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Differences in learning and gene expression in brains of male and female Bicyclus anynana

Gabrielle Agcaoili

Citation

Differences in learning and gene expression in brains of male and female *Bicyclus anynana*

An Honors Thesis submitted in partial fulfillment of the requirements of Honors Studies

in Biological Sciences

By

Gabrielle A. Agcaoili

Spring 2020

Biological Sciences

J. William Fulbright College of Arts and Sciences

The University of Arkansas

Acknowledgements

  I would like to thank Matthew Murphy, Grace Hirzel, D. Nikki Robertson, and Sushant

Potdar for animal husbandry, and the University of Arkansas, the Arkansas Biosciences

Institute, and the University of Arkansas Honors College for funding. I would not have

been able to complete this project without the guidance of my research mentor, Dr. Erica

Westerman. She has expanded my knowledge of the scientific world and continuously

encouraged me to seek more. I would also like to express the utmost gratitude to Dr.

David Ernst, a postdoctoral fellow in the Westerman lab. He spent many hours working

with me in learning the techniques needed for my project, and was a resource I could turn

to for my many questions. Being a part of the Westermna lab the past few years has

grown me as a student and as a person.

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Abstract

One way to understand the variation in the behavior of animals is by looking at the genes involved. We were particularly interested in behavioral differences between the sexes. How might these differences be manifested in the brain? This study worked to answer this question by using male and female butterflies of the species *Bicyclus anynana*, examining what is going on in the brains of males and females as they learned from a social exposure. We focused in on how sex plays a role in behavior and learning; it has been seen that males and females respond to the same social experience in similar yet different ways. *B. anynana* males and females learn from the same experience but exhibit sex biases in the traits they look for as well as in what each are good at learning. We explored whether sex specific biases in learning are associated with sex specific variation in gene expression in perception or higher processing. Males and females were given the same social experience, and the behaviors they exhibited during this training period were recorded. Each treatment - naive versus learning and male versus female - consisted of 10 individuals for a total sample size of 40. At the end of the training period, their heads were flash frozen for later dissection and RNA extraction for eyes and brains. A total of 40 eyes and 40 brains were collected. The RNA was sequenced to look for differentially expressed genes between the sexes. We found 18 differentially expressed genes in the eyes of naïve individuals, 18 differentially expressed genes in the eyes of trained individuals, 19 differentially expressed genes in the brains of naïve individuals, and eight differentially expressed genes in the brains of trained individuals. Genes differentially expressed included ones that control X-box binding protein, circadian clock rhythms, sex peptide receptor, and vitellogenin expression. These genes looked to be

differentially expressed as effects of sex, training, or an interactive effect of sex and training. Butterflies, like many species, including humans, are social animals. So, the conclusions drawn from this experiment could be applied to better understanding differences in behavior and genetics of many animals, possibly including humans. This can help in understanding more about human biology. This is especially important in today's world as individualized medicine becomes more and more prevalent.

Introduction

Learning is defined as a change in behavior in response to an experience (Galaf and Laland 2005). It occurs across most animal taxa with learned behavior playing a role in sexual selection (Verzijden et al 2012). Learned mate choice behavior is influenced by social behavior and can affect the genetic patterns of a population (Verzijden et al 2012). Learning can impact evolutionary processes such as food choice, predator avoidance, and mate choice; this is why exploring further what may be behind this learning, such as differentially expressed genes, is important. The conclusions that may be made in studying how and which genes play a part in learning could help in our understanding of why animals behave in certain ways as well as recognizing the advantages and disadvantages in learning and behavior between animals. This could also further our understanding of the evolution of populations as learning impacts the choices animals make in their daily lives.

Changes in behavior in a social situation are due to not only the processing of the social information that occurs during experiences, but are also due to genetic variations in the

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brain (Robinson et al 2008). In humans, learning has been extensively studied, especially looking at the relationship between social cognition and interactions. It has been seen that processing of social signals begins at a young age, with 18 month old children showing signs of implicit awareness and are able to pretend play (Frith 2007). In addition to humans, learning in various animals has been studied as well. For example, Norway rats learn food choice and poison avoidance, and brown-headed cowbirds learn songs for courting (reviewed in Galef and Laland 2005).

We also know a bit about brain gene expression changes during learning and other situations. For example, brain gene expression has been studied in contexts such as comparing between different species in different environments, and looking at a single animal. Brain gene expression in domesticated dogs, pigs, and rabbits is specific to events, and a digital atlas of the genes in a mouse brain has been made (Albert et al 2012, Lein et al 2007). The genes associated with what is occurring in the brain during learning, however, have not been studied.

