated in triplicate for experimental control.

Plates were analyzed using a Bio-Rad CFX Connect Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA). Analyses consisted of a 10 minute reverse transcription

phase at 50˚ C, followed by a 1 minute enzymatic activation phase at 95˚ C, and thirty-nine following cycles of denaturation, annealing and extension. Cycle threshold (CT) will be recorded for each sample.

CHAPTER III

FINDINGS

For each experiment, data were initially analyzed in IBM SPSS version 24 (Armonk, NY). Statistical tests consisted of ANOVA, t-test, and correlation analyses, dependent on the research question inherent in each experiment. However, due to widespread criticism regarding the replicability and validity of null hypothesis significance testing present in modern statistical analyses, the current work elected to elaborate on traditional statistical models using a non-parametric alternative (Grice, 2011; Grice et al., 2012; Maxwell et al., 2015; Grice, et al. 2017). These subsequent analyses were conducted using the Observation Oriented Modeling (OOM) software, and were tailored to the respective experiments (Grice, 2011).

The OOM software allows researchers to analyze data in order to uncover extant patterns within the data set, or compare a given set of data to a predicted pattern based on an *a priori* hypothesis (Grice, 2011; 2015). For each of the experiments in the present work, the latter method was employed. Patterns of expression data between all experimental groups were hypothesized based on the present understanding of the effects of learning and physiological stress, as well as the context of the experiment. Specific hypotheses are outlined below (See Appendix A, Table 2).

Unlike traditional statistical analyses, OOM is not bound to assumptions of normality, random sampling, or homogeneity of samples, making it an ideal tool to use for analyses of highly variable biomarkers. To test specific directional hypotheses, a crossed ordering ordinal analysis was conducted. This analysis incorporated pairwise analyses between behavioral conditions to determine which data dyads fit the expected pattern. Patterns must be defined so that each group is presented within relation to another. Based on group assignment, each data point is compared to data points from each other group to determine what percentage of each pairwise comparison fits the anticipated pattern. Such analyses are comparable to single tailed t-tests or ANOVA analyses with pairwise post hoc tests.

Results of this model fit are presented as Percent Correct Classification (PCC), a percentage of which dyads fit the expected pattern. For all analyses, a 1000 trial randomization test was also conducted. These randomization tests randomly assign each data point to a new location within the data and produce a c-value, corresponding to the percentage of cases wherein the original PCC was equaled or exceeded by the randomized data set. As such, c-values are a probability value obtained from the randomization test. For purposes of these experiments, PCC values above 60% were considered moderate model fit, while those above 70% were considered good model fit. (Raw data presented in Appendix C)

Experiment 1

Data Preparation

The shuttle box apparatus used in experiment 1 automatically records positional data for the duration of the experimental trial for both subjects present. Positional data

was used to calculate percentage of time spent in each half of the chamber for each individual at 30 second intervals. Baseline bees which died while within the apparatus (n = 3) were removed. In the event of experimental or yoked bees dying within the apparatus (n = 4) both individuals from the pair were removed in order to maintain uniform pairs.

In order to assess expression representative of behavioral performance for each experimental role, a subset of 10 individuals were selected form each. Baseline samples were selected based on degree of separation from probabilistic chance at the final time point. Experimental-yoked dyads were selected in pairs, based on performance of the experimental bee at the final time point. This ensures that comparison of expression between experimental and yoked bees are consistent on the amount of shock experienced by both. Cycle threshold data for each gene of interest for each individual was averaged across triplicate technical replicates in order to generate a representative level of gene expression.

Summary of Findings

Behavioral data show patterns of behavior consistent with past shuttle box research (Dinges et al., 2013; Dinges et al., 2017; Black et al., 2018; Black et al., 2021; See Appendix B, Figure 4). Baseline individuals were tested for color bias, and showed no behavioral preference for either yellow or blue portions of the apparatus, suggesting no color bias present within the sample. Experimental bees displayed passive avoidance learning, spending a significantly higher proportion of time within the apparatus on the side not associated with shock when compared to yoked and baseline bees. Yoked and

baseline conditions did not deviate from random chance, spending equivalent time on both sides of the apparatus.

Traditional parametric statistics revealed no significant difference between experimental roles on expression of 5HT2A, DOP2 or miR932. However, due to the non-random nature of sampling method, and unequal variance between samples, non-parametric statistics are more informative in this instance. OOM analyses suggest moderate adherence to predicted expression patterns for 5HT2A, and miR932, and questionable adherence for DOP2 (See Appendix A Table 2; Appendix B, Figure 5). Such results suggest that the majority of cases fit the prescribed pattern for 5HT2A and DOP2. The differences between parametric and non-parametric outcomes is likely due to the wide degree of variance observed in yoked individuals in DOP2 and 5HT2A, as well as the very small differences between expression levels of all groups in miR932.

Traditional Analyses

To assess the possibility of pre-existing bias toward one color, behavioral data for baseline bees were compared using a single sample t-test to probabilistic chance of time spent on either side of the apparatus (0.5) at each time point (Black et al., 2018). To adjust for α inflation inherent within repeated t-tests, a Bonferroni adjustment was conducted, yielding an actual α value of 0.005. Results indicated that at no statistically significant differences from the test value at any time point (30 sec: $t(16) = .144$, $p = .887$; 60 sec: $t(16) = -.431$, $p = .672$; 90 sec: $t(16) = -.156$, $p = .878$; 120 sec: $t(16) = .010$, $p = .992$; 150 sec: $t(16) = .247$, $p = .808$; 180 sec: $t(16) = .294$, $p = .773$; 210 sec: $t(16) = .162$, $p = .874$; 240 sec: $t(16) = .340$, $p = .738$; 270 sec: $t(16) = .205$, $p = .841$; 300 sec:

$t(16) = .086$, $p = .933$). These results suggest that no color bias was present within the sample.

To ensure behavioral performance was consistent with expected behavioral patterns within a shuttle box experiment (Dinges et al., 2013; Dinges et al., 2017; Black et al., 2018; Black et al., 2021), a split plot ANOVA was conducted comparing performance of each experimental role at each time point, with a Tukey HSD post hoc. To adjust for α inflation inherent within repeated tests, a Bonferroni adjustment was conducted, yielding an actual α value of 0.005. For all time points, results indicated a significant difference between groups (See Appendix A, Table 4). The Tukey HSD Post hoc test indicated that for each time point, the experimental group differed significantly from both baseline and yoked groups, possessing a higher percentage of time spent on the correct portion of the apparatus (See Appendix A, Table 5; Appendix B, Figure 4).These results indicate a pattern of behavior between experimental groups consistent with past shuttle box research.

Analysis of genes of interest was conducted using a one-way ANOVA to compare expression across each experimental role. Post hoc analyses were conducted using a Tukey HSD. Data for 5HT2A displayed no significant differences between groups ($F(2, 26) = 1.716$, $p = .199$). Similarly, no significant results were observed for DOP2 ($F(2, 26) = 1.361$, $p = .274$) or miR932 ($F(2, 26) = .097$, $p = .908$), suggesting no difference in expression across these experimental groups (See Appendix B, Figure 5).

To examine the specific effect of unavoidable shock on expression, an independent samples t-test was conducted comparing expression of yoked individuals to baseline individuals. Data indicated no significant difference between yoked and baseline

individuals for 5HT2A ($t(17) = -1.879$, $p = .078$), DOP2 ($t(17) = -1.552$, $p = .139$), or

miR932 ($t(17) = -.387$, $p = .703$). These data suggest that for all genes of interest,

inescapable physiological stress does not significantly alter expression.

OOM Analyses

In addition to the traditional ANOVA analysis of gene expression, data were

analyzed using the Observation Oriented Modeling (OOM) software for goodness of fit

when compared to a specific *a priori* hypothesis. Based on previous research, there were

three hypotheses for expression within Experiment 1. H1: for 5HT2A, it was expected

that learning and physiological stress would both down-regulate expression. As such,

experimental bees were expected to display highest cycle threshold of 5HT2A, followed

by yoked bees, with baseline bees displaying the lowest cycle threshold. H2: for miR932,

it was expected that learning and physiological stress would both upregulate expression.

As such, experimental bees were expected to display lowest cycle threshold of miR932,

followed by yoked bees, with baseline bees displaying the highest cycle threshold. H3:

for DOP2, it was expected that physiological stress would decrease expression, while

learning would increase it. As such, yoked bees were expected to display the highest

cycle threshold of DOP2, followed by experimental bees, with baseline bees displaying

the lowest cycle threshold (See Appendix A, Table 2).

To test these hypotheses, a crossed ordering ordinal analysis was conducted.

Results for 5HT2A displayed moderate model fit (PCC = 63.21%, c-value = .10).

Pairwise comparisons revealed moderate model adherence for the baseline-experimental

dyad, bees (PCC = 36.18%, c-value = .88). Good model fit was observed for the baseline-

yoked dyad, such that yoked bees possessed on average higher expression than baseline

bees (PCC = 75.56%, c-value = .06). A questionable model fit for the experimental-yoked dyad, showing that expression was not consistently higher between either group (PCC = 48.00%, c-value = .56).

OOM results for DOP2 indicated questionable model fit overall (PCC = 57.86%, c-value = .18). Pairwise analyses showed moderate model fit for baseline-experimental dyads (PCC = 63.33%, c-value = .19), baseline-yoked dyads (PCC = 61.11%, c-value = .21) and moderate fit for the experimental- yoked dyads (PCC = 50.00%, c-value = .49).

For miR932, OOM results indicated questionable model fit overall (PCC = 48.21%, c-value = .57). Pairwise analyses showed questionable model fit for baseline-experimental dyads (PCC = 52.22%, c-value = .42), baseline-yoked dyads (PCC = 41.11%, c-value = .75) and for the experimental-yoked dyads (PCC = 51.00%, c-value = .44). These results indicate that overall, no clear pattern of expression is present between experimental groups for miR932.

Experiment 2

Data preparation

For Experiment 2, behavioral data were collected and used to subdivide subjects based on foraging pattern. Past research has shown two distinct behavioral syndromes in foraging within honey bees: generalist foragers, which forage on all available food sources within a patch, and specialist foragers, which will adapt their foraging preference based on the energy cost and reward associated with specific food sources within a patch (Hill, Wells, & Wells, 1997). As such, bees which increased their visitation to the high concentration wells in phase 2 when compared to phase 1 were considered specialist foragers (n = 6), while those that maintained a consistent proportion or decreased

visitation to the high concentration flowers were considered generalist foragers (n = 5, see Appendix B, Figure 7).

Summary of Findings

Behavioral data indicated a wide variety of visitation formats, with 6 individuals fitting the prescribed criteria of increasing visitation to the flowers designated as high reward in the second phase to be classified as specialist foragers. Results of traditional statistics indicate no difference between foraging strategies in metrics such as number of visits to the flower patch or visit duration across all experimental phases.

Results of traditional parametric analysis revealed no significant differences between foraging strategy on expression of 5HT2A, DOP2, or miR932. In order to identify potential behavioral predictors of gene expression, cycle threshold data was correlated with metrics including number of visits, visit duration, and percentage of visits to high reward flowers across all three phases. No correlation existed between candidate predictor variables and gene expression for any gene.

Due to the non-random sample and wide degree of variance, OOM analyses were employed to examine expression data in further detail. Results indicate that moderate adherence to expected expression models fit was present for 5HT2A and DOP2, and good model fit was present for expression of miR932 (See Appendix B, Figure 8: Appendix A, Table 2). Such results indicate that expression for all genes adheres to predicted patterns, with the majority of miR2 cases showing predicted expression. The lack of potential behavioral predictors suggests that expression of these genes in natural contexts may be linked more to internal phenotypes than learning or experience based expression.

