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Effects of aversive conditioning on expression of physiological stress in honey bees (*Apis mellifera*)

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ABSTRACT

Stress is defined as any deviation from an organism's baseline physiological levels. Therefore, introduction of new stimuli and information, such as in learning, can be defined as a stressor. 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 study used a wide variety of learning centric stress responses. Researchers examined changes in expression of ten stress and learning related genes in various physiological systems in domesticated honey bees (*Apis mellifera*) as a result of exposure to an aversive conditioning task. Gene expression was examined using quantitative real-time polymerase chain reaction following the learning task. Results indicate that learning affects expression of some stress related genes.

1. Introduction

The purpose of this experiment is to examine the effects of aversive conditioning on the expression of a number of neural proteins in the honey bee brain. This study seeks to understand the effects of physiological stress in association with the acquisition of new behaviors in an invertebrate model. Currently, there is little research indicating the effect learning plays on the expression of physiological stress markers in vertebrates, and no literature examining these effects in invertebrate brains. Aversive conditioning was selected for this study as a method of tiered experimental control to observe differences between gross physiological stress, and stress in the context of learning.

Stress is defined as any deviation from an organism's baseline physiological levels (Selye, 1950). Along with these deviations, organisms possess a suite of adaptations in order to combat stressors, and return physiological systems to their baseline levels in the general adaptation syndrome (Selye, 1950). This syndrome includes a wide variety of adaptations including cellular changes (Kültz, 2005), proteomic shifts (Hranitz, Abramson, & Carter, 2010; Kültz, 2005), neural adaptations (Niewalda et al., 2015), and hormonal alterations (Kapan, Lushchak, Luo, & Nässel, 2012; Sapolsky & Meaney, 1986). These varied physiological responses to stress are necessary due to the wide range of stressors potentially altering an organism's homeostatic balance. For example, a startling stimuli may not pose a threat to cellular structure, but must evoke a rapid shift in an organism's hormonal profile in order to maintain survival (Sapolsky, 1990). Conversely, a virus attempting to invade a cell elicits a cellular stress response, while potentially not causing organismal stress responses (Kültz, 2005). Due to this, it is important to select the appropriate physiological measure when examining stress responses.

Considering stress as any deviation from baseline levels, behavioral and neurological processes that result in physiological changes may also be classified as such. In particular, learning has been linked to the generation of a number of neurological changes, including development of new neural connections, new cellular projections, and increased synaptic activity (Houweling, Daffertshofer, Van Dijk, & Beek, 2008; Kami et al., 1995; Kelly & Garavan, 2005; Lisberger, 1988). As such, learning is known to exert a physiological toll on the cells involved in the creation or modification of the neural pathways involved. Similar physiological occurrences, such as changes in neurohormonal sensitivity and alterations in neuronal protein structures have been observed as a result of stress exposure (Kapan et al., 2012; Niewalda et al., 2015; Sapolsky & Meaney, 1986). This suggests that the link between stress and learning may be more dynamic than previously thought.

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For example, Lisberger (1988) noted distinct changes in the firing rates of neurons controlling eye movements in non-human primates trained to an eye movement task after learning of the task was complete. This result suggests that the cells involved can make a physiological change to respond more efficiently to a given stimulus. Likewise, this suggests an increased rate of cellular metabolism, as more energy must be expended in order to maintain a higher rate of neuronal activity. This in turn, opens the cells up to a higher rate of oxidative stress as a result of the increased metabolism.

Additional studies have corroborated this effect using magnetic resonance imaging (MRI) and electroencephalograph (EEG) readings (Houweling et al., 2008; Kami et al., 1995; Kelly & Garavan, 2005) in regards to neural pathways corresponding to repeated motor skills in humans. As a result, we can argue that neural systems are extremely plastic, and subject to conformational and chemoreceptive changes as a result of learning.

In the psychological literature, there exist a number of studies suggesting that the expression of physiological stress may alter an individual's ability to learn. The nature of the stress has been shown to both increase and decrease recall depending on the task, the timing of the stressor in relation to the task, and the organism engaged in learning (Joëls, Pu, Wiegert, Oitzl, & Krugers, 2006; Kim & Diamond, 2002; Smeets et al., 2009).

A consistent finding regarding the role of stress in learning performance is that of dose dependency. Studies conducted in both rodents and humans show that moderate levels of stress are likely to improve learning performance, while both high and low levels are likely to have an impairing effect (Joëls et al., 2006; Kim & Diamond, 2002).

Using a Morris water maze, Sandi, Loscertales, and Guaza (1997) conducted a study in rats to examine the effect of cortisol in conjunction with performance. Results indicated that animals which had received a dosage of cortisol displayed higher learning acquisition and better recall over time than control animals. In addition, when examining the effects of environment, the researchers noted that animals subjected to cooler water temperatures expressed better performance, and higher levels of cortisol post-trial. These results suggest that the glucocorticoid system in vertebrates, and its homologous system in invertebrates, may promote increased learning performance when subjects are placed under moderate stress.

Despite this wealth of literature proposing stress impairs or enhances learning, fairly little research has been conducted examining the relationship in the reverse. The current study proposes to fill this gap by examining the effects of a learning protocol on a suite of physiological stress markers, while controlling for levels of stress across subjects.

Honey bees (*Apis mellifera*) offer a unique opportunity to explore the junction of stress and learning, as they are a model system for many learning paradigms within the study of insect behavior. Behaviors such as foraging choice (Amaya-Márquez, Hill, Abramson, & Wells, 2014; Hill, Wells, & Wells, 1997; Menzel & Erber, 1978; Menzel, 1999), avoidance (Agarwal et al., 2011; Dinges, Varnon, Cota, Slykerman, & Abramson, 2017), appetitive and aversive conditioning (Abramson, 1986; Abramson, Mixson, Çakmak, Place, & Wells, 2008; Dinges et al., 2013) can all be readily observed within honey bees and their various subspecies.

In free flight experiments, honey bees rapidly display associations between a number of stimuli and a potential reward. Bees have been shown to not only use color and odor to predict the strength of reward, but to alter their foraging behavior when the pattern of reward is no longer as pronounced, or a greater reward is available (Giray et al., 2015; Hill et al., 1997; Sanderson, Orozco, Hill, & Wells, 2006). This consistent pattern of behavior in response to the potential changes in environmental cues suggests that bees possess a remarkable neural plasticity and an ability to rapidly acquire learned associations.