It is known that male and female *B. anynana* learn, and that they learn differently (Westerman et al 2014). However, it is not known why there are these differences. This experiment dove into whether such differences are associated with differential gene expression. What is happening in the brain during the training period that is causing the associated behavior was looked at. Differentially expressed genes in the butterfly *B. anynana* during a social experience were identified. It is not known what exactly is going on in male and female brains when learning, but some studies delve into that. A couple of

studies have suggested that hormones, specifically vasopressin and oxytocin and their metabolites, modulate social learning in rats (Dluzen et al 1998, Bunsey and Strupp 1990). In another study, it was found that the anatomical structure of the hippocampus in female rats differed from that of males because of increased neuron density as a result of quicker learning (Dalla et al 2009). However, in studies of learning, most of the time one sex or the other is prefaced; learning is rarely studied in both sexes at the same time.

Experience affects behavior, as can be seen in the influence of sensory physiology on swordtail fish learning and mate choice (Cummings et al 2008, Cui et al 2017). But in addition to experience, genes can play an even larger role in determining behavior; this can be seen in the context of division of labour. The presence of a genetic toolkit for division of labour in paper wasps may suggest the possibility of a genetic toolkit for learning (Toth et al 2010). Association of differentially expressed genes with specific tasks demonstrates the influence of genes in processing of information and so determination of behavior.

While we often look at genes when examining causes of behavior, evidence shows that hormones may be tied into this as well. Work with *Drosophila melanogaster* has shown that hormones in the brain influence learning (Schwaerzel et al 2003), and suggest that differentiation in the sexes and between the individuals who should learn and who should not learn may be associated with hormones. Dopamine was found to be important in aversive stimulus learning (electroshock) and octopamine in appetitive learning (sugar award) in the *Drosophila* during training. The changes that occurred (hormones given) in

the *Drosophila* training period affected their memory. What occurs in the brain in the training time frame is crucial in long term memory. Based on this research, changes in the brain during training determine whether animals will learn. This is why the brains of *B. anynana* were looked at during training as opposed to any other point in time of the experiment.

My experiment looked at brain gene expression, looking at what is different between the brains of males and females. Genomics and bioinformatics allow for specific targeting of genetic sequences and even whole organism genome analysis. This study utilized these powerful tools to examine the genetic basis for the difference in learning of male and female *B. anynana*. RNA-Seq allowed us to identify all of the genes expressed in the eyes and brains for each experimental treatment group. Genomics and bioinformatics contribute to behavioral research, molecular biology, and computational biology. The vast amount of knowledge that we can learn from studying genes in association with behavior makes it even more imperative that we continue to do such studies as this one. Furthermore, such studies looking specifically at differences in males and females can be applicable to the expanding field of individualized medicine. Awareness of sex-related differences is critical in determining patient needs so that individualized care can be established; treatment outcome predictions can be linked to the genetic variation as well as the sex of a patient.

The goal of this study is to determine the gene(s) and mRNA expression patterns that are differentially expressed during training of female and male butterfly *Bicyclus*

anynana. If differences are seen in gene expression in the eyes of males and females, but not the central brain, that would suggest that processing of learning in the brain is identical for males and females of *B. anynana* but sensory outputs are different. However, if differentially expressed genes are seen in the central brain, that would suggest that there is indeed a difference in processing of learning between males and females.

Materials and Methods

Study Species and Husbandry

Figure 1: *B. anynana*

  Bicyclus anynana is a sub-tropical African butterfly that has a form for the wet season (Figure 1) and a form for the dry season (Brakefield and Reistma 1991). This species has been reared in the lab since 1988. The colony at the University of Arkansas was started in spring 2017 with hundreds of eggs from a population in Singapore. Butterflies at the University of Arkansas are reared in a climate-controlled greenhouse at 27°C, 80 percent humidity, and a 13:11h light:dark photoperiod to mimic wet season conditions and ensure production of the wet season form. Their diet consists of young corn plants as larvae and moist banana slices as adults (Westerman et al 2014). Butterflies bred in the lab have

similar levels of genetic diversity as those in natural populations, as suggested by similar single-nucleotide polymorphism frequencies found in laboratory and natural populations (Beldade et al 2006 and de Jong et al 2013).

This is how butterflies were reared; eggs were laid on young corn plants in the colony cages containing both male and female butterflies. Plants with eggs were moved to net cages with two flats of corn plants for larvae consumption. New plants were added to larval cages every two days. Pupae from these cages were placed in net cages until emergence. Upon emergence, butterflies were put in sex-specific cages, and were also kept by age. Cages were separated by pieces of white corrugated plastic so butterflies could not see the opposite sex. All butterflies were given wetted cotton and banana daily. Butterflies were painted the day prior to behavioral watches.