Data on individual performance was assessed across groups using a series of independent samples t-tests. To control for inflated α values inherent in repeated tests, a Bonferroni adjustment was employed, yielding an actual α value of 0.006. Individuals were compared based on foraging group classification on number of visits to the flower patch, average visit duration, and percentage of time spent on the flowers designated as high reward. Metrics were compared for each experimental phase.

Results indicate no statistically significant difference in number of visits between groups for all phases (Phase 1: $t(9) = 2.729$, $p = .023$; Phase 2: $t(9) = 1.458$, $p = .179$; Phase 3: $t(9) = 3.427$, $p = .008$), though phase three shows marginal significance with specialists displaying more visits on average (MD $= 4.883$, SE $= 1.340$). No significant difference was observed in average duration of visit for all phases (Phase 1: $t(9) = -.550$, $p = .596$; Phase 2: $t(9) = -.924$, $p = .399$; Phase 3: $t(9) = -.586$, $p = .572$). Similarly, no significant difference was observed in percentage of time spent on high reward flowers for all phases (Phase 1: $t(9) = -1.419$, $p = .190$; Phase 2: $t(9) = -2.033$, $p = .073$; Phase 3: $t(9) = 1.258$, $p = .240$).

Expression data was compared based on foraging strategy using an independent samples t-test. Results indicated no significant difference in expression between foraging groups for 5HT2A ($t(9) = .557$, $p = .591$), DOP2 ($t(9) = -.690$, $p = .508$), or miR932 ($t(9) = .979$, $p = .353$). These results suggest that adapting to changes in environmental contexts do not significantly alter expression (See Appendix B, Figure 8).

In order to identify behavioral factors that predicted expression, a correlation matrix was conducted. Significant correlations would be grounds to perform regression

analyses using the candidate variable as a predictor and expression of genes of interest as criterion variables. Expression data was correlated with number of visits to the flower patch per phase, proportion of visitation to high concentration per phase, and average visit duration per phase. Results indicated no significant correlations between any gene and these candidate predictor variables (See Appendix A, Table 5), and as such, no regression analyses were conducted.

OOM Analyses

For experiment 2, results were examined in OOM using a crossed orderings ordinal analysis. Expression of the genes of interest were expected to follow patterns of expression based on learning within the specialist foragers. As such, three hypotheses were generated as follows. H1: Cycle threshold of 5HT2A will be greater in specialist foragers than in generalist foragers. H2: Cycle threshold of DOP2 will be higher in specialist foragers than in generalist foragers. H3: Cycle threshold of miR932 will be higher in specialist foragers than in generalist foragers.

Results for 5HT2A displayed moderate model fit (PCC = 64.29%, c-value = .28). While the PCC indicates a relatively high degree of model fit, it is important to note that the c-value is relatively high. This indicates that the randomized data are capable of replicating the observed PCC to a relatively high degree. As such, while the data observed does fit the expected pattern, generalization should be done with care.

Moderate model fit was observed within DOP2 (PCC = 60.71%, c-value = .36). This indicates, that DOP2 shows higher expression in specialist individuals. Finally, good model fit was observed in expression of miR932 (PCC = 71.43, c-value = 0.15). Results

indicate that on average, specialist foragers show higher expression of miR932 than generalist foragers.

Experiment 3

Data Preparation

For experiment 3, a performance score was determined for each individual. This performance score was determined by ranking individuals based on percentage correct choices within the test trials, such that individuals which completed all 6 test trials by selecting the well associated with the sucrose solution were ranked higher than those that selected properly 5 out of 6 times. In the event of a tie between two individuals, the individual with a shorter average time before interaction was ranked higher. The highest ranked individuals (n = 5) and lowest ranked individuals (n = 5) were selected for brain dissection and expression analysis.

Summary of Findings

Behavioral data displays results consistent with effective conditioning. During training phases across subjects number of errors declines (See Appendix B, Figure 9), indicating learning of association between color and reward. Data indicated no differences across the sample between individuals associating blue or yellow with reward.

Results of traditional parametric tests revealed no significant difference between high performing and low performing groups on expression of 5HT2A, DOP2, or miR932. Similar to previous experiments, due to non-random selection and high degree of variability within individual results, non-parametric tests offer a better insight into expression data. OOM results indicate questionable adherence to the expected patterns of

expression in 5HT2A and DOP2A, and good model fit in expression of miR932. These results suggest that the hypothesized relationship between 5HT2A and DOP2 and learning is likely not an adequate explanation for variation seen within the genes. The hypothesized relationship between learning and expression of miR932 however, appears to be supported by these results (See Appendix B, Figure 10; Appendix A, Table 2).

Traditional Analysis

To test for color bias within the sample, bees which received sucrose paired with blue and those which received sucrose paired with yellow were compared on average time before interaction in the test trials as well as average proportion of correct selection within the test trial using independent samples t-tests. Results indicated no significant difference for color on proportion of correct selections ($t(18) = -1.118$, $p = .278$) or average time before interaction ($t(18) = .936$, $p = .362$), indicating no difference in performance based on paired color.

Samples selected based on performance score were likewise compared on expression of genes of interest using an independent samples t-test. Results indicated no significant difference between high performing and low performing individuals on 5HT2A ($t(4.428) = -.794$, $p = .463$), DOP2 ($t(9) = -.757$, $p = .470$) or miR932 ($t(9) = -.800$, $p = .447$, See Appendix B, Figure 10).

OOM Analyses

Similarly to experiments 1 and 2, hypotheses were generated for expression resulting from the Y-Maze protocol based on learning related expression expected from past literature. These hypotheses are as follows. H1: High performance bees will express higher cycle thresholds of 5HT2A than do low performance bees. H2: High performance

bees will express lower cycle thresholds of DOP2 than do low performance bees. H3: High performance bees will possess lower cycle thresholds of miR932 than do low performance bees. Hypotheses were tested in OOM using a crossed orderings ordinal analysis.

OOM results indicate questionable model fit for expression of 5HT2A (PCC = 44.00%, c-value = .66), with the slight minority of analyzed dyads fitting the predicted pattern of expression. Results also indicate a questionable to poor model fit for expression of DOP2 (PCC = 40.00%, c-value = .67). Finally, results indicate good model fit for expression of miR932 (PCC= 72.00%, c-value = .19), indicating the majority of high performing bees show higher expression of miR932 than low performing bees in the Y-Maze task.

CHAPTER IV

CONCLUSIONS

The purpose of the current work was threefold: To explore the effects of a variety of learning paradigms on expression of three genes of interest, to examine whether or not physiological and environmental stressors affect genes linked to learning, and to replicate findings of a previous studies examining the same genes of interest.

Experiment 1 hypothesized that the presence of physiological stress would result in upregulation of DOP2 and miR932, while downregulating expression of 5HT2A. While results of parametric tests display no significant results, OOM analyses suggest that this hypothesis is supported for both 5HT2A and DOP2. Results from 5HT2A in particular suggest a substantial effect of physiological stress in downregulating the gene, while a similar, albeit smaller effect is seen in expression of DOP2. Expression of miR932 conversely, does not appear to be affected by physiological stress (See Table 7).

The secondary hypothesis from experiment 1 was that learning would downregulate 5HT2A in a similar fashion as stress, while DOP2 and miR 932 would be upregulated. Results for all genes suggest that, in the context of aversive conditioning, these patterns of expression are not applicable. In genes such as 5HT2A and DOP2, this may be due to the combination of learning and physiological stress. Observed in the case

of both genes, the effect of physiological stressors appears to affect expression is much a much stronger fashion (See Appendix A, Table 7; Appendix B, Figure 5).

Experiment 2 sought to explore the effects of learning under naturalistic conditions on expression of the genes of interest. It was hypothesized that individuals displaying a specialist foraging strategy would display increased expression of DOP2 and miR932, and decreased expression of 5HT2A. While parametric statistics indicate no significant difference between foraging strategies, non-parametric statistics indicate strong support for these hypotheses. DOP2 and 5HT2A both displayed moderate model fit, indicating a majority of individuals fit the expected pattern, while miR932 displayed good model fit, with over 70% of individuals falling within the expected pattern. These results suggest that within a naturalistic context, learning can affect expression in the expected fashion (See Appendix A, Table 7; Appendix B, Figure 8).

Finally, experiment 3 sought to identify the effect of learning absent external stressors on expression of the genes of interest. As with experiment 2, it was hypothesized that individuals displaying a high learning performance would display increased expression of DOP2 and miR932, and decreased expression of 5HT2A. Of these genes, data only supports this hypothesis for miR932, which displayed good model fit for the predicted pattern (See Appendix A, Table 7; Appendix B, Figure 8).

Discussion

Collectively, results for 5HT2A do not fully support the findings of Black et al., (2021). As was seen with the previous study, the effect of physiological stress was capable of profoundly altering the expression of 5HT2A, while expression of the same gene followed separate patterns between the two experiments. This discrepancy is

potentially due to the variation in subspecies (*Apis mellifera ligustica* in the present study, *Apis mellifera mellifera/scutellata* hybrid in Black et al. (2021)). Variation in genetic composition of subspecies is a widely known phenomenon, with honey bees in particular noting profound behavioral differences in factors such as foraging strategies (Chicas-Mosier et al., 2017), learning ability (Claudio et al., 2018), aggression (Gross et al., 2019), and even susceptibility to toxins (Chicas-Mosier et al., Under Review) across subspecies.

In addition, environmental factors may affect the expression of 5HT2A across these two subspecies. Factors such as predictable and unpredictable weather patterns (Love et al., 2013) differ greatly between the two research sites, and may result in heightened baseline allostatic load in one species or the other. Similarly, presence of agricultural activity, including pesticides and fungicides is likely varied between the two sites as well.

It would appear that the effect of learning on expression of 5HT2A is also a conditional one. Results from experiments 1 and 3 show increased expression of 5HT2A in learning and high performing individuals, rather than the down regulation hypothesized. This result differs however, under the conditions of experiment 2, wherein expression of 5HT2A is upregulated. As such, it appears that environmental context plays a role in the expression of 5HT2A in conjunction with learning.

Downregulation of 5HT2A as expected based on research in *D. melanogaster* (Sitaraman et al., 2008; Johnson et al., 2011) and rats (McKittrick et al., 2000; Majlessi et al., 2003) may occur primarily under conditions of adequate physiological arousal. Serotonin across taxa is linked to alertness, and as such, environmental contexts with the

wide variety of stimuli present may be necessary to maintain the arousal required to alter physiological expression of this particular gene. It is further possible that 5HT2A is subject to the same form of differential regulation seen in DOP2 in the results of Black et al. (2020). This would be supported by results similar to those seen by Izquierdo et al. (2012), which indicate that behavioral persistence and reward motivation are both effected in different ways by serotonin signaling. Regardless of mechanism, it can safely be concluded that expression of serotonin is not affected by learning and stress in the same manner, and as such learning is unlikely to exhibit a stress-like response in this system

Results of DOP2 appear to mirror those of 5HT2A, expressing notable down regulation as a result of physiological stress in experiment 1, and upregulation as a result of learning in environmental contexts. Similarly, the results here only partially support the hypothesis offered by Black et al. (2020). As with 5HT2A, differences expression may be explained by both location and sub species.

However, it is possible that the expression of DOP2 seen in experiment 2 is a result expression of DOP2's role in neuroplasticity (Humphries et al., 2003). Experimental manipulations in both experiment 1 and experiment 3 were constant throughout experiment duration, whereas reward valence changed as a result of experimental phase in experiment 2. It is likely that this, coupled with the density of novel stimuli encountered within foraging trips was sufficient to promote neuroplastic changes. This conclusion is further strengthened by the corresponding upregulation of miR932 under the same circumstances.