Of particular interest to the present study, are the results of the learned helplessness paradigm designed by Dinges et al. (2013). This aversive conditioning paradigm pairs "master" and "yoked" bees together for the duration of a learning session. The master bee is trained to avoid a colored portion of a chamber through exposure to shock upon entering the assigned portion. The yoked bee then is subjected to shock at any point the master bee is, regardless of position. This paradigm allows researchers to examine learning in conjunction with existing physiological stress, as well as the effects of a similar stressor (e.g. shock) without the presence of learning.

In addition to their wealth of behavioral advantages, honey bees possess an extensively mapped genome. Much of their genome is shared with another highly researched organism, *Drosophila melanogaster*. A survey of the genomes of both animals by Walldorf, Fleig, and Gehring (1989) suggested that honey bees possess a genome with 90% similarity of homeobox regions to that of *D. melanogaster*. This result has been corroborated by recent honey bee genomic sequencing efforts (Honeybee Genome Sequencing Consortium, 2006). As such, where genetic information is missing from models such as the honey bee stress response (Even, Devaud, & Barron, 2012), researchers may look to *D. melanogaster* to provide an educated launching point for exploration of genetic material.

Due to the nature of stress as a varied physiological process and the broad spectrum scope of this experiment, it is necessary to examine a suite of genetic markers in order to fully assess the extent to which learning affects physiological stress. As such, 10 genes associated with varying phases of the stress response were selected for examination (See Table 1). Heat Shock Protein 70 (HSP70), a chaperone protein and measure of oxidative stress (Hranitz et al., 2010), Protein Kinase A (PKA; Horiuchi, Yamazaki, Naganos, Aigaki, & Saitoe, 2008; Li, Tully, & Kalderon, 1996; Yamazaki, Horiuchi, Miyashita, & Saitoe, 2010) and the calcium/calmodulin-dependent protein kinase II (CaMKII; Santalla et al., 2014; Kadas, Tzortzopoulos, Skoulakis, & Consoulas, 2012), both g-protein coupled receptors, Diuretic Hormone 44 (DH44; Cannell et al., 2016; Kapan et al., 2012), a vasodilator and invertebrate homologue of corticosteroids, the Diuretic Hormone 44 receptor protein (DH44R; Dus et al., 2015; King et al., 2017), Dopamine receptor 2 (DOP2), a receptor for an insect dopamine (Humphries et al., 2003; Mustard, Pham, & Smith, 2010), the insect serotonin receptor (5HT2A; Nichols, 2007), the

Table 1
Summary of genes.

Gene	Abbreviation	Function	Experimental Role
Small Ribosomal Sub-unit	RS5	Illustration of baseline transcription	Control
Heat Shock Protein 70	HSP70	Chaperone Protein; Measure of cellular and oxidative stress	Experimental
Protein Kinase A	PKA	cAMP binding protein; neural signaling mechanism	Experimental
Calcium/ Calmodulin Dependent Protein Kinase	CaMKII	cAMP binding protein; cardiovascular and acetylcholine signaling mechanism	Experimental
Diuretic Hormone	DH44	Binds to CRF to perform corticosteroid-like functions; vasodilator	Experimental
Diuretic Hormone Receptor	DH44R	Receptor in gastrointestinal and locomotor function	Experimental
Dopamine Receptor 2	DOP2	Receptor for Dopamine; associated with spatial memory and motor control	Experimental
Serotonin Receptor	5HT2A	Receptor for serotonin; downregulation associated with improved recall	Experimental
Discs Large Homologue 1	DLG1	Cytoskeletal scaffold protein	Experimental
Pumilio	PUM	Ion channel implicated in inhibitory neural signaling	Experimental
Bruchpilot	BRP	Presynaptic anchor protein	Experimental

discs large homologue, a cytoskeletal scaffolding protein, (DLG1; Mauri, Reichardt, Mummery-Widmer, Yamazaki, & Knoblich, 2014; Noseda et al., 2016), pumilio, a neural ion channel protein (PUM; Burow et al., 2015; Schweers, Walters, & Stern, 2002; Stern, Blake, Zondlo, & Walters, 1995), and bruchpilot, a presynaptic anchor protein (BRP; Gehring et al., 2017; Honeybee Genome Sequencing Consortium, 2006).

The current experiment examines the effects of an aversive learning paradigm on the expression of genetic products associated with a suite of physiological stress related genes. Due to the varied nature of these genes, the study possesses two hypotheses. H_1: honey bees that undergo a learning task should express higher levels of HSP70, PKA, II, DH44, DH44R, and DOP2 and lower levels of BPR, PUM, 5HT2A, and DLG1 when compared to bees who have not undergone a learning task. H_2: bees that are not exposed to shock will express the lowest levels of HSP70, PKA, II, DH44, DH44R, and DOP2 and the highest levels of BPR, PUM, 5HT2A, and DLG1.

2. Methods

2.1. Subjects

Subjects consisted of 74 "gentle Africanized" honey bees, a hybrid of *Apis mellifera* and *Apis mellifera scutellata* native to the Island of Puerto Rico (Avalos et al., 2017). All subjects were collected from an observation hive at the Gurabo Agricultural Research Station of the University of Puerto Rico – Rio Piedras in Gurabo, Puerto Rico. Bees were collected by placing a mesh screen over the entrance to the hive, preventing bees from exiting the hive, while foraging bees were unable to re-enter the hive, and collected in a handheld vacuum tube. Bees collected in this manner were exclusively forager bees, and were collected as such to ensure all subjects were within the last 2–3 weeks of their lifespan (Seeley, 2009). All subjects collected in this manner were assumed to be naïve to the current experiment.

Once collected, subjects were transferred to a communal wire mesh cage with access to a 1 M sucrose solution. The cage was stored in an incubation chamber to maintain consistent temperature of 35 °C and 42% humidity. Bees remained in the cage for 24 h prior to the onset of experimental sessions.

2.2. Apparatus

Behavioral assays were conducted using the shuttle box apparatus designed by Dinges et al. (2013). The apparatus itself consists of two chambers affixed to a single shock grid. The chambers consist of a 3d-printed acrylonitryle butadiene styrene compartment measuring 135 mm × 20 mm × 5 mm, with a Plexiglas cover. The shock grid consists of a series of 2.5 mm diameter pins, spaced at 2.5 mm intervals. Bees touching consecutive pins would complete the circuit, resulting in the administration of a shock when current was supplied to the grid (See Fig. 1). Current was supplied to the grid using a 7.0 V, 0.05 A DC power supply.