Behavioral Assays

Behavioral assays consisted of one newly emerged male paired with a two-day old, four spot female or one newly emerged female paired with a two-day old, four spot male. Control groups consisted of one newly emerged male or one newly emerged female. Watches were conducted for three hours starting within one hour of dawn. Behavioral data observed was inputted into SpectatorGo!, a software used to record behavior. Behaviors included: *flutter, fly, walk, rest, bask, antenna wiggle, court,* and *copulate* (which only happened once). After each three hour watch, the butterflies' heads were cut off and immediately flash frozen in liquid nitrogen, and then moved to a -80 freezer until RNA extraction. In determining sample size, it was important to take into account that B.

anynana at the University of Arkansas have shown that 75 percent of females and 80 percent of males learn. So, there must be a large enough sample size – 10 per group – to account for the portion of butterflies that do not learn.

RNA Library Preparation

We separated mRNA and small RNA from total RNA using a Machery-Nagel miRNA isolation kit. One day prior to RNA extraction, pestles were made using 1000 ml pipette tips and 1.5 ml microcentrifuge tubes. Additionally, 500 µl RNALater-ICE was added to frozen tissue and the frozen tissue was placed in -20°C freezer for approximately 18 hours prior to dissection.

Equipment and work areas were cleaned with 70% ethanol followed by RNase AWAY. Frozen individuals were dissected under a dissecting microscope while in RNAlater ice for removal of the eyes, brain, and antennae. Eye and brain tissues were mechanically disrupted using the pestles, lysed in 300 µl Buffer ML, and incubated at room temperature for 5 minutes according to Machery-Nagel protocol. The lysate was loaded onto a NucleoSpin filter column and centrifuged for 1 minute at 11,000 x g. 150 µl 96-100% ethanol was added to the flowthrough and vortexed for 5 seconds to adjust the binding conditions of large RNA and DNA and incubated at room temperature for 5 minutes. The sample was then loaded onto a NucleoSpin RNA column and centrifuged for 1 minute at 11,000 x g to bind large RNA and DNA. To desalt the silica membrane of the large RNA, 350 µl Buffer MDB was added to the filter and then centrifuged for 1

minute at 11,000 x g. 100 µl rDNase was placed into the NucleoSpin RNA column to digest the DNA; this was incubated at room temperature for at least 15 minutes.

Precipitating the protein, 300 µl Buffer MP was added to the flowthrough of the small RNA and then vortexed for 5 seconds and centrifuged for 3 minutes at 11,000 x g. The supernatant was loaded onto a NucleoSpin Protein Removal Column and centrifuged for 1 minute at 11,000 x g. 800 µl Buffer MX was added to the flowthrough and vortexed for 5 seconds to adjust binding conditions for small RNA. To bind small RNA, 600 µl of the sample was loaded onto a NucleoSpin RNA column and centrifuged for 30 seconds at 11,000 x g; flowthrough was discarded and the column placed back in the collection tube. This was repeated two more times.

Washing of large RNA and small RNA columns were done in parallel. For the first wash, 600 µl Buffer MW1 was added to the columns and then centrifuged for 30 seconds at 11,000 x g. Flowthrough was discarded and the columns placed back into the collection tubes. For the second wash, 700 µl Buffer MW2 was added to the columns and then centrifuged for 30 seconds at 11,000 x g. Flowthrough was discarded and the columns placed back into the collection tubes. For the third wash, 250 µl Buffer MW2 was added to the columns and then centrifuged for 2 minutes at 11,000 x g. The NucleoSpin RNA columns were moved into new collections tubes. RNA elution was done by adding 50 µl 90°C RNase-free H_2O was to the columns, allowing them to incubate at room temperature for 1 minute, and centrifuging them for 30 seconds at 11,000 x g.

Small RNA was saved in -80 freezer for later analysis. RNA quality and amount for both small and mRNA were checked on Thermo Scientific nanodrop and Agilent tape station to ensure enough quality and amount for library preparation. High throughput sequencing was done on the mRNA using the KAPA mRNA HyperPrep Kit. Illumina sequencing on a high seq, single read 50 base pairs was done at the genomics facility at the University of Chicago.

Bioinformatics

Read Quality

We concatenated all fastq files for each sample. There was a total of eight lanes of sequencing for 79 samples, for a total of 115 million single-end reads 50 base pairs in length. We checked the quality of each raw sample using FastQC v0.11.5 (Andrews 2010). Reads with low quality and/or ambiguous barcodes were thrown out. It was clear that one sample, TMB_E2, labeled as a brain sample was actually an eye sample, and so we discarded this sample (Figure 2). We used Trimmomatic v0.38 to remove sequencing adapters from the raw reads; trimmed sequences were one to fifty base pairs in length (Bolger et al 2014). Approximately 18,000 reads were removed, so we retained a total of about 115 million (99.98%) filtered single-end reads. We ran the now trimmed reads through FastQC again.