It is also possible that, as we only see upregulation of DOP2 in experiment 2, wherein honey bees were subject to free-flight, that expression is more highly tied to motor behavior than learning. Dopamine has been linked directly to motor performance in honey bees in repeated studies (Mustard et al., 2010; Agarwal et al., 2011). This link and subsequent upregulation are potentially due to increased motor activity demanded by flight behaviors. These results corroborate past literature indicating DOP2 to be linked with reward and learning systems in honey bees (Mustard et al., 2010) as well as neural plasticity (Humphries et al. 2003).

Similar to the results seen in 5HT2A, these results suggest that DOP2 does not display a link between physiological stress and learning. In juxtaposition to the results seen by Black et al., 2020), it appears that under these experimental conditions, DOP2 is also more likely linked to neuroplastic changes or motor regulation than learning outcomes. Results for both studies may differ further as a result of genetic analysis methodology. Black et al. (2021) made use of a method of analysis known as the ddCt method (Schmittgen & Livak, 2008), which incorporates use of a housekeeping gene to serve as a control of baseline transcription. While the baseline transcription level serves as a covariate, the method requires additional sample handling, which may destabilize RNA products within the tissue. This analysis was forgone by the present study to reduce risk of RNA contamination and destabilization, in favor of non-parametric methods.

Of all genes of interest, results of miR932 were most consistent across all three experiments. In experiments 2 and 3, miR932 displayed the expected pattern of expression, being upregulated as a result of learning based behaviors. Interestingly, this pattern is not present in experiment 1.

The results seen in experiment 1 suggest two possibilities: that miR932 is not affected by physiological stress, or that miR932's learning expression counteracts expression based on physiological stress. While miR932's link to actin and neuroplasticity would suggest the latter (Li et al., 2008; Cristino et al., 2014), a recent study by Black et al. (Under Review) illustrated that the role of miR932 is more closely linked to memory consolidation, rather than learning acquisition. The results seen in experiment 1, in the context of experiments 2 and 3, lend credence to this conclusion. Results from Black et al. (under Review) showed upregulation of miR932 following a long term memory task in honey bees, but not following short term acquisition. The length of training and experimental manipulation found in experiments 2 and 3 (2.25 and 1 hours respectively) may result in sufficient time for memory consolidation to begin, and exhibiting expression of miR932.

It is worth nothing that the expression of DOP2 and miR932 in experiment 2, as well as expression of miR932 in experiment three may be due to an alternative hypothesis. Taking into account the shared role of both genes in neuroplasticity, and overall impressive model fit seen in OOM results for experiment 2, it is possible that expression of these genes is based more closely to a phenotype than experimental outcome. Neuroplasticity systems have been linked to success in novel situations (VanElzakker et al., 2008) and have even been linked to alterations in mRNA transcription and translation in vertebrates such as mice (Puighermanal et al., 2017).

While expression of miR932 does appear to be linked to learning outcomes, as seen in experiments 2 and 3 as well as past research (Cristino et al, 2014; Lamprecht et al., 2016), it does not appear to be linked to physiological stress. Closer examination of

pairwise comparisons within the OOM analysis of experiment 1 indicate overall poor model fit between the baseline and yoked dyads. This indicates that individuals subject to inescapable shock did not display altered expression when compared to un-shocked individuals.

The present work, while informative, is not without its limitations. Most notably the sample sizes were relatively small, with experiment 2 possessing only 11 individuals which completed the behavioral task. While not inherently a problem, as results were assessed primarily through non-parametric means which do not depend on normally distributed or random samples, interpretation of results must be done with care. This likely explains the non-significant results within the traditional parametric statistics as well, as all studies were under powered.

Individuals were also collected from a mixed hive apiary, which indicates there is a possibility of differential baseline expression of all gene across individuals. It should be noted however that within a given hive, individuals are nearly all within 75% relatedness due to guaranteed parentage, of the queen. Further, results from Baudry et al. (1998) suggest that queens within a given geographical area share a large degree of interrelatedness with nearby queens, and all hives local hives are equally represented in nuptial flights. As such, the degree of genetic variation within hives maintained in the same apiary is relatively low.

Future Directions

While the present experiments examined stress in the context of an inescapable noxious stimulus, the effects of stress on gene expression still require further study. In particular, the variation in 5HT2A expression seen in experiment 2, suggests that

extended periods of heightened arousal in honey bees may potentially decrease 5HT2A expression, as is seen in chronic stress exposure in rats (McKittrick et al., 2000). Subjecting insect models to a chronic stress manipulation may lend insight into how serotonergic systems are affected by environmental factors, as well as how stress may moderate learning outcomes in invertebrate and vertebrate systems. Likewise, further examination into how chronic stress suppresses serotonergic systems may add to the growing body of literature using honey bees and bumble bees as analogues for human depression, anxiety, and mood (Mendl et al., 2010; Bateson et al., 2011).

Further, the role of environmental stressors may be more robust than imagined. In many studies, effects of expression as a result of infection with viruses or parasites are used to simulate increased environmental strain on individual honey bees (Kuster et al., 2014; Fanny et al., 2020; Morfin et al., 2020). Morfin et al. (2020) in particular have illustrate that combined effects of local pesticide use and infection with the widespread mite *Varroa destructor* are shown to significantly decrease expression of genes associated with learning, memory, and motor pathways. Such forces may drastically change expression of the genes of interest, particular 5HT2A, and DOP2, resulting in differing learning and performance outcomes.

Additionally, further evidence regarding the role of miR932 on the regulation of actin and its related proteins should be gathered. Presently, little is known about the function of microRNA, other than that it consists of small non-coding fragments capable of enzymatic, or cofactor roles (Bushati & Cohen, 2007; Ghildiyal & Zamore, 2009). The link between actin and its related family of proteins is well known, but miR932 is only newly being investigated in conjunction with them. While the present work and that of

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Black et al (Under Review) suggest that miR932 is responsible for regulation of actin in its role as a cytoskeletal scaffold responsible for neuroplasticity, further data is required to identify the functional role of the fragment. Enzymatic analysis and x-ray crystallography of actin family proteins, or examination of expression of coding mRNA fragments with miR932 controlled as a co factor may provide more insight into the fragment's role in actin regulation.

The role of DOP2 and miR932 as predictors of a neuroplastic phenotype is worth further investigation. In the event that baseline DOP2 and miR932 levels are capable of predicting an individual's learning outcomes honey bees may be selectively bred to improve experimental and agricultural outcomes. Likewise, further investigation of allelic variation may lead to insights in the role of both DOP2 and miR932 on learning and neural restructuring outcomes. Concrete evidence linking DOP2 to a neuroplastic phenotype in particular may highlight new information in conjunction with the works of Humphries et al. (2003), Mustard et al. (2010), and Agarwal et al. (2011), allowing for mechanistic conclusions regarding why dopaminergic signaling is linked to learning outcomes.

In addition, the role of brain region on expression must be explored. For this work, whole brain extraction was used in order to ensure examination of expression across brain regions. However, studies have noted that distribution of coding mRNA and their respective products differs throughout regions of the honey bee brain. In particular, DOP2 and 5HT2A have been shown to be more highly expressed in the mushroom bodies of honey bees, a region analogous to the vertebrate cerebral cortex (Humphries et al., 2003; Thamm et al., 2013). Investigation of individual brain regions may allow for

increased accuracy in quantification of RNA expression as a result of neurological changes.

Finally, it should be reiterated that many of the analyses present in the current work made use of a non-parametric design: that of Observation Oriented Modelling (Grice, 2011). All experiments were designed with this software and analysis system in mind and included specific, *a priori* hypotheses about direction of expression of all RNA products. Few past studies have made use of this methodology in conjunction with molecular or genetic measures. In particular, it must be pointed out that the OOM system was capable of detecting variation within the observed data set that was not seen in traditional parametric statistics.

Traditional statistics largely make use of comparison of mean values (such as ANOVA and t-test statistics), which are subject to large degrees of variation based on outliers present within the data set. OOM eschews this by comparing each data point to all other data points within a given data set (Grice, 2015). As such, the system is more likely to detect consistent variation within a data set, even if the variation between groups is small, as was seen in the expression data for miR932.

Further, traditional statistics are tied to assumptions often violated by researchers, such as homogeneity of variance and the assumption of random sampling or normal distributions of data within one's samples (Grice et al., 2017). As mentioned previously, the OOM software by virtue of being a data driven approach eschews the need for these assumption. Instead the approach requires a specific understanding of the proposed mechanisms involved within a given experiment's manipulations, and corresponding directional hypotheses. This collective approach allows for researchers to ensure that the

results observed are pointing them toward the appropriate inference: that which best explains the patterns present within the data. While OOM is not presently seen in widespread use of genetic or other molecular markers, the present study provides strong indication that it is a system capable of illustrating changes in genetic expression that would potentially go unnoticed.

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APPENDICES

Table 1

Gene	Abbreviation	Summary of Function
Serotonin Receptor	5HT2A	Receptor for serotonin; downregulation associated with improved recall and elevated stress
Dopamine Receptor 2	DOP2	Receptor for Dopamine; associated with spatial memory and motor control
microRNA fragment 932	miR932	Non-coding fragment of RNA; linked to neuroplasticity through regulation of actin and related proteins

Table 1. Summary of genes of interest including respective abbreviations and functional grounds for inclusion in the current inquiries.

Table 2

Experiment	Hypothesis	Behavioral Predictions	Gene of Interest	Expression Prediction
Experiment 1 – Shuttle Box	Learning and physiological stress will affect 5HT2A and miR932 in the same direction, while DOP2 will display differential effects of learning and physiological stress	Experimental bees will learn to avoid color paired with shock, yoked and baseline bees will spend equal amounts of time on both sides of the apparatus	5HT2A	Stress and learning will downregulate 5HT2A expression
			DOP2	Stress will downregulate DOP2 expression, learning will upregulate DOP2 expression
			miR932	Stress and learning will upregulate miR932 expression
Experiment 2 – Flower Patch	Specialist foragers will display increased expression DOP2 and miR932, and decreased expression of 5HT2A	Specialist foragers will increase visitation to high reward flowers, while generalist foragers will not	5HT2A	Specialist foragers will display downregulated 5HT2A when compared to generalists
			DOP2	Specialist foragers will display upregulated DOP2 when compared to generalists
			miR932	Specialist foragers will display upregulated miR932 when compared to generalists
Experiment 3 – Y-Maze	Higher performance will correspond to increased expression DOP2 and miR932, and decreased expression of 5HT2A	In test trials, individuals will select a well associated with a color. High performing individuals will select the color associated with a sucrose reward with fewer errors and faster speed	5HT2A	High performing individuals will display down regulation of 5HT2A when compared to low performing individuals
			DOP2	High performing individuals will display upregulation of DOP2 when compared to low performing individuals
			miR932	High performing individuals will display upregulation of miR932 when compared to low performing individuals

Table 2. Table presenting hypotheses regarding expression of genes of interest as well as expected behavioral outcomes of each experiment

Table 3

Gene	Forward Sequence	Reverse Sequence
5HT2A	GCAAAGAATCCCGAGAAGAA	GTTACAACGACCACACCTC
DOP2	ACCTCGGATACCTCATCTTC	ATTTCAGGCTCTTGGTCTG
miR932	ACCACCGCGCATTATCCAAA	GCGTATCAAATTCCCAGCGT

Table 3. Forward and reverse sequences for primers used in RT-qPCR analysis.

Table 4

Time Point	df	F-value	p
30 sec	2, 66	8.741	<.001
60 sec	2, 66	6.975	.002
90 sec	2, 66	10.695	<.001
120 sec	2, 66	13.075	<.001
150 sec	2, 66	12.263	<.001
180 sec	2, 66	12.088	<.001
210 sec	2, 66	16.194	<.001
240 sec	2, 66	9.837	<.001
270 sec	2, 66	18.760	<.001
300 sec	2, 66	20.943	<.001

Table 4. ANOVA results for behavioral performance across experimental groups per each time point. All time points returned statistically significant differences between groups.