Discrimination stimuli were placed under the shock grid and visible to the subjects. The stimuli consisted of a yellow or blue paint swatch, each corresponding to half of the compartment. These colors were chosen as they have been shown to be readably distinguishable from one another by foraging insects, such as honey bees, even in aversive conditions (Dinges et al., 2013; Hill et al., 1997).

Each shuttle box was connected to a control unit consisting of a Propeller experiment controller (Varnon & Abramson, 2013, 2018) and user interface. Infrared beams placed inside each shuttle box detected the locations of subjects, activated experiment protocols, and recorded data, in accordance with the protocol set forth by Dinges et al. (2013).

2.3. Behavioral protocol

Subjects were randomly assigned to one of four groups, each corresponding to their role in the behavioral assay. Bees assigned to naïve, baseline and yoked groups served as varying experimental controls, while bees assigned to the master condition served as the experimental group.

Naïve bees (n = 18) were used as an overall control group. These subjects were collected simultaneously with the other bees and housed in the communal wire mesh cage. Subjects in this condition were randomly selected and removed from the communal cage one at a time. Upon removal, these bees were immediately submerged in liquid nitrogen to await dissection for the neurological assay.

Baseline bees (n = 16) were used as a control for handling and measurement variables. Subjects were removed from the communal housing cage, and placed into the shuttle box apparatus. While subjects in this condition experienced the same amount of time in the apparatus

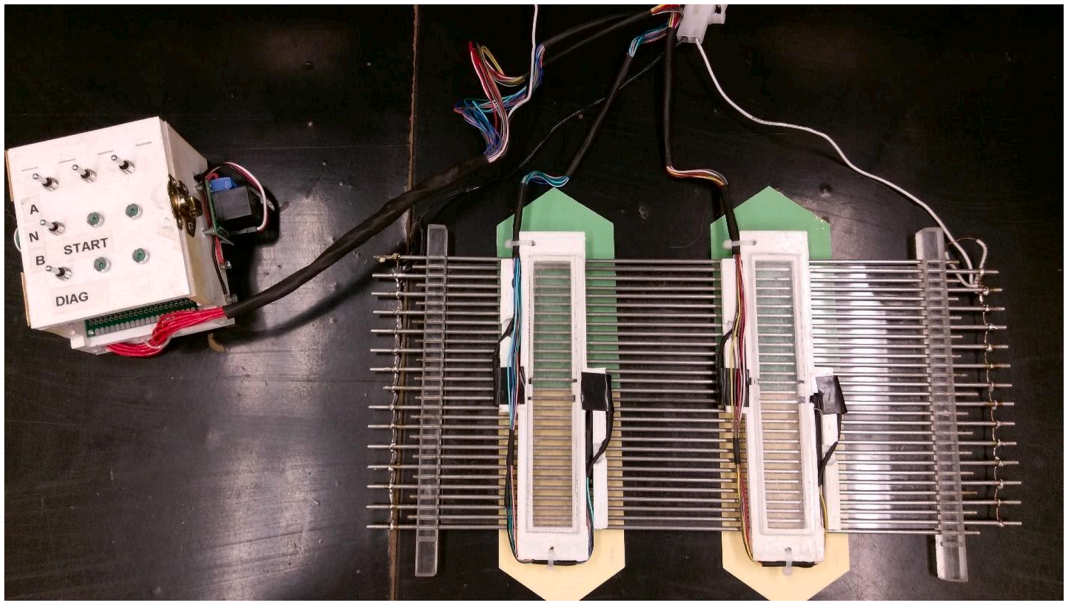

Fig. 1. Shuttle box apparatus with alternate color stimuli.

as those in other conditions, they experienced no shock while present in the apparatus.

The remaining bees were placed into master and yoked pairs, and introduced into the apparatus in such pairs. Master bees (n = 29) were those that underwent the aversive conditioning avoidance task, while yoked bees experienced a shock whenever the master bee experienced shock. As such, yoked bees (n = 29) served as a control for the amount of shock received by an individual subject (See Table 2).

Prior to the introduction of subjects, each compartment of the apparatus was cleaned with 70% ethanol in order to remove any waste, detritus, or pheromones left by the previous subject. For each session, two bees were randomly selected from the communal housing cage, and placed into the apparatus, one in each compartment. Bees were given a five minute recovery period, wherein no shock was supplied to the grid. Experimentation began after the recovery period concluded pending two additional criteria: that both subjects were detected by the apparatus, and that the bee labeled as the Session's master had crossed wholly from one side of the compartment to the other.

Each experimental session consisted of two five minute periods, the second starting immediately after the conclusion of the first. During each experimental session one of the two halves of the compartment, yellow or blue, was designated as the correct side. Whenever the master bee was in the correct portion of the apparatus, no shock was administered. When the master bee crossed into the incorrect portion, current was passed through the shock grid, administering shock to both the master and yoked bees. For each session, the correct portion of the apparatus was randomized such that 14 master/yoked pairs experienced sessions with yellow as the correct color, and 15 pairs experienced sessions with blue as the correct color. This was done to control for any effects of color bias. Upon completion of the second period, bees were removed from the apparatus for the neurological assay.

2.4. Neurological assay

Upon their removal from the apparatus, individual subjects were each placed in a 15 mL Falcon tube with access to a 1 M sucrose solution. Bees remained in this containment for 45 min, to allow expression of the mRNA for the proteins of interest. This time unit was selected, as research has shown that in honey bees, immediate early genes (IEGs) reach peak expression of transcriptional products roughly 30 min after exposure to the driving stimulus (Alaux & Robinson, 2007). While the genes of interest themselves are not IEGs, most possess relatively rapid transcription. Due to this, and the relatively large number of genes investigated, a 45 min expression period was selected to allow for effective transcription of all genes of interest. While not necessarily the peak expression point for the genes of interest, the 45 min period allows for adequate expression to determine differential effects of behavioral outcomes. After the 45 min expression period, subjects were sacrificed by submersion in liquid nitrogen. This method arrests neurological and proteomic function without degrading mRNA products. Subjects were removed from the liquid nitrogen, and placed in a −80 °C freezer until dissection.

During dissection, heads were removed and placed on dry ice. Bodies were discarded. Heads were dissected, discarding ocular and hypopharyngeal tissue. Whole brains consisting of mushroom body, ocular lobes and antennal lobes were stored in 40 μL RNA*later*-ICE Frozen Tissue Transition solution (Ambion, Life Technologies, Carlsbad, CA), at −20 °C for approximately 24 h, after which mRNA extraction and qPCR analysis were performed.