Reference Alignment

We created a genome index using the most recent *B. anynana* genome (Challis et al 2016). We aligned the adapter-trimmed reads for each sample to this most recent *B. anynana* genome using STAR v2.7.1a, and quantified using the "GeneCounts" flag (Dobin et al 2013).

Analysis of Gene Expression Profiles

Finally, we used the "ReadsPerGene.out.tab" counts files generated with STAR for differential expression analysis in R using the DESeq2 v1.24.0 package. We used the Gene Ontology (GO) to obtain sets of terms that described the genes (Blake et al 2015). This Principal component analysis (PCA) allowed for visualization of all 79 expression profiles, showing any differentially expressed genes in eyes and brains. Comparisons were made between eyes and brains, and males and females. We used heatmaps to look at the genes that were upregulated and downregulated between the sexes (i.e., eyes of naïve males versus females, brains of naïve males versus females, and eyes of trained males versus females, and brains of trained males versus females). We used boxplots to show statistically differentially expressed genes. Genes such as octopamine receptors and choline o-acetyltransferase were paid particular attention to because they have been implicated in learning (Schwaerzel et al 2003). While these gene candidates were looked for, RNAseq looked at all of the differentially expressed genes in the brains of male and female *B. anynana* so that an unbiased approach was taken.

Ethical Note

All *B. anynana* butterflies were maintained in laboratory conditions as specified by U.S. Department of Agriculture permit P526P-17-00343. Butterflies not used for this experiment were maintained with ample food and water until natural death.

Results

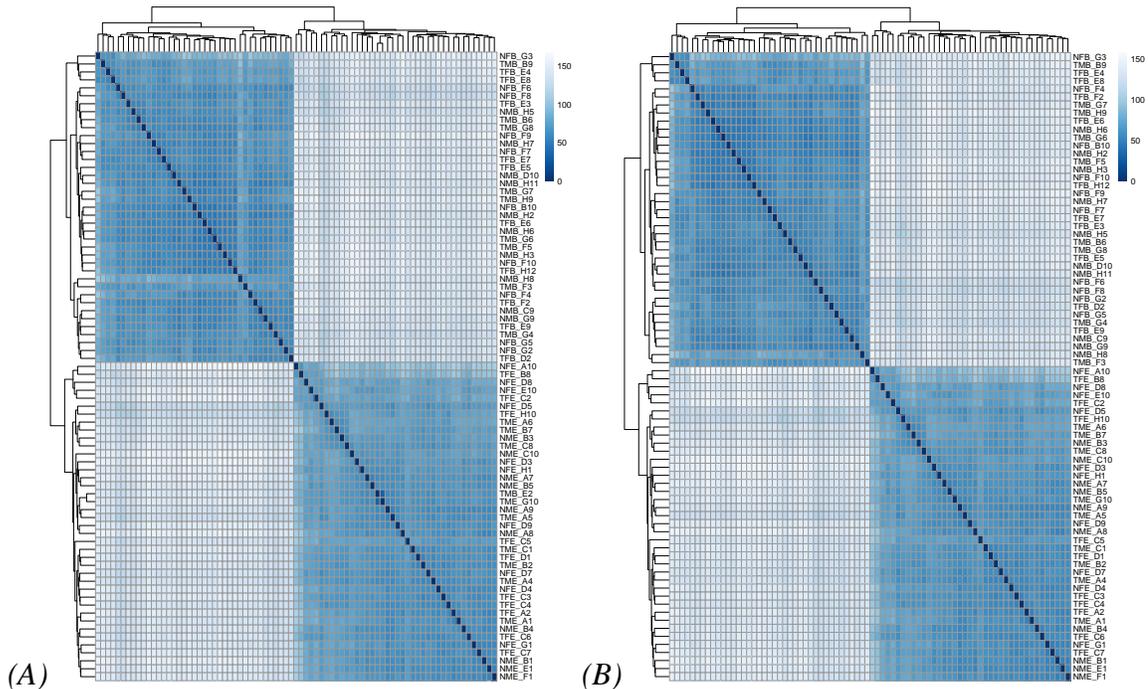*Eyes and Brains had distinct expression profiles*

(A)   (B)

Figure 2: Heatmap of all samples *(A)* with discarded sample TMB_E2 and *(B)* and without discarded sample TMB_E2 visibly showing that eyes and brains have different expression patterns.

We threw out sample TMB_E2 as it was clearly mislabeled, clustering with eyes instead of brains (2A). Removing this sample, all other samples cluster with their respective tissue (2B). In addition, it can be seen that brains have a sample distance of about 100

while eyes have a sample distance of about 50. Samples were labeled by condition, sex, and tissue (N = naïve, T = trained, M = male, F = female, E = eye, B = brain).

Eyes of naïve males and females had distinct expression patterns