Table 5

Time Point			Mean Difference	*p*
30 sec	Experimental	Baseline	.206	.008
		Yoked	.232	.001
60 sec	Experimental	Baseline	.195	.007
		Yoked	.175	.006
90 sec	Experimental	Baseline	.196	.001
		Yoked	.192	<.001
120 sec	Experimental	Baseline	.199	<.001
		Yoked	.191	<.001
150 sec	Experimental	Baseline	.204	<.001
		Yoked	.182	<.001
180 sec	Experimental	Baseline	.209	<.001
		Yoked	.187	<.001
210 sec	Experimental	Baseline	.230	<.001
		Yoked	.215	<.001
240 sec	Experimental	Baseline	.196	.001
		Yoked	.182	.001
270 sec	Experimental	Baseline	.241	<.001
		Yoked	.222	<.001
300 sec	Experimental	Baseline	.249	<.001
		Yoked	.231	<.001

Table 5. Post hoc results using a Tukey HSD, showing a comparison of behavioral performance at each time point between experimental bees and their corresponding baseline and yoked counterparts.

Table 6

Gene		Visits Phase 1	Visits Phase 2	Visits Phase 3	Avg. Visit Duration Phase 1	Avg. Visit Duration Phase 2	Av. Visit Duration Phase 3	Proportion on High Concentration Phase 1	Proportion on High Concentration Phase 2	Proportion on High Concentration Phase 3
5HT2A	r	.011	-.173	.102	-.228	-.029	-.450	.198	.076	-.189
	p	.975	.610	.766	.499	.932	.165	.560	.825	.576
DOP2	r	-.100	-.176	-.037	-.178	.033	-.001	.536	.014	-.221
	p	.771	.604	.915	.601	.924	.998	.089	.968	.514
miR932	r	.106	-.158	.331	.108	.007	-.477	-.020	-.011	-.270
	p	.755	.643	.320	.753	.983	.138	.955	.976	.422

Table 6. Correlation coefficients and corresponding significance of candidate predictor variables for regression analyses using genes of interest as criterion variables.

Table 7

Experiment	Hypothesis	Gene of Interest	Expression Prediction	Supported by Data?
Experiment 1 – Shuttle Box	Learning and physiological stress will affect 5HT2A and miR932 in the same direction, while DOP2 will display differential effects of learning and physiological stress	5HT2A	Stress and learning will downregulate 5HT2A expression	Partial Support
		DOP2	Stress will downregulate DOP2 expression, learning will upregulate DOP2 expression	Partial Support
		miR932	Stress and learning will upregulate miR932 expression	Partial Support
Experiment 2 – Flower Patch	Specialist foragers will display increased expression of DOP2 and miR932, and decreased expression of 5HT2A	5HT2A	Specialist foragers will display downregulated 5HT2A when compared to generalists	Yes
		DOP2	Specialist foragers will display upregulated DOP2 when compared to generalists	Yes
		miR932	Specialist foragers will display upregulated miR932 when compared to generalists	Yes
Experiment 3 – Y-Maze	Higher performance will correspond to increased expression of DOP2 and miR932, and decreased expression of 5HT2A	5HT2A	High performing individuals will display down regulation of 5HT2A when compared to low performing individuals	No
		DOP2	High performing individuals will display up regulation of DOP2 when compared to low performing individuals	No
		miR932	High performing individuals will display up regulation of miR932 when compared to low performing individuals	Yes

Table 7. Summary of hypothesis of gene expression, including summary of which hypotheses were supported by results of each experiment

Figure 1
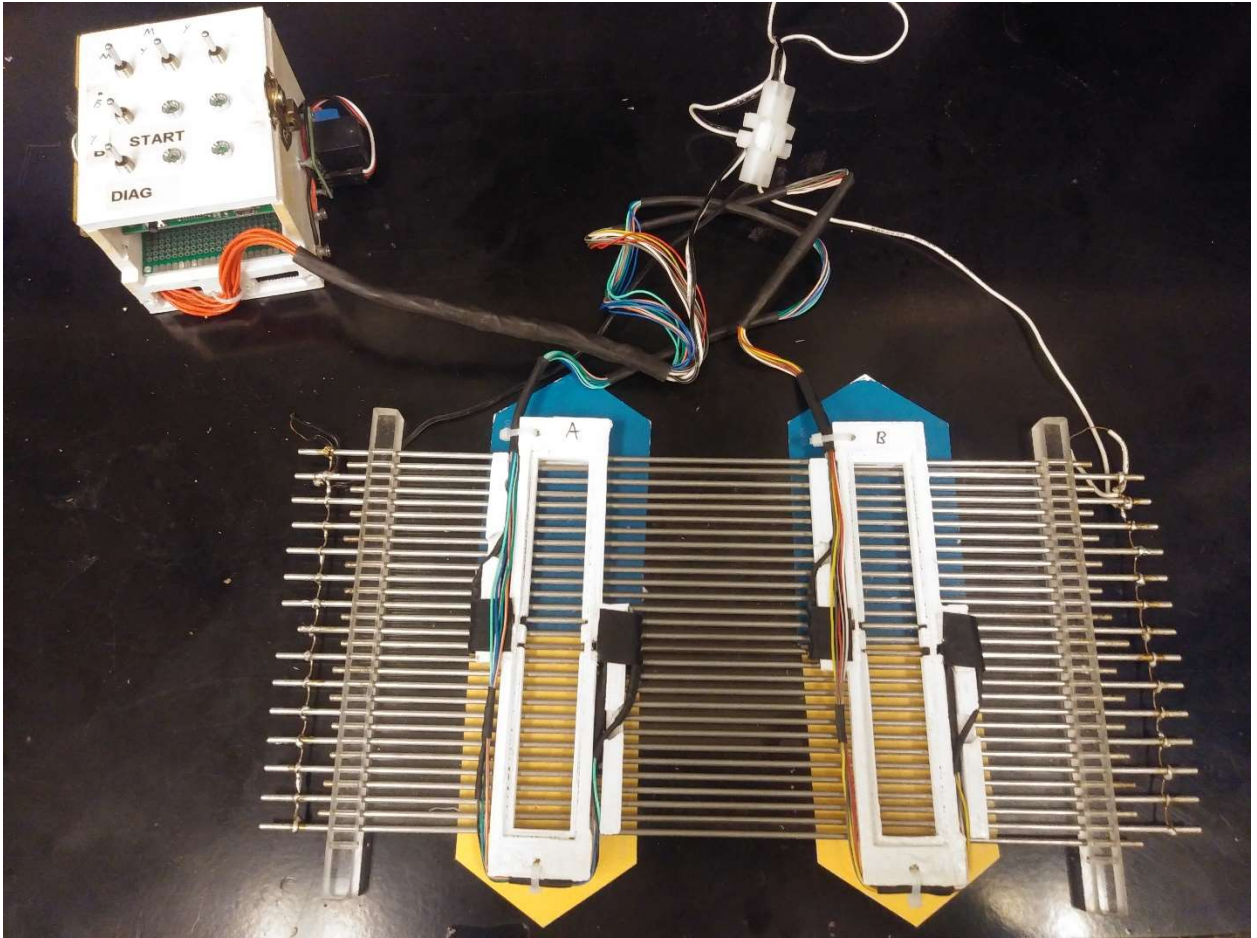
Figure 1: Shuttle box apparatus with colored visual stimuli. The experimental controller is in the upper left corner.

Figure 2
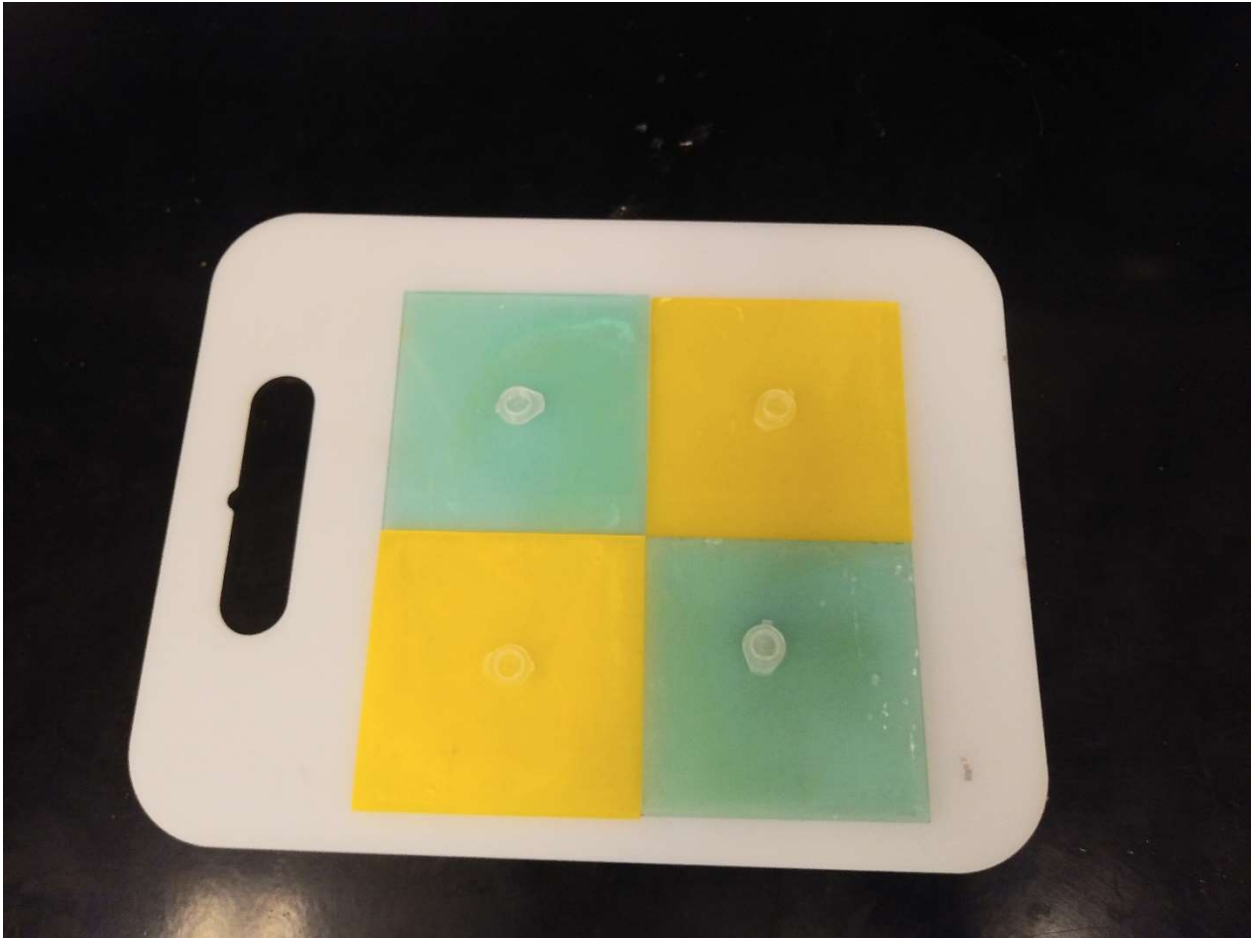
Figure 2. Artificial flower patch apparatus. Wells consist of a small reservoir and were dilled with 40 µL of either a 1.5M or 2 M sucrose solution depending upon the experimental session.