RNA extraction and qPCR analysis were conducted on a subset of each of the experimental conditions, with 5 bees each selected from both the naïve and baseline conditions, and 10 bees selected from the master and yoked groups. In total, RNA extraction was conducted on 30 individual subjects. Bees were randomly selected from the naïve condition, but for each other experimental conditions, bees exhibiting behavioral outcomes typical to those expected from the experimental condition were selected. As such, bees were selected from the yoked and baseline groups which displayed roughly 50% of overall time spent in each side of the apparatus, while master bees were selected based on overall ability to avoid shock. This was done to ensure genetic expression that was most representative of behavioral outcomes.

Brains were mechanically homogenized in 1 mL of Trizol using a syringe. Upon completion of homogenization, 100 μL of Bromo-3-chloropropane (BCP) was added. Samples were vortexed, and let incubate at room temperature for 15 min. After incubation, samples were centrifuged at 14,000 rpm for 15 min at 4 °C. Centrifugation in this manner created a two phase mixture, with RNA suspended in the upper, aqueous phase. The aqueous phase was removed, and transferred to a sterile tube stored on ice.

Next, 500 μL of isopropyl alcohol was added to each RNA sample, inverted repeatedly for 10 s, and let incubate for 10 min at room temperature. After incubation, samples were again vortexed at 14,000 rpm for 10 min at 4 °C in order to produce an RNA pellet. Pellets were washed twice with 1 mL 75% ethanol and air dried. The pellet was re-suspended in 40 μL DEPC water and incubated at 65 °C in a water bath for 10 min and stored at −20 °C after the incubation period. Each sample was examined for initial RNA concentration and purity using a Nanodrop 1000 (BioAnalytical). Three samples were discarded due to low RNA concentrations leaving 4 remaining naïve samples, 4 baseline samples, 9 yoked samples, and 10 master samples. mRNA samples were normalized and reverse transcribed to cDNA using the iScript Reverse Transcription Supermix (BioRad Laboratories, Hercules, CA).

For the qPCR analyses reactions contained 5 μL of the fluorescent agent SyBR green (BioRad Laboratories, Hercules, CA), 1 μL of forward and reverse primers for one of the genes of interest (TaqMan® probes, Premier Biosoft, Palo Alto, CA; See Table 3), 1 μL of cDNA in a concentration of 100 μg/mL in a final volume of 10 μL. qPCR cycles were conducted using an Eppendorf Mastercycler® RealPlex^2 (Eppendorf, Hamburg, Germany). Plates consisted of two samples, each with three technical replicates. Samples for qPCR analysis included the housekeeping gene (RS5; small ribosomal subunit) and all ten genes of interest.

2.5. Data preparation

The shuttle box apparatus (Dinges et al., 2013) digitally records data

Table 2
Subject group by exposure and experimental role.

Group	Handled	Placed in Apparatus	Exposed to Shock	Able to Avoid Shock	Experimental Role
Naïve	Yes	No	No	–	Control
Baseline	Yes	Yes	No	–	Control
Yoked	Yes	Yes	Yes	No	Control
Master	Yes	Yes	Yes	Yes	Experimental

Table 3
Sequences for forward and reverse primers used in qPCR analyses.

Gene	Forward Sequence	Reverse Sequence
RS5	TAACGTCCAGCAGAATGTGGTA	AATTATTTGGTCGCTGGAATTG
PUM	GTGTCGGAACTGGATACCATC	AATCTTTGGGTGTCGTAGGAC
PKA	GCAGACCACCACTTCTAC	TCATCTTCTTCATCTTCCTCC
HSP70	GTGATGAACGATGGAGGAAA	GAAATATGCTGGGACGGTAATA
DLG1	CACACGACCTGTCATTATCC	ATGTCCCTCTCCATCTGTT
DH44	TGTAGCATTCAGAAAGGGAAG	GTGGGATCACAACGCATAA
DH44R	ACTCAGCAATACAGGAAAGC	ACAATGCCACGAACAGTC
DOP2	ACCTCGGATACCTCATCTTC	ATTTCAGGCTCTTGGTCTG
CaMKII	GACAAGAGACTGTCGATTGC	TGATGCTCCGACTGGAAA
BRP	ACAACGAGAACGAGCATCTG	CCCTTTGTTTTGGAGCATCTC
5HT2A	GCAAAGAATCCCGAGAAGAA	GTTACAACGACCACACCTC

each time a subject crosses the infrared beams. Data is collected on total time each subject spends in each portion of the apparatus, and at what point the subject crossed the center threshold from one portion to the other. Data for the amount of time spent on the correct portion of the apparatus was manually tallied from this collected data at 60 s intervals throughout both periods. For ease of interpretation, a percent correct value was calculated for each time point by dividing the total amount of time spent in the correct portion by the time point in the session. Data were initially analyzed in IBM SPSS version 24 (Armonk, NY). One sample t-tests were used to determine whether or not performance differed from chance on the behavioral task, followed by a split-plot ANOVA in order to assess where experimental groups differed.

Gene expression was calculated using the ΔΔCt method (Schmittgen & Livak, 2008). Naïve subjects served as the control group. We examined gene expression using linear model fitting of the ΔΔCt value (N = 26) against our variables of interest (behavioral role, color of shock side). For each gene we conducted a stepwise regression approach which considered models fitting each variable independently and together with their interaction term. Across all models the one with the lowest Akaike Information Criteria, representing the best fit, was considered. Regressions were conducted using the glm() function in R and post-hoc tests were conducted with glht() using appropriate contrast matrices.

To test if gene expression covariance is able to separate samples by treatment groups, we conducted a Linear Discriminant Analysis (Fig. 4). We followed a conservative approach to retain as much information as possible using the data that we had. Therefore, we opted to remove genes from the analysis (5HT2A and CaMKII) to incorporate the highest number of individuals (N = 23).

To examine associations of genes across our two focal groups (Master, Yoked) we conducted a network analysis following the approach outlined by (Solomon-Lane & Hofmann, 2019). This approach first builds a partial correlation matrix across genes. In this way we examined possible correlations between individual genes that take into account covariation from their epistatic interaction with all other genes we observed. We used an arbitrary but conservative threshold (R2 >= 0.8) to construct an adjacency matrix and network (Fig. 5). Due to missing values in our data set we opted to mean-impute gene-wise. Following imputation we subset the data set to Master (n = 10) and Yoked (n = 10) individuals and for each built a partial correlation matrix. This partial correlation matrix was used to build an adjacency matrix that considered an edge only between those genes whose partial correlation coefficient was at or above 0.8. Data on gene expression was analyzed using the statistical program R (RStudio, Inc.) v. 3.5.2 (2018–12-20). Graphs and figures were done in Graph Pad Prism 8.0, (GraphPad software, La Jolla California USA).