Figure 3
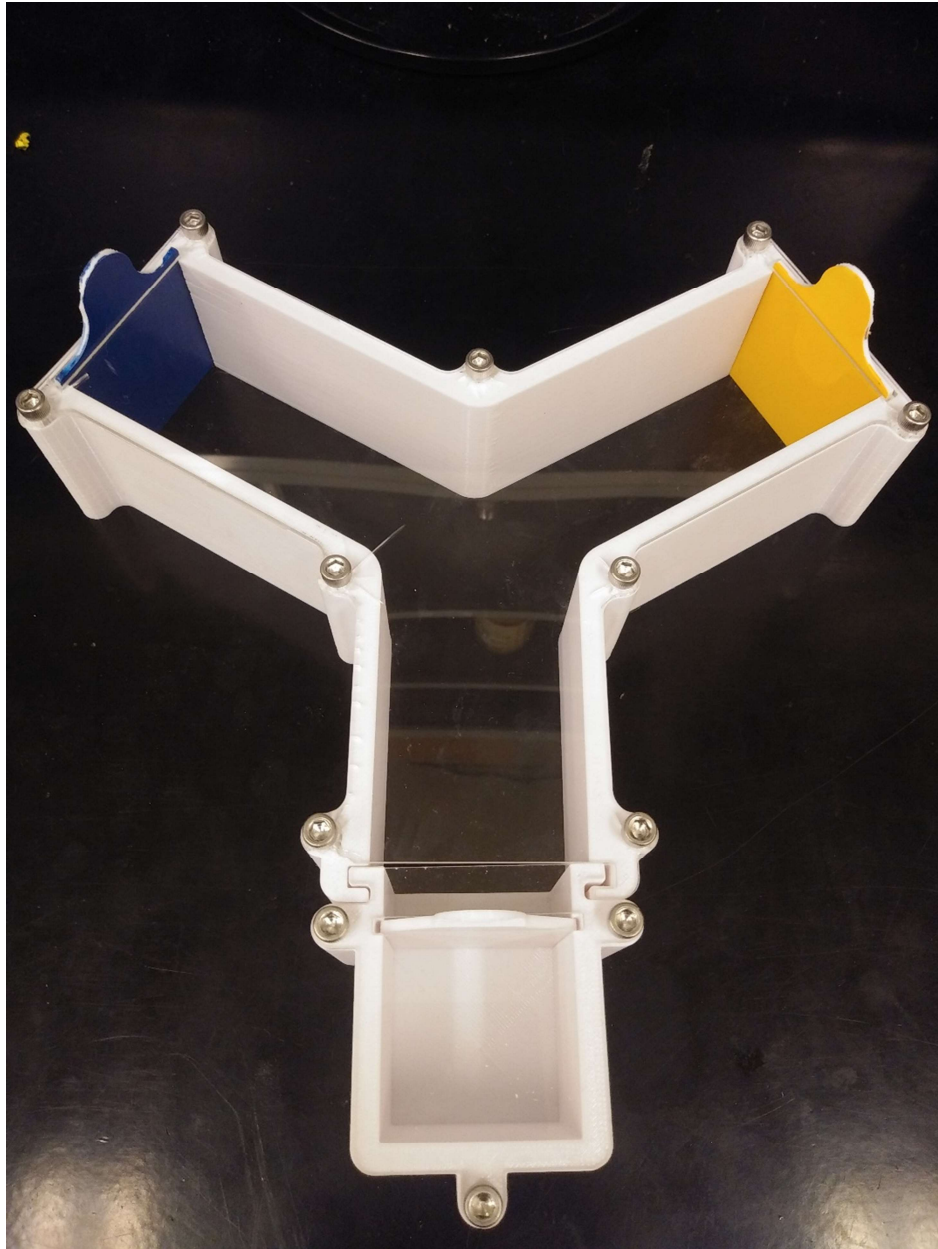
Figure 3. Y-Maze apparatus. Located at the top of the apparatus are the two choice arms with visual stimuli. Located at the bottom is the containment chamber with removable gate to allow for release and recapture of the experimental subject

Figure 4
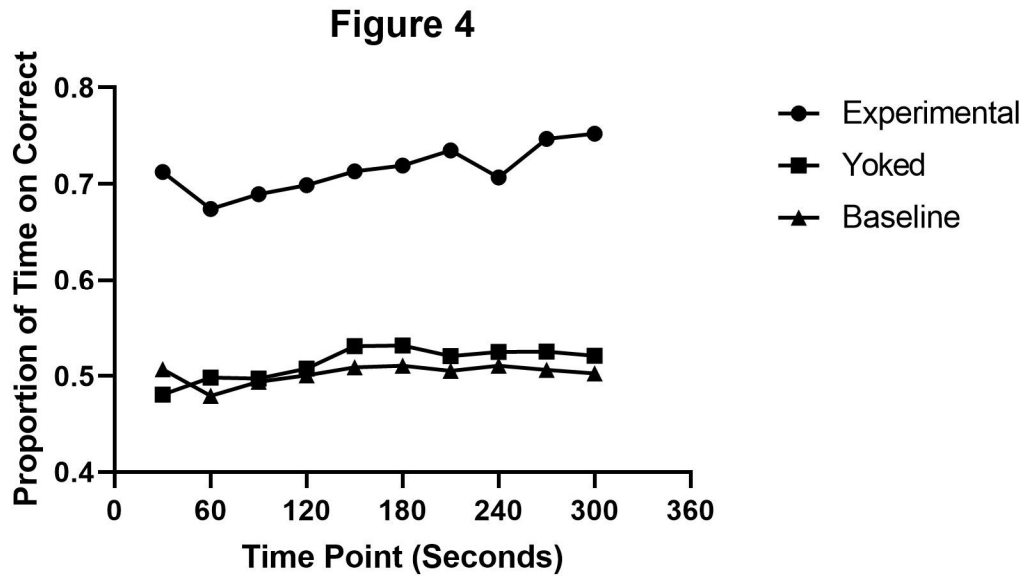
Figure 4. Behavioral results for experiment 1 showing average performance for each experimental role across all experimental time points.

Figure 5
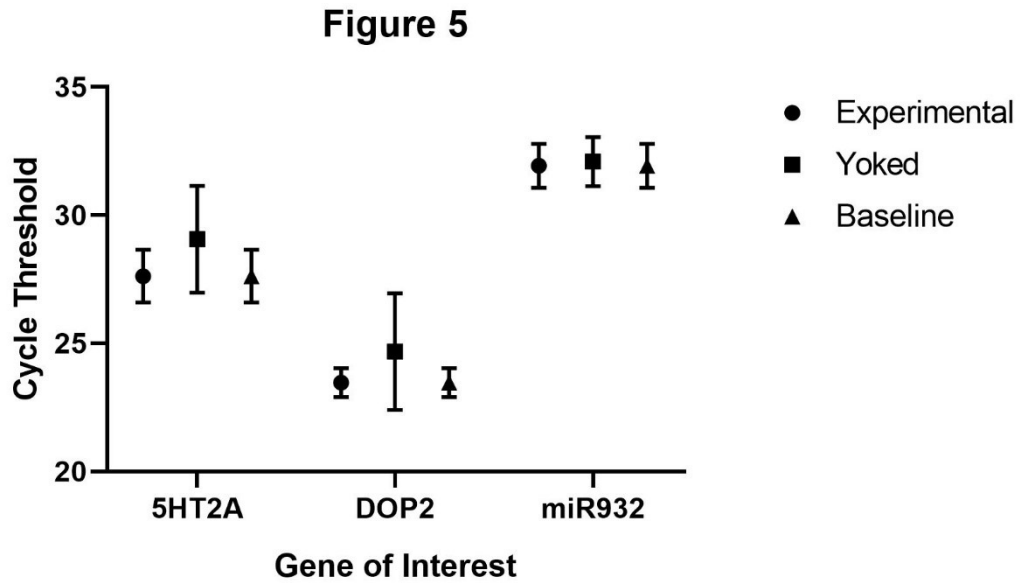
Figure 5. Expression data for each experimental role and gene of interest. Note: higher CT values correspond to lower genetic expression. Mean data are plotted with standard deviation error bars.

Figure 6
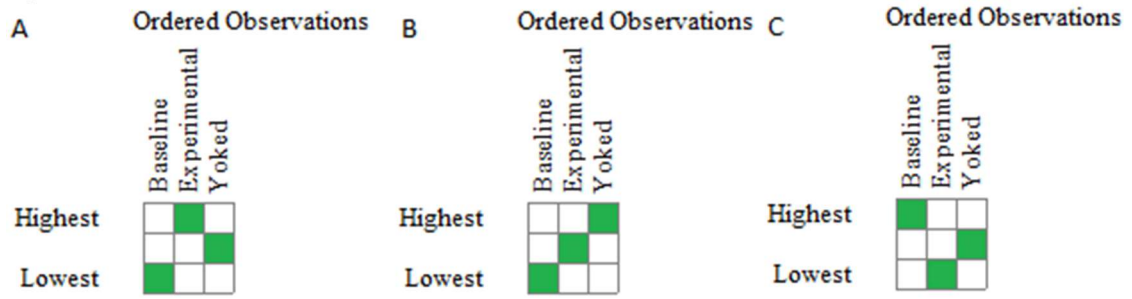
Figure 6. Crossed orderings ordinal analysis patterns for genes of interest expression based on experimental role. A) pattern of expected cycle threshold distribution for 5HT2A expression. B) pattern of expected cycle threshold distribution for DOP2. C) Pattern of expected cycle threshold distribution for miR932 expression.

Figure 7
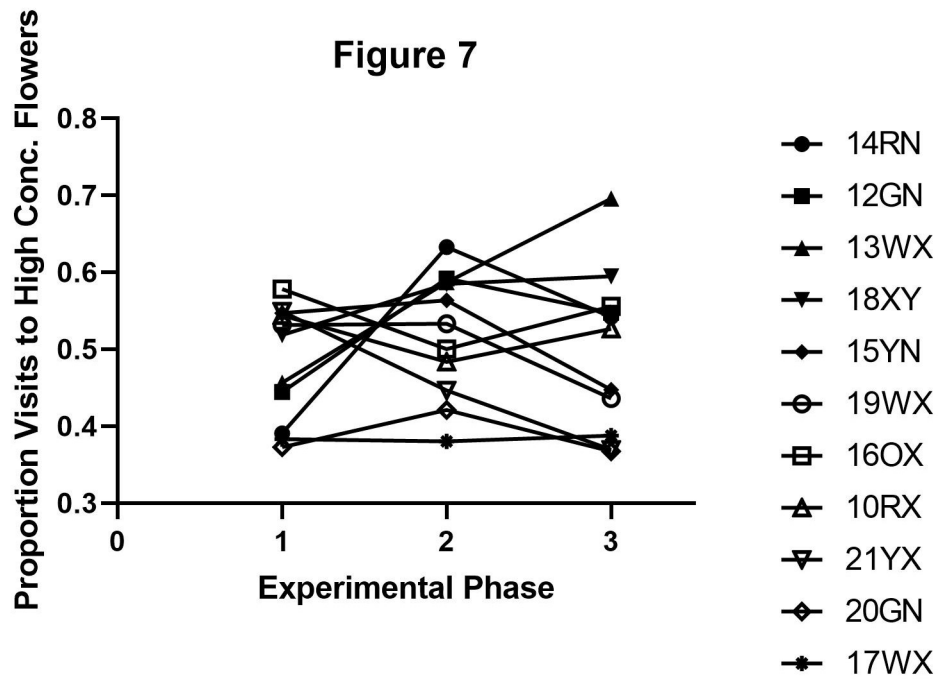
Figure 7. Proportion of visits to the wells designated to contain high sucrose concentration in phase 2 for each experimental phase. Data were used to determine individual foraging patterns and designate individuals as specialist or generalist foragers.