3. Results

The initial *t*-test for baseline bees compared percent time spent in the correct portion of the apparatus to an expected random chance of 50%. Data indicate that for all time points in both periods, there was no significant difference from chance. As such, baseline bees are representative of normal behavior when exposed to the apparatus under no aversive conditions (See Table 4). These results also indicate that no color preference was present in the present sample. Color bias data was assessed, as past research has indicated that honey bee heavier in the face of aversive conditions can be influenced by previous exposure to salient color stimuli (Black et al., 2018; Dinges et al., 2017). By assessing Baseline and Yoked bees for color bias, we can determine that Master bees are not likely to differ in performance based on the color stimulus paired with shock.

A second *t*-test was used to examine the behavior of yoked bees. Like the baseline bees, yoked bees displayed no significant differences from the expected 50% chance. As such, yoked bees can be said to not have undergone learning in the presence of shock (See Table 5).

Master bees displayed significant differences from chance for every time point during the first period (60 s, $t(27) = 3.123$, $p = .004$; 120 s, $t(27) = 2.932$, $p = .007$; 180 s, $t(27) = 2.832$, $p = .009$; 240 s, $t(27) = 3.072$, $p = .005$, 300 s, $t(27) = 3.060$, $p = .005$). During the second period, master bees displayed no statistical difference from 50% chance. As such, master bees can be said to have undergone learning, at least in the first period (See Table 6).

A split-plot ANOVA was conducted examining role (master, yoked or baseline) and time point. For these analyses, experimental role was the independent variable, while performance at each point was the dependent variable. Significant differences were identified for the 180 s ($F(2, 69) = 3.444$, $p = .038$), 240 s ($F(2, 69) = 4.609$, $p = .013$) and 300 s time points of the first period ($F(2, 69) = 4.311$, $p = .017$). No statistical differences occurred for time points of the second period (See Fig. 2).

Post hoc analyses consisting of a Tukey HSD test were used to identify the nature of the differences. For the 180 time point, Master bees were observed to spend significantly more time in the correct portion of the apparatus (MD = 0.11882, $p = .03$), as expected. This pattern is repeated in the 240 s time point (MD = 0.12377, $p = .011$), and the 300 time point (MD = 0.11510, $p = .011$).

From the gene expression analysis, only two genes showed significant expression patterns DOP2 and HSP70 (Fig. 3A). We found a significant effect of Role only in DOP2 (Fig. 3B: Fst = 83.54, p-value = 0.047). A post hoc test showed that expression of DOP2 was significantly lower in the Yoked group when compared to Baseline (z = 2.57, p-value = 0.027) with no differences between Master group and Baseline (z = 1.10, p-value = 0.510) or between the two experimental groups (Master v. Yoked, z = 1.88, p-value = 0.144). We also found a significant interaction effect in HSP70 (Fig. 3C; Fst = 19.00, p-value = 0.013) largely driven by distinct responses from Master group individuals that received shock in Color1 vs those from the same group in Color2 (z = 3.321, p-value = 0.012).

To explore if orthogonalized covariance of genes clearly separate treatment groups, we ran a canonical discriminant analysis (Fig. 4). It also shows separation between baseline and the master/yoked groups, and was able to segregate Master from Yoked gene expression profile. Genes whose expression pattern appear to contribute to this separation include members of learning, stress, and neuromodulation genes.

Gene network analysis shows different sets of genes to be connected

Table 4
One sample *t*-test results for Baseline bees compared to random chance.

Time Point (s)	*t* value	df	Sig.
60	0.681	13	0.508
120	0.322	13	0.753
180	0.707	13	0.492
240	0.272	13	0.790
300	0.113	13	0.912
360	1.287	13	0.221
420	1.016	13	0.328
480	0.733	13	0.477
540	0.978	13	0.346
600	1.015	13	0.329

Table 5
One sample *t*-test results for Yoked bees compared to random chance.

Time Point (s)	*t* value	df	Sig.
60	0.474	29	0.639
120	0.132	29	0.896
180	−0.403	29	0.690
240	−0.769	29	0.448
300	−0.725	29	0.475
360	−0.110	29	0.913
420	0.141	29	0.889
480	−0.042	29	0.967
540	−0.285	29	0.778
600	−0.013	29	0.990

Table 6
One sample *t*-test results for Master bees compared to statistical chance.

Time Point (s)	*t* value	df	Sig.
60	3.123	27	0.004*
120	2.932	27	0.007*
180	2.832	27	0.009*
240	3.072	27	0.005*
300	3.060	27	0.005*
360	−0.786	27	0.439
420	0.049	27	0.961
480	0.748	27	0.461
540	1.134	27	0.267
600	1.185	27	0.246

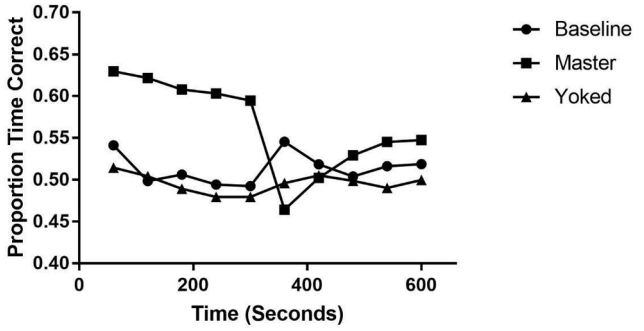

Fig. 2. Plot of proportion of time spent on the correct portion of the shuttle box apparatus over the span of two consecutive 300 s experimental periods for master, yoked, and baseline bees.

in master vs yoked bees (Fig. 5). Specifically, neuromodulation and learning genes in the Master group, with one stress gene with R2 >= 0.8 or higher partial correlations. In contrast, in the yoked individuals several stress genes and one neuromodulator gene is highlighted. In the Master group, DOP2 expression seems to positively correlate with BRP expression but this relationship reverses in the Yoked group. Similarly, DOP2 expression only strongly correlates with PKA expression in the Master group. Contrastingly the PUM gene seems to have more strong correlations in the Yoked group than in the Master group but maintains a consistent strong negative correlation with 5HT2A across groups. Different genes associated with synaptic plasticity are in the two networks.

4. Discussion

The goal of the current experiment sought to assess to what extent learning in an aversive conditioning paradigm altered expression of physiological stress related genetic products on a suite of genes.

Data from the behavioral assay suggests that learning occurred early on, in the first 5 min of the experimental protocol. We see this by the elevated percent time on the correct portion of the apparatus for master bees while yoked and baseline bees remain consistently near the 50% chance measure. After this point, the acquisition decays, and behavior reverts to similar patterns seen in the non-experimental groups. This corroborates past research in shuttle box aversive conditioning paradigms within honey bees (Black et al., 2018; Dinges et al., 2013, 2017). This also demonstrates that the master bees are, as expected, initially learning to avoid the incorrect side of the apparatus. It is expected that, since the learning in this group is driven by avoidance of an aversive physiological consequence (shock) that is administered to both master and yoked bees, that both of these groups experience higher rates of physiological stress when compared to baseline bees.

Of note, however, is the apparent loss of learning observed in the second period. The decline in performance observed in the first five minute phase suggests that bees may be displaying a fatigue effect following the extended period in the shuttle box. Similar results have been observed in some experiments using the same apparatus (Black et al., 2018), but run counter to results displayed in others (Dinges et al., 2017). In addition to experimental fatigue, honey bees may be displaying preferential activity for a certain time period. Bees examined in this study were examined throughout the day, while some research suggests that honey bees have differential activity, and foraging patterns throughout the day (Baum, Rubink, Coulson, & Bryant, 2011; Linsley, 1978).

When examining gene expression data, we see that only DOP2 showed significant changes in expression as a result of experimental role (Fig. 3). Counter to prediction, baseline bees, which were not exposed to shock, expressed the highest level of DOP2, indicating that in both yoked and master groups, subjects displayed decreased expression of DOP2. It is also worth noting that the pattern of DOP2 expression, while not statistically significant, is varied between yoked and master bees (see Fig. 3B). Master bees in particular display higher variance as well as a higher average expression.

This data, coupled with the large change in expression between baseline bees and experimental bees suggests that the relationship between stress, learning, and gene expression is more nuanced than hypothesized, namely, that a physical stressor (in this case shock) may result in decreased expression, while learning and acquisition of new information in turn results in increased expression. This corroborates past research which implicates the DOP2 gene in neuroplastic processes (Humphries et al., 2003), particularly in foraging bees. Likewise, this data supports previous research suggesting that higher levels of dopamine correspond with better avoidance learning (Agarwal et al., 2011). The present data, coupled with previous results suggest that increased dopamine sensitivity in honey bees may in fact be driving avoidance learning, as both artificially elevated levels of biogenic amine, and increased expression of its receptor correspond with shock avoidance.

It is possible that, due to age related expression of the DOP2 gene, variance may exist even within foraging bees. This may account for some of the observed variance in the expression of the gene, as the method used to collect animals only ensures that they are foragers, not that individuals are truly age matched (Seeley, 2009). Therefore, future research should include age tagged animals in order to control for differential expression based on subject age.

There remains the further possibility that the current statistical analyses used are insufficient to detect differences between groups across the remaining genes examined. For example, data from 5HT2A (Serotonin Receptor) showed marginal significance (ANOVA: $p = .073$), which suggests that differences might be more readily observed under conditions of larger sample sizes. This corroborates past findings in vertebrate systems, which suggest that serotonin is linked to not only flight related behaviors, but is affected by both escapable and inescapable shock (Maier & Watkins, 2005).

While past research in honey bees have indicated that the species does not display the expected behavioral outcomes of learned helplessness seen in vertebrates (Dinges et al., 2017), the pattern of 5HT2A expression is similar to results seen in the experiment conducted by Maier and Watkins (2005). Master bees (M = 1.165) and yoked bees (M = 1.991) displayed lower median 5HT2A expression than did baseline bees (M = 3.017). Maier and Watkins (2005) noted that inescapable shock mounted an increased release of serotonin from serotonergic neurons, which may directly correspond to down regulation of serotonin receptors to avoid excitotoxicity. While this may explain the decreased expression, it is worth nothing that the median expression between master and yoked subjects in the present experiment do not differ substantially. This is likely due to one of two plausible explanations. The first is that the present study made use of whole brain analyses, whereas the study by Maier and Watkins (2005) engaged in specific analysis of serotonergic neurons. This may suggest that, in areas of active neurosecretory function, the patterns of receptor expression are more susceptible to changes as a result of avoidable or unavoidable stress, while