Figure 8
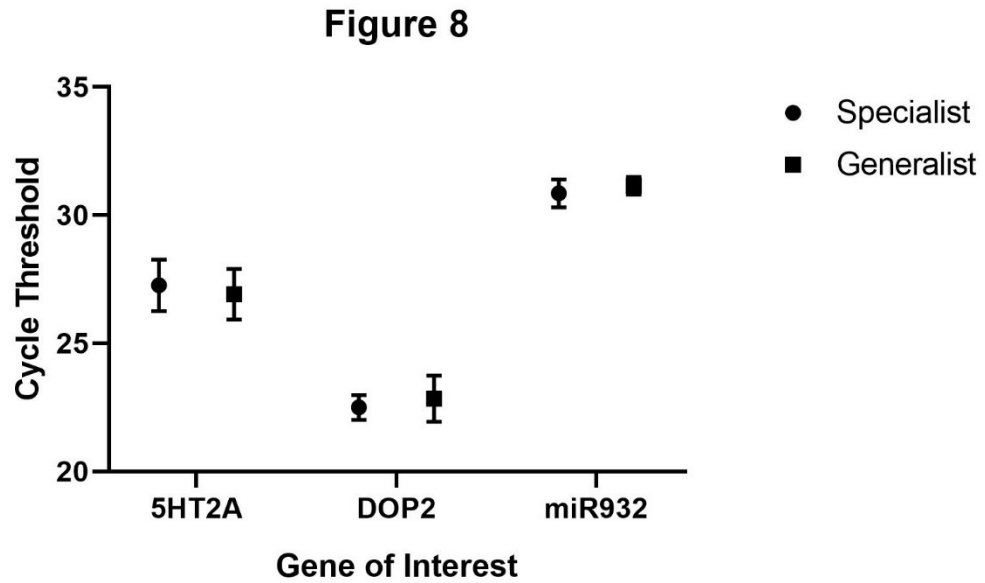
Figure 8. Gene expression data for experiment 2. Data represents average expression based on behavioral pattern presented in the foraging of specialist and generalist foragers for each gene of interest. Mean data are plotted with standard deviation error bars.

Figure 9
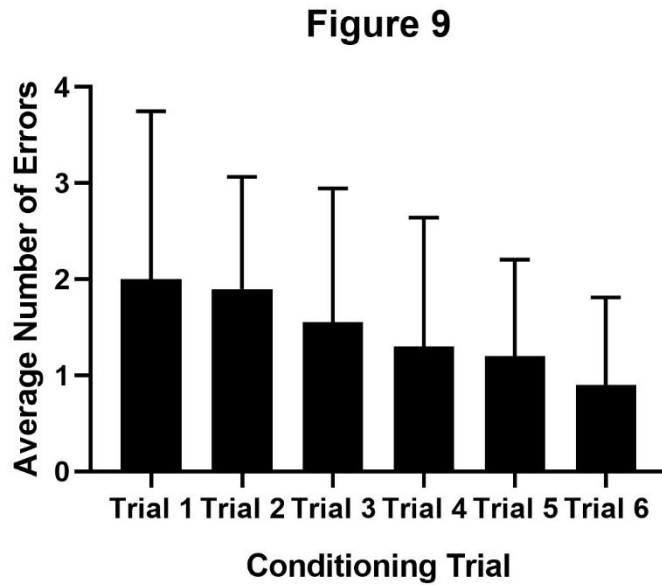
Figure 9

Figure 9. Graphical display of average number of errors in subject selection experiment 3 throughout 6 training trials. Errors were defined as drinking behavior from the well filled with a 1.5M NaCl solution. Results indicate behavior consistent with learning acquisition. Mean data are plotted with standard deviation error bars.

Figure 10
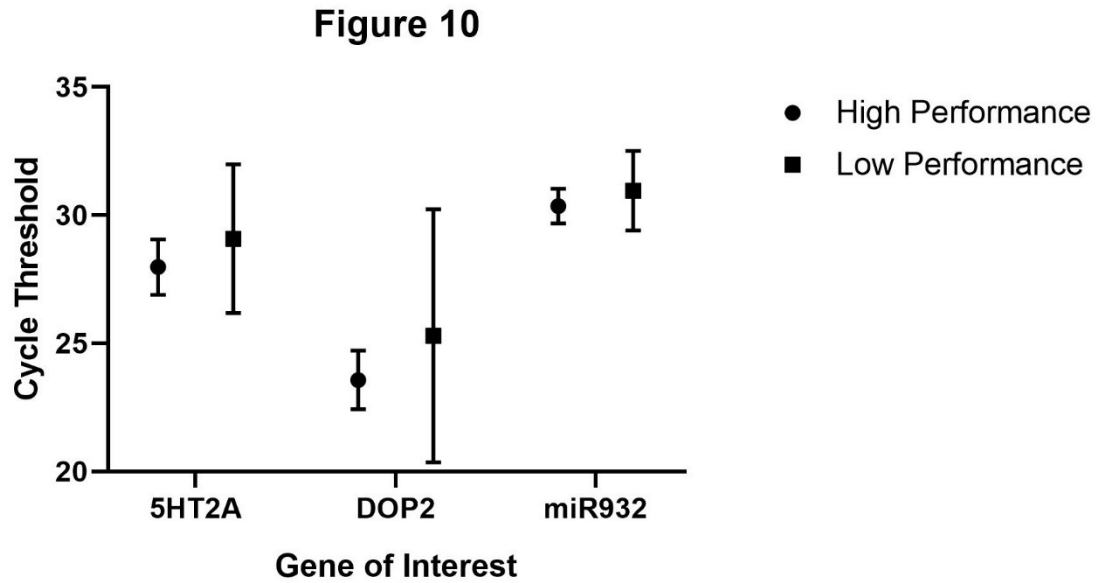
Figure 10. Gene expression data for experiment 3. Expression data presented for high performance and low performance individuals as designated by percent correct choice and average time of first interaction within test trials. Mean data are plotted with standard deviation error bars.

Appendix C: Data

Raw data of total time in seconds spent on correct portion of the apparatus for

experiment 1

Data Label	Bee	Role	Shock Color	tc.30	tc.60	tc.90	tc.120	tc.150	tc.180	tc.210	tc.240	tc.270	tc.300
MB07	A	B	B	15.994	45.994	60.561	79.468	99.786	129.786	148.81	180.538	188.565	206.566
MB11	A	B	B	15.401	28.952	51.216	71.164	89.083	103.019	118.741	137.985	155.774	176.665
MB11	B	B	B	15.631	38.115	58.416	75.943	96.32	122.467	144.704	159.572	178.027	201.139
MY04	B	B	Y	12.529	20.181	39.837	50.134	60.584	68.699	85.187	103.844	121.429	141.663
MY04	A	B	Y	25.153	38.911	57.123	74.242	100.695	120.031	124.384	124.384	146.196	165.12
MY08	B	B	Y	16.071	35.127	46.829	61.815	83.117	100.394	109.013	128.313	151.89	164.808
MY08	A	B	Y	4.256	4.256	14.291	27.277	43.394	52.757	72.996	85.755	85.755	85.755
MY10	A	B	Y	23.286	30.225	49.366	63.064	78.648	94.082	108.839	127.254	143.322	156.4
MY10	B	B	Y	15.511	36.084	51.145	73.189	98.081	117.486	133.82	155.212	167.926	182.059
YB05	A	B	B	13.9	29.485	40.478	42.72	66.143	81.653	101.2	119.188	130.847	139.323
YB05	B	B	B	18.173	38.774	53.617	73.067	93.151	105.413	121.775	142.643	156.445	171.526
YB09	B	B	B	19.651	36.962	52.455	67.893	77.544	91.915	106.887	124.923	144.811	161.144
YB09	A	B	B	15.075	30.047	42.704	53.724	63.168	77.157	91.754	106.745	113.466	120.535
YY06	B	B	Y	19.435	34.413	57.888	72.652	86.556	105.059	120.813	134.771	143.203	157.193
YY06	A	B	Y	13.495	20.145	37.254	66.348	84.635	100.024	103.584	122.874	149.986	167.345
YY11	A	B	Y	13.222	16.416	30.109	43.926	51.31	57.887	63.773	68.218	76.082	87.907
YY11	B	B	Y	1.636	4.777	12.499	24.082	24.937	34.335	47.065	61.216	69.818	78.343
MB04	A	M	B	25.971	55.761	71.199	84.613	114.631	144.299	174.299	202.504	214.937	228.571
MB05	A	M	B	24.743	54.011	82.966	112.736	142.736	172.736	202.736	231.97	252.797	262.354
MB06	A	M	B	25.035	43.133	70.148	98.352	126.4	153.919	183.919	213.919	243.919	273.919
MB08	A	M	B	26.09	56.09	85.388	115.388	145.388	174.505	199.206	226.689	256.689	286.689
MB09	A	M	B	28.876	54.808	54.808	54.808	54.808	55.397	55.397	55.397	66.378	73.199
MB10	A	M	B	23.441	37.986	58.286	72.221	90.614	104.914	130.412	147.649	170.261	197.725
MB12	A	M	B	19.128	47.965	75.974	102.761	128.021	155.506	194.473	214.045	241.831	271.831
MY05	A	M	Y	15.649	25.984	52.211	59.411	65.997	81.041	106.109	113.542	128.692	155.915
MY06	A	M	Y	22.206	33.004	49.674	79.674	109.674	139.674	169.674	199.674	229.674	259.674
MY07	A	M	Y	13.646	22.807	41.206	71.206	101.206	101.844	101.844	116.323	145.933	175.933
MY09	A	M	Y	26.246	49.796	75.975	105.975	135.975	165.975	195.975	225.975	255.539	285.539
MY11	A	M	Y	29	55.05	84.232	112.427	136.791	153.238	176.026	206.026	236.023	265.788
MY12	A	M	Y	8.313	11.831	23.58	47.832	75.724	105.724	135.724	158.494	175.147	191.256
YB04	B	M	B	25.558	34.822	44.039	61.405	81.116	103.531	131.734	161.734	180.056	208.067
YB06	B	M	B	17.607	26.187	41.612	67.278	82.99	101.695	117.166	134.493	158.75	175.051
YB07	B	M	B	20.193	41.914	65.03	79.096	94.308	120.012	148.352	175.658	203.004	230.622
YB08	B	M	B	15.034	27.83	40.36	46.878	61.197	70.59	94.483	105.519	121.711	128.576
YB10	B	M	B	21.637	51.637	69.518	94.888	105.403	124.301	141.756	158.098	177.732	204.054
YB11	B	M	B	30	59.195	89.195	109.195	139.195	169.195	209.015	234.133	264.133	294.133
YB12	B	M	B	24.186	53.108	80.834	110.354	138.6	168.6	198.6	22.017	254.121	284.121
YY04	B	M	Y	7.843	17.348	45.893	75.271	105.271	134.081	164.081	193.889	223.889	253.889
YY05	B	M	Y	25.966	39.711	63.009	85.294	115.294	133.47	144.108	163.561	182.896	193.413
YY07	B	M	Y	26.696	42.075	69.001	90.577	118.059	142.848	171.15	200.45	229.489	259.489
YY08	B	M	Y	28.285	45.434	64.321	72.222	100.261	130.261	155.094	184.473	214.473	244.473
YY09	B	M	Y	19.359	41.484	62.597	89.119	100.146	116.339	139.878	162.867	183.751	207.6
YY10	B	M	Y	5.161	22.724	52.724	81.569	111.569	141.569	171.569	200.841	230.841	256.219
MB04	B	Y	B	5.529	19.996	39.326	59.126	74.771	92.607	99.297	111.161	126.825	136.113
MB05	B	Y	B	4.972	21.517	28.625	37.417	50.48	58.111	70.995	84.463	91.104	103.545
MB06	B	Y	B	12.99	36.67	42.688	53.394	72.122	86.828	100.607	111.209	131.153	140.059
MB08	B	Y	B	8.518	18.793	26.328	49.986	73.82	90.373	103.279	118.31	137.552	147.711
MB09	B	Y	B	14.21	29.78	50.561	65.544	84.986	101.941	105.001	125.77	135.068	148.78
MB10	B	Y	B	17.488	27.774	46.492	66.431	84.473	104.218	110.845	128.742	139.944	142.117
MB12	B	Y	B	20.334	35.322	58.817	84.29	111.709	129.677	151.259	170.784	188.843	207.423
MY05	B	Y	Y	23.816	46.862	71.231	95.095	123.572	149.076	167.981	186.421	215.564	241.4
MY06	B	Y	Y	16.346	32.399	62.399	91.421	121.421	139.751	143.336	153.091	167.489	184.378
MY07	B	Y	Y	16.697	30.494	47.162	64.244	81.756	89.124	110.213	121.818	141.536	157.523
MY09	B	Y	Y	21.405	44.21	67.164	89.804	116.663	139.592	154.763	179.654	197.568	218.86
MY11	B	Y	Y	18.366	23.817	43.851	58.085	67.264	81.484	99.729	112.793	124.499	138.28
MY12	B	Y	Y	18.052	26.625	39.647	58.343	75.121	94.493	110.081	126.125	140.997	143.384
YB04	A	Y	B	7.618	24.594	38.044	45.936	65.423	79.416	96.442	115.687	136.68	149.379
YB06	A	Y	B	20.476	41.589	51.611	61.056	72.363	88.795	96.561	112.299	130.71	130.71
YB07	A	Y	B	19.611	43.278	59.738	76.935	101.392	128.467	165.843	180.82	208.19	238.19
YB08	A	Y	B	13.734	27.853	34.146	44.835	66.84	85.203	89.878	119.878	149.878	170.985
YB10	A	Y	B	8.676	30.445	47.208	60.647	72.253	78.539	85.938	91.167	106.876	133.29
YB11	A	Y	B	22.309	39.995	54.168	57.288	66.244	79.939	90.572	101.7	119.04	135.121
YB12	A	Y	B	2.245	2.267	6.293	35.067	54.026	54.026	69.669	88.861	103.76	118.433
YY04	A	Y	Y	15.617	28.643	46.259	59.396	77.784	95.884	109.963	127.129	139.551	158.96
YY05	A	Y	Y	10.947	22.825	29.674	29.674	29.674	29.674	39.487	60.12	61.8	91.099
YY07	A	Y	Y	13.383	29.492	38.15	54.602	76.044	93.696	106.122	117.581	124.69	128.741
YY08	A	Y	Y	1.756	15.408	33.075	50.196	80.196	110.196	121.309	144.958	156.736	166.526
YY09	A	Y	Y	20.862	38.117	50.342	68.068	82.673	101.818	121.207	142.588	154.885	159.506
YY10	A	Y	Y	18.958	38.61	50.526	66.432	87.752	104.852	120.836	142.425	156.292	172.563