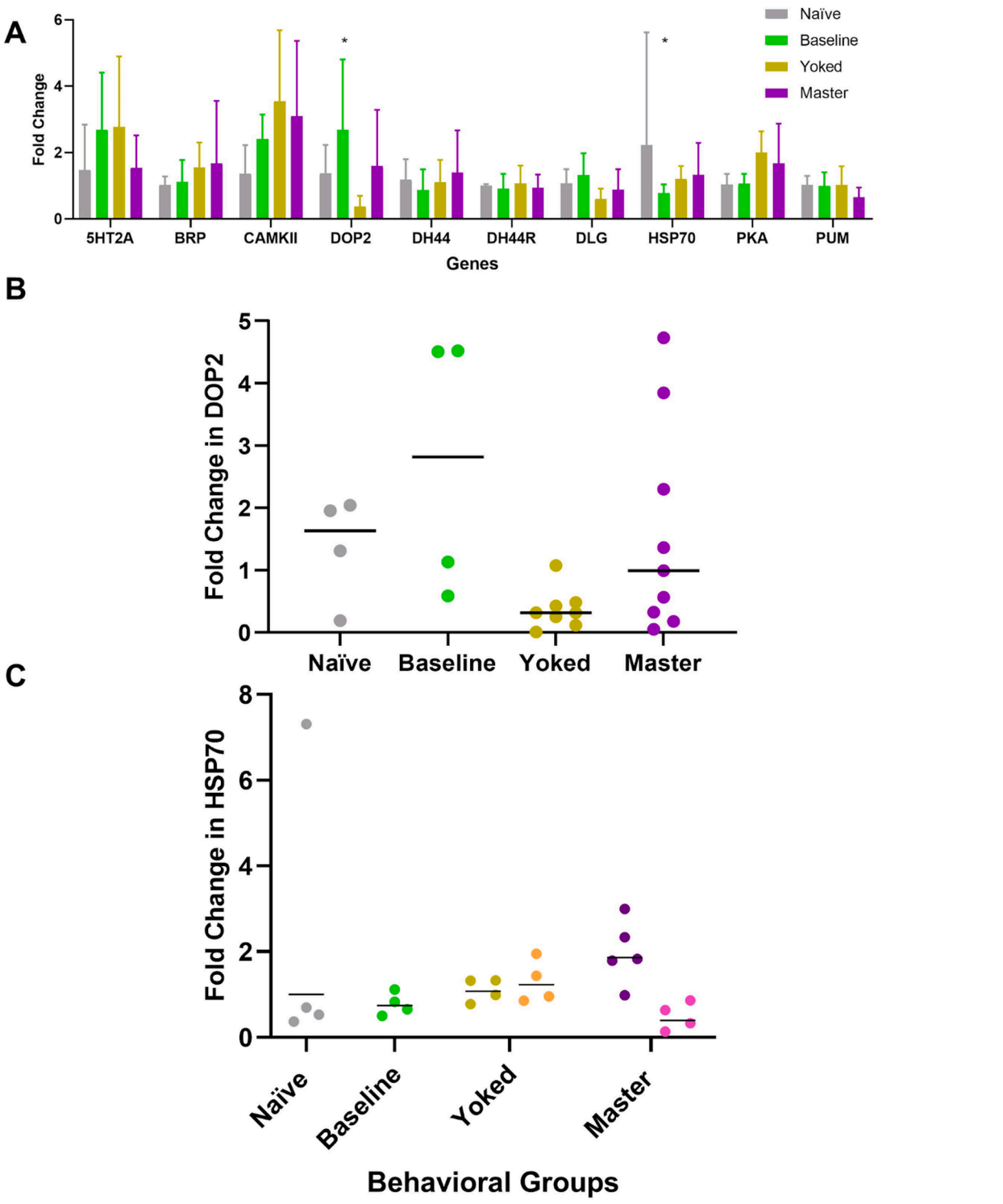

Fig. 3. *Gene expression analysis.* A. Summary of gene expression. The plot summarizes gene expression patterns for all genes across our behavioral groups. Asterisk (*) refers to which set of genes shows a significant difference. B. Gene expression of DOP2. The plot summarizes gene expression patterns of DOP2 gene across our behavioral groups. There is a significant difference between Yoked and Baseline ($z = 2.57$, p-value = 0.027), however no differences between Yoked and Master ($z = 1.88$, p-value = 0.144) or Master and Baseline ($z = 1.10$, p-value = 0.510). The y-axis represents fold change in gene expression while the x-axis defines our behavioral groups. Each point in the plot represents a sample with color correlating to group: grey = Naïve, green = Control, gold = Yoked, magenta = Master. Horizontal black bars denote the group mean fold change. C. Gene expression pattern of HSP70. The plot summarizes gene expression patterns of the HSP70 gene across behavioral group and color at which shock was applied. There is a significant difference in the color association in the Master group ($z = 3.321$, p-value = 0.012) The y-axis represents fold change in gene expression while the x-axis defines our behavioral groups. Each point in the plot represents a sample with color correlating to group: grey = Naïve, green = Control, gold = Yoked, magenta = Master. The Yoked and Master groups are separated by color in which shock (punishment) was presented, with darker colors corresponding to Color 1 and lighter colors with Color 2. Horizontal black bars denote the group mean fold change. Relative expression was measured using the ΔΔCt method. The relative expression values presented are relative to the control group (Naïve subjects).

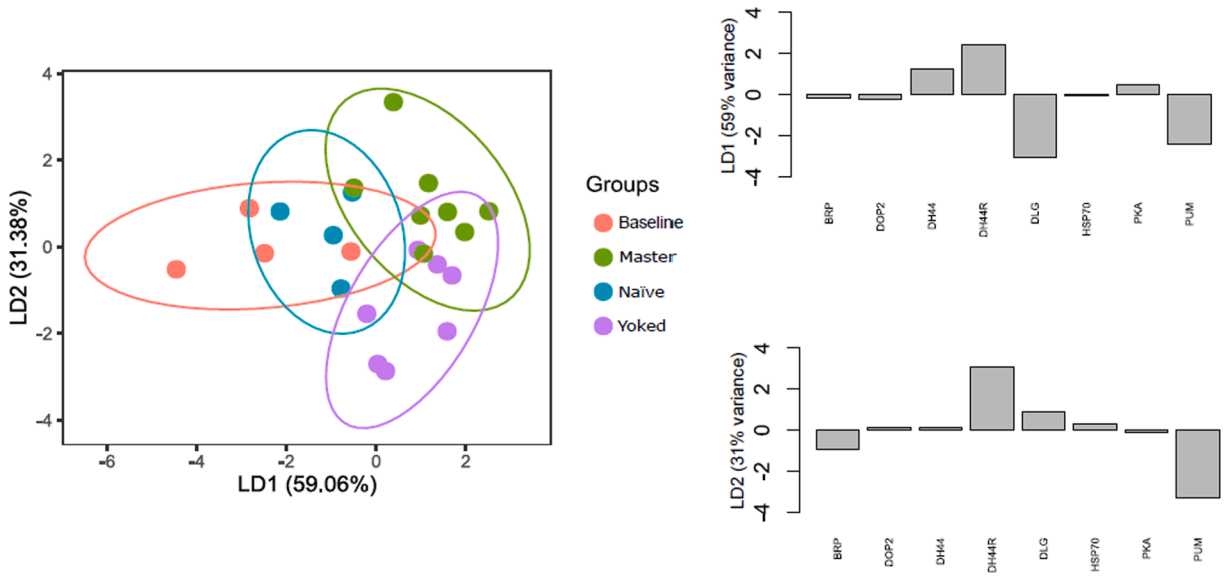

Fig. 4. *Linear Discriminant Analysis.* This figure shows the gene expression profile for each individual (8 genes, 23 individuals) summarized as a single point, scattered around two canonical axes. Pink circles = Baseline, Green circles = Master, Blue circles = Naïve and Purple circles = Yoked. We used a Wilk's Lambda test calculated from the overall pooled covariance matrix, using type of training as source. The analysis explains 63.92% of the variation ($R^2 = 0.84$, Approx. F = 1.11, Prob > F = 0.44, −2LogLikelihood = 12.24). Analysis produces four clusters which are centered on the distribution of each data set per group. The bar plots show the most contributive loadings (genes) to the value in the LDA. 5HT2A and CaMKII were removed from the canonical discriminant analysis in order to ensure accurate data was present for as many subjects and genes as possible.

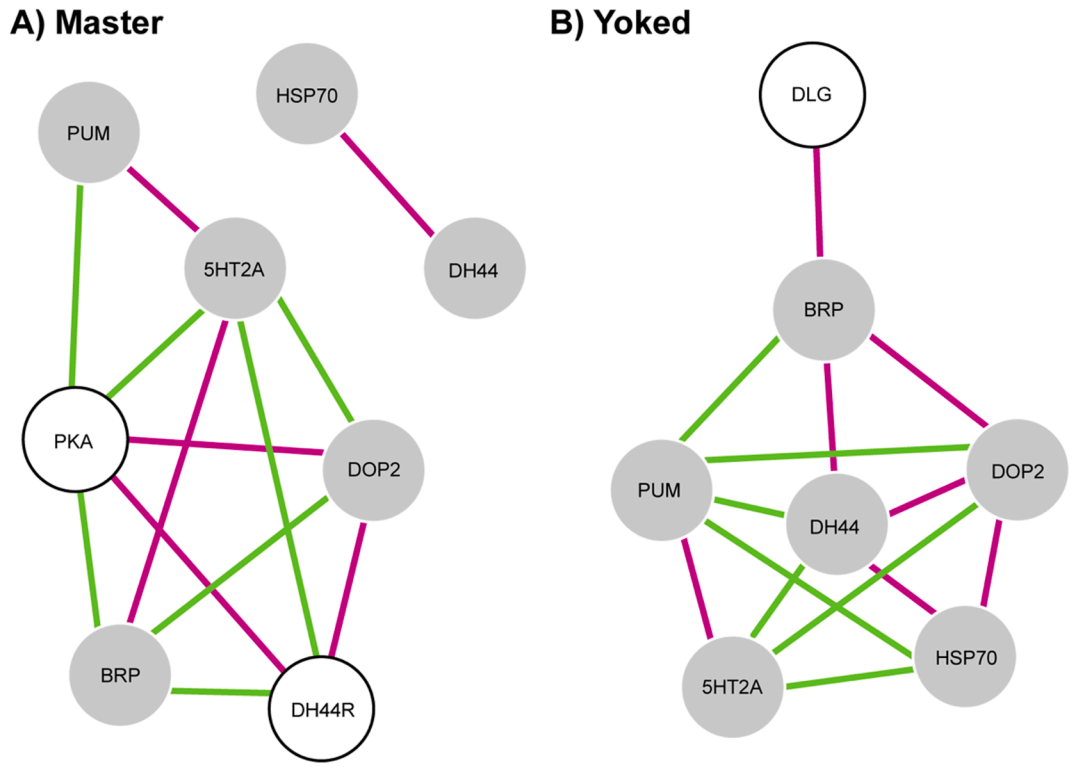

Fig. 5. *Gene expression networks between behavioral groups.* The figure summarizes networks built from partial correlations of gene expression within Master and Yoked behavioral groups. Genes are denoted by circles which are colored grey if the gene is present across both networks or white if only found within one of the networks. Green lines correspond to positive partial correlations with R2 >= 0.8 between genes, while magenta-colored lines highlight negative partial correlations at the same threshold.

the nervous system as a whole may not display a difference. Secondly, this lends may lend further credence to the conclusion by Dinges et al. (2017) that insects do not possess a learned helplessness response as we understand it in vertebrate systems. The lack of difference in expression of 5HT2A suggests that honey bees in particular may not possess the necessary neural complexity to identify differences between avoidable and unavoidable shock.

Of further interest, are the results for the CaMKII gene. Past research

in *Drosophila melanogaster* has identified CaMKII as a major influence of neuroplasticity and synaptic regulation in a number of neuromuscular circuits, and as such, we would expect to see a large difference in expression between groups, particularly between master and yoked bees (Kadas et al., 2012). However, the observed results suggest little to no difference. This difference may be due to this study's use of whole brain dissections, as in honey bees, CaMKII is primarily expressed in the mushroom body (Pasch, Muenz, & Rössler, 2011). Inclusion of ocular and antennal lobes may normalize expression across conditions, resulting in the observed non-significant difference. Future analysis of individual brain regions may yield the expected differences in expression.

It is likewise possible that these genetic products are outside the window of peak expression. Methods were conducted based on the peak expression of immediate early genes in insects (Alaux & Robinson, 2007). However, none of the genes analyzed were themselves IEGs, and as such, estimates of peak genetic expression may be flawed.

Nonetheless, when we consider the expression profile of baseline, master and yoked subjects, we can observe three distinct clusters that represent each of the groups (Fig. 4). Differences in gene expression might not be detected throughout many of the genes examined, but the collective expression between groups seems to be indicative of master and yoked profile. Therefore, conditioning events lead to very clear gene expression patterns associated with learning vs. uncontrollable stress.

It is likely that any effects were masked by fatigue on the part of research subjects, as behavioral performance normalizes across groups during the second 5 min period. Similarly, the fatigue effect resulted in master bees experiencing a level of shock more characteristic of yoked bees, and as such, experiencing higher levels of physiological stress during the second period. This exposure to the physiological stressor likely altered expression of the genes of interest, and as such, results may not be truly representative of changes based solely on learning.

It is important to note that of the examined genes, HSP70, PKA, and 5HT2A are all greatly conserved between insect and human systems (Bechtel et al., 2007; Choi et al., 2018; Manchev et al., 2014; Mano et al., 2018). As such, results directly translate to how learning affects stress levels of human populations. While more research is required in vertebrates to fully understand how these same proteins affect changes in human brains as a result of learning, we can assume that similar effects are likely. A likely candidate to begin such studies would be in rodent systems, as they have been widely used to model gross vertebrate behavior and neural systems, and have shown similar results, with improved learning performance in systems of reduced serotonergic activity (Meneses, 1998).

CRediT authorship contribution statement

Timothy E. Black: Conceptualization, Methodology, Formal analysis, Investigation, Validation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Ova Fofah:** Formal analysis, Investigation, Data curation. **Christopher W. Dinges:** Conceptualization, Methodology, Software. **Carlos A. Ortiz-Alvarado:** Formal analysis, Investigation, Validation, Resources, Data curation, Writing - review & editing, Visualization. **Arian Avalos:** Conceptualization, Resources, Writing - review & editing, Visualization. **Yarira Ortiz-Alvarado:** Formal analysis, Investigation, Writing - review & editing, Visualization. **Charles I. Abramson:** Conceptualization, Methodology, Validation, Resources, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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