Note: Role B refers to baseline bees, M to experimental bees, and Y to Yoked bees.

Raw data for proportion of time spent on correct portion of the apparatus in experiment

1

Data Label	Bee	Role	Shock Color	pc.30	pc.60	pc.90	pc.120	pc.150	pc.180	pc.210	pc.240	pc.270	pc.300
MB07	A	B	B	0.533133	0.766567	0.6729	0.662233	0.66524	0.721033	0.708619	0.752242	0.698389	0.688553
MB11	A	B	B	0.513367	0.482533	0.569067	0.593033	0.593887	0.572328	0.565433	0.574938	0.576941	0.588883
MB11	B	B	B	0.521033	0.63525	0.649067	0.632858	0.642133	0.680372	0.689067	0.664883	0.659359	0.670463
MY04	B	B	Y	0.417633	0.33635	0.442633	0.417783	0.403893	0.381661	0.405652	0.432683	0.449737	0.47221
MY04	A	B	Y	0.838433	0.648517	0.6347	0.618683	0.6713	0.666839	0.592305	0.518267	0.541467	0.5504
MY08	B	B	Y	0.5357	0.58545	0.520322	0.515125	0.554113	0.557744	0.51911	0.534638	0.562556	0.54936
MY08	A	B	Y	0.141867	0.070933	0.158789	0.227308	0.289293	0.293094	0.3476	0.357313	0.317611	0.28585
MY10	A	B	Y	0.7762	0.50375	0.548511	0.525533	0.52432	0.522678	0.518281	0.530225	0.530822	0.521333
MY10	B	B	Y	0.517033	0.6014	0.568278	0.609908	0.653873	0.6527	0.637238	0.646717	0.621948	0.606863
YB05	A	B	B	0.463333	0.491417	0.449756	0.356	0.440953	0.453628	0.481905	0.496617	0.484619	0.46441
YB05	B	B	B	0.605767	0.646233	0.595744	0.608892	0.621007	0.585628	0.579881	0.594346	0.579426	0.571753
YB09	B	B	B	0.655033	0.616033	0.582833	0.565775	0.51696	0.510639	0.508986	0.520513	0.536337	0.537147
YB09	A	B	B	0.5025	0.500783	0.474489	0.4477	0.42112	0.42865	0.436924	0.444771	0.420244	0.401783
YY06	B	B	Y	0.647833	0.57355	0.6432	0.605433	0.57704	0.583661	0.5753	0.561546	0.530381	0.523977
YY06	A	B	Y	0.449833	0.33575	0.413933	0.5529	0.564233	0.555689	0.493257	0.511975	0.555504	0.557817
YY11	A	B	Y	0.440733	0.2736	0.334544	0.36605	0.342067	0.321594	0.303681	0.284242	0.281785	0.293023
YY11	B	B	Y	0.054533	0.079617	0.138878	0.200683	0.166247	0.19075	0.224119	0.255067	0.258585	0.261143
MB04	A	M	B	0.8657	0.92935	0.7911	0.705108	0.764207	0.801661	0.829995	0.843767	0.796063	0.761903
MB05	A	M	B	0.824767	0.900183	0.921844	0.939467	0.951573	0.959644	0.96541	0.966542	0.936285	0.874513
MB06	A	M	B	0.8345	0.718883	0.779422	0.8196	0.842667	0.855106	0.875805	0.891329	0.903404	0.913063
MB08	A	M	B	0.869667	0.934833	0.948756	0.961567	0.969253	0.969472	0.9486	0.944538	0.9507	0.95563
MB09	A	M	B	0.962533	0.913467	0.608978	0.456733	0.365387	0.307761	0.263795	0.230821	0.245844	0.243997
MB10	A	M	B	0.781367	0.6331	0.647622	0.601842	0.604093	0.582856	0.62101	0.615204	0.630596	0.659083
MB12	A	M	B	0.6376	0.799417	0.844156	0.856342	0.853473	0.863922	0.926062	0.891854	0.89567	0.906103
MY05	A	M	Y	0.521633	0.433067	0.580122	0.495092	0.43998	0.450228	0.505281	0.473092	0.476637	0.519717
MY06	A	M	Y	0.7402	0.550067	0.551933	0.66395	0.73116	0.775967	0.807971	0.831975	0.850644	0.86558
MY07	A	M	Y	0.454867	0.380117	0.457844	0.593383	0.674707	0.5658	0.484971	0.484679	0.540493	0.586443
MY09	A	M	Y	0.874867	0.829933	0.844167	0.883125	0.9065	0.922083	0.933214	0.941563	0.946441	0.951797
MY11	A	M	Y	0.966667	0.9175	0.935911	0.936892	0.91194	0.851322	0.838219	0.858442	0.874159	0.88596
MY12	A	M	Y	0.2771	0.197183	0.262	0.3986	0.504827	0.587356	0.646305	0.660392	0.648693	0.63752
YB04	B	M	B	0.851933	0.580367	0.489322	0.511708	0.540773	0.575172	0.627305	0.673892	0.666874	0.693557
YB06	B	M	B	0.5869	0.43645	0.462356	0.56065	0.553267	0.564972	0.557933	0.560388	0.587963	0.583503
YB07	B	M	B	0.6731	0.698567	0.722556	0.659133	0.62872	0.666733	0.706438	0.731908	0.751867	0.76874
YB08	B	M	B	0.501133	0.463833	0.448444	0.39065	0.40798	0.392167	0.449919	0.439663	0.450781	0.428587
YB10	B	M	B	0.721233	0.860617	0.772422	0.790733	0.702687	0.690561	0.675029	0.658742	0.658267	0.68018
YB11	B	M	B	1	0.986583	0.991056	0.909958	0.927967	0.939972	0.99531	0.975554	0.97827	0.980443
YB12	B	M	B	0.8062	0.885133	0.898156	0.919617	0.924	0.936667	0.945714	0.091738	0.941189	0.94707
YY04	B	M	Y	0.261433	0.289133	0.509922	0.627258	0.701807	0.744894	0.781338	0.807871	0.829219	0.846297
YY05	B	M	Y	0.865533	0.66185	0.7001	0.710783	0.768627	0.7415	0.686229	0.681504	0.677393	0.64471
YY07	B	M	Y	0.889867	0.70125	0.766678	0.754808	0.78706	0.7936	0.815	0.835208	0.849959	0.864963
YY08	B	M	Y	0.942833	0.757233	0.714678	0.60185	0.668407	0.723672	0.738543	0.768638	0.794344	0.81491
YY09	B	M	Y	0.6453	0.6914	0.695522	0.742658	0.66764	0.646328	0.666086	0.678613	0.680559	0.692
YY10	B	M	Y	0.172033	0.378733	0.585822	0.679742	0.743793	0.786494	0.816995	0.836838	0.854967	0.854063
MB04	B	Y	B	0.1843	0.333267	0.436956	0.492717	0.498473	0.514483	0.472843	0.463171	0.469722	0.45371
MB05	B	Y	B	0.165733	0.358617	0.318056	0.311808	0.336533	0.322839	0.338071	0.351929	0.337422	0.34515
MB06	B	Y	B	0.433	0.611167	0.474311	0.44495	0.480813	0.482378	0.479081	0.463371	0.485752	0.466863
MB08	B	Y	B	0.283933	0.313217	0.292533	0.41655	0.492133	0.502072	0.491805	0.492958	0.509452	0.49237
MB09	B	Y	B	0.473667	0.496333	0.561789	0.5462	0.566573	0.500005	0.524042	0.500252	0.495933
MB10	B	Y	B	0.582933	0.4629	0.516578	0.553592	0.563153	0.578989	0.527833	0.536425	0.518311	0.473723
MB12	B	Y	B	0.6778	0.5887	0.653522	0.702417	0.744727	0.720428	0.720281	0.7116	0.699419	0.69141
MY05	B	Y	Y	0.793867	0.781033	0.791456	0.792458	0.823813	0.8282	0.79991	0.776754	0.798385	0.804667
MY06	B	Y	Y	0.544867	0.539983	0.693322	0.761842	0.809473	0.776394	0.682552	0.637879	0.62033	0.614593
MY07	B	Y	Y	0.556567	0.508233	0.524022	0.535367	0.54504	0.495133	0.524824	0.507575	0.524207	0.525077
MY09	B	Y	Y	0.7135	0.736833	0.746267	0.748367	0.777753	0.775511	0.736967	0.748558	0.731733	0.729533
MY11	B	Y	Y	0.6122	0.39695	0.487233	0.484042	0.448427	0.452689	0.4749	0.469971	0.461107	0.460933
MY12	B	Y	Y	0.601733	0.44375	0.440522	0.486192	0.500807	0.524961	0.524195	0.525521	0.522211	0.477947
YB04	A	Y	B	0.253933	0.4099	0.422711	0.3828	0.436153	0.4412	0.459248	0.482029	0.506222	0.49793
YB06	A	Y	B	0.682533	0.69315	0.573456	0.5088	0.48242	0.493306	0.459814	0.467913	0.484111	0.4357
YB07	A	Y	B	0.6537	0.7213	0.663756	0.641125	0.675947	0.713706	0.789729	0.753417	0.771074	0.793967
YB08	A	Y	B	0.4578	0.464217	0.3794	0.373625	0.4456	0.47335	0.42799	0.499492	0.555104	0.56995
YB10	A	Y	B	0.2892	0.507417	0.524533	0.505392	0.481687	0.436328	0.409229	0.379863	0.395837	0.4443
YB11	A	Y	B	0.743633	0.666583	0.601867	0.4774	0.441627	0.444106	0.431295	0.42375	0.440889	0.450403
YB12	A	Y	B	0.074833	0.037783	0.069922	0.292225	0.360173	0.300144	0.331757	0.370254	0.384296	0.394777
YY04	A	Y	Y	0.520567	0.477383	0.513989	0.494967	0.51856	0.532689	0.523633	0.529704	0.516856	0.529867
YY05	A	Y	Y	0.3649	0.380417	0.329711	0.247283	0.197827	0.164856	0.188033	0.2505	0.228889	0.303663
YY07	A	Y	Y	0.4461	0.491533	0.423889	0.455017	0.50696	0.520533	0.505343	0.489921	0.461815	0.429137
YY08	A	Y	Y	0.058533	0.2568	0.3675	0.4183	0.53464	0.6122	0.577662	0.603992	0.580504	0.555087
YY09	A	Y	Y	0.6954	0.635283	0.559356	0.567233	0.551153	0.565656	0.577176	0.594117	0.573648	0.531687
YY10	A	Y	Y	0.631933	0.6435	0.5614	0.5536	0.585013	0.582511	0.57541	0.593438	0.578859	0.57521

Note: Role B refers to baseline bees, M to experimental bees, and Y to Yoked bees.

Raw data for average gene expression of selected individuals for experiment 1

Data Label	Bee	Role	Shock Color	5HT2A.AVG	DOP2.AVG	MIR932.AVG
MB07	A	B	B			
MB11	A	B	B	26.30333	22.48333	32.28
MB11	B	B	B			
MY04	B	B	Y	26.985	23.35	30.64
MY04	A	B	Y	28.70333	24.14	32.52667
MY08	B	B	Y	26.58	23.13	31.35333
MY08	A	B	Y			
MY10	A	B	Y	28.44	23.88	33.14333
MY10	B	B	Y			
YB05	A	B	B	29.27	24.12333	32.47
YB05	B	B	B			
YB09	B	B	B	28.09667	23.86667	30.70333
YB09	A	B	B			
YY06	B	B	Y	27.11333	23.11333	32.10667
YY06	A	B	Y	27.11333	23.11333	32.10667
YY11	A	B	Y			
YY11	B	B	Y			
MB04	A	M	B	31.78333	26.5	33.43
MB05	A	M	B			
MB06	A	M	B	27.52	23.35	30.96333
MB08	A	M	B	28.9	24.24667	31.60333
MB09	A	M	B			
MB10	A	M	B			
MB12	A	M	B			
MY05	A	M	Y			
MY06	A	M	Y	27.3	28.87	31.32333
MY07	A	M	Y			
MY09	A	M	Y			
MY11	A	M	Y	28.02	23.95667	31.625
MY12	A	M	Y			
YB04	B	M	B			
YB06	B	M	B			
YB07	B	M	B			
YB08	B	M	B			
YB10	B	M	B			
YB11	B	M	B	26.61667	22.96	31.18333
YB12	B	M	B			
YY04	B	M	Y	28.80333	24.05667	31.74
YY05	B	M	Y			
YY07	B	M	Y	27.79667	22.87333	33.12333
YY08	B	M	Y	32.13	27.61667	32.75333
YY09	B	M	Y			
YY10	B	M	Y	27.77	22.87	31.69
MB04	B	Y	B	28.88	24.45	32.01667
MB05	B	Y	B			
MB06	B	Y	B	28.69	24.025	31.39
MB08	B	Y	B	27.25	23.86667	31.13333
MB09	B	Y	B			
MB10	B	Y	B			
MB12	B	Y	B			
MY05	B	Y	Y			
MY06	B	Y	Y	30.97667	27.33	33.08333
MY07	B	Y	Y			
MY09	B	Y	Y			
MY11	B	Y	Y	27.58	23.06	30.74333
MY12	B	Y	Y			
YB04	A	Y	B			
YB06	A	Y	B			
YB07	A	Y	B			
YB08	A	Y	B			
YB10	A	Y	B			
YB11	A	Y	B	30.49	24.95333	32.57667
YB12	A	Y	B			
YY04	A	Y	Y	27.22	22.77	33.3
YY05	A	Y	Y			
YY07	A	Y	Y	28.38667	23.31667	32.58667
YY08	A	Y	Y	33.68	23.08	30.98667
YY09	A	Y	Y			
YY10	A	Y	Y	27.50333	29.935	33.06

Note: Role B refers to baseline bees, M to experimental bees, and Y to Yoked bees.

Raw behavioral data for experiment 2

Subject.Label	Phase 2 Condition	Phase 1 visits	Phase 2 visits	Phase 3 visits	Phase 1 interactions	Phase 1 on correct	pc. phase1	P1.Avg. visit.duration	Phase 2 itneractions	Phase 2 on correct	pc. phase2	P2.Avg. visit.duration	Phase 3 Interactions	Phase 3 on correct	pc. phase3	P3.avg.visit.duration
14RN	1	17	16	20	87	34	0.390805	98.067	79	50	0.632911	72.063	96	52	0.541667	59.947
12GN	1	17	19	19	108	48	0.444444	63	125	74	0.592	60.211	148	81	0.547297	59
13WX	2	18	20	20	92	42	0.456522	54.706	75	44	0.586667	48.4	46	32	0.695652	50.55
18XY	2	12	13	13	54	28	0.518519	83.833	65	38	0.584615	67.231	74	44	0.594595	75.462
15YN	1	14	17	19	64	35	0.546875	66.5	71	40	0.56338	59	76	34	0.447368	85.316
19WX	1	8	10	12	30	16	0.533333	81.625	32	17	0.53125	72.9	39	17	0.435897	64
16OX	2	12	11	15	64	37	0.578125	98.083	64	32	0.5	81.462	81	45	0.555556	70.368
10RX	1	11	18	14	57	31	0.54386	70.455	91	44	0.483516	55.5	95	50	0.526316	76.286
21YX	2	11	11	13	51	28	0.549020	81.909	56	25	0.446429	66.909	46	17	0.369565	60.538
20GN	1	11	12	16	51	19	0.372549	97.091	38	16	0.421053	61.667	49	18	0.367347	51.313
17WX	1	12	16	11	60	23	0.383333	79.333	61	11	0.180328	56.813	49	19	0.387755	66.545

Raw data for gene expression in experiment 2

Subject.Label	5HT2A.AVG	DOP2.AVG	MIR932.AVG	Pattern
14RN	25.82333	21.86	30.99	1
12GN	28.18	22.99333	30.74667	1
13WX	27.54	22.68667	31.33667	1
18XY	26.11667	22.17	30.03333	1
15YN	26.84	22.99	31.10667	1
19WX	27.12667	22.23333	30.72	2
16OX	27.16333	23.09	31.20667	2
10RX	26.22333	22.79667	30.62667	2
21YX	28.895	24.63667	31.71667	2
20GN	27.515	22.47333	31.52333	1
17WX	26.05	21.98333	30.54333	2

Note: pattern 1 refers to specialist foragers, while pattern 2 refers to generalist foragers

Raw behavioral data for experiment 3

Subject.ID	Sugar.Start	Correct.Color	Visit.count.Tr1	Correct.visit.Tr1	Visit.count.Tr2	Correct.visit.Tr2	Visit.count.Tr3	Correct.visit.Tr3	Visit.count.Tr4	Correct.visit.Tr4	Visit.count.Tr5	Correct.visit.Tr5	Visit.count.Tr6	Correct.visit.Tr6	Test Trials visited	Correct in test	pc.int est	Avg time of first visit.TT
2BR5	1	1	2	1	1	1	2	1	1	0	2	0	2	1	6	5	0.83333	97.833
2BL5	2	1	3	0	5	4	2	2	1	1	2	1	4	2	5	5	1	89.2
2YL4	2	2	9	4	7	3	4	2	3	2	2	0	4	4	4	4	1	40.75
2BL3	2	1	3	1	5	3	3	2	1	1	2	1	2	1	6	4	0.66667	35
2YR1	1	2	6	4	3	2	2	1	2	1	4	4	3	2	6	4	0.66667	63.333
2BR2	1	1	7	2	6	4	2	0	1	0	1	1	2	1	6	3	0.5	130.667
2BR1	1	1	1	1	3	0	1	0	4	1	3	2	3	2	6	2	0.33333	71.333
2YR3	1	2	2	2	2	1	3	2	3	3	2	0	5	2	6	2	0.33333	94.833
2BR4	1	1	5	5	3	2	2	0	1	1	1	1	1	1	4	2	0.5	113.5
2YL5	2	2	5	2	6	3	9	3	3	0	8	7	2	2	6	1	0.16667	70.833
2YR5	1	2	7	6	8	6	4	3	3	2	2	2	1	1	6	4	0.66667	64.667
2YL1	2	2	9	7	7	5	5	4	1	1	6	3	3	1	6	4	0.66667	95.333
2YL2	2	2	3	2	5	1	5	2	3	1	1	1	3	2	6	4	0.66667	108.5
2BL4	2	1	6	1	5	4	5	2	7	4	3	2	3	1	6	4	0.66667	124.5
2BL1	2	1	5	1	2	1	2	0	2	1	3	1	2	2	5	4	0.8	164.2
2BR3	1	1	2	2	2	0	3	1	3	1	2	2	1	0	6	4	0.66667	179.167
2BL2	2	1	5	5	1	0	2	2	1	1	4	2	0	0	6	3	0.5	83
2YL3	2	2	10	7	6	4	5	3	8	3	9	6	1	1	6	3	0.5	85
2YR2	1	2	4	3	3	2	1	1	3	2	2	1	0	0	6	3	0.5	107
2YR4	1	2	3	1	5	1	1	1	3	2	3	1	3	1	5	3	0.6	128

Note: Sugar.Start 1 indicates sucrose/color paring was located to the left in trial 1.
Correct.Color 1 indicates sucrose was paired with yellow.

Raw data for gene expression in experiment 3

Subject.ID	5HT2A.AVG	DOP2.AVG	MIR932.AVG	Performance
2BR5	27.54333	22.845	30.50333	1
2BL5	27.20333	22.95667	30.74667	1
2YL4	27.47667	23.18	30.37667	1
2BL3	27.79667	23.28667	29.21	1
2YR1	29.87333	25.59333	30.92667	1
2BR2	32.74	33.7	28.63333	2
2BR1	27.14	21.98	31.33667	2
2YR3	31.54667	25.75333	32.86	2
2BR4	26.09333	22.76667	30.52	2
2YL5	27.86667	22.24667	31.43333	2
2YR5				
2YL1				
2YL2				
2BL4				
2BL1				
2BR3				
2BL2				
2YL3				
2YR2				
2YR4				

Note: Performance 1 indicates designation as a high performance individual

VITA

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Black, T. E., Fofah, O., Giray, T., Wells, H., Le Conte, Y., & Abramson, C. I. (2018). Influence of environmental experience on aversive conditioning in honey bees (Apis mellifera L.). Apidologie, 49(5), 647-659.

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Society for Neuroscience   2019-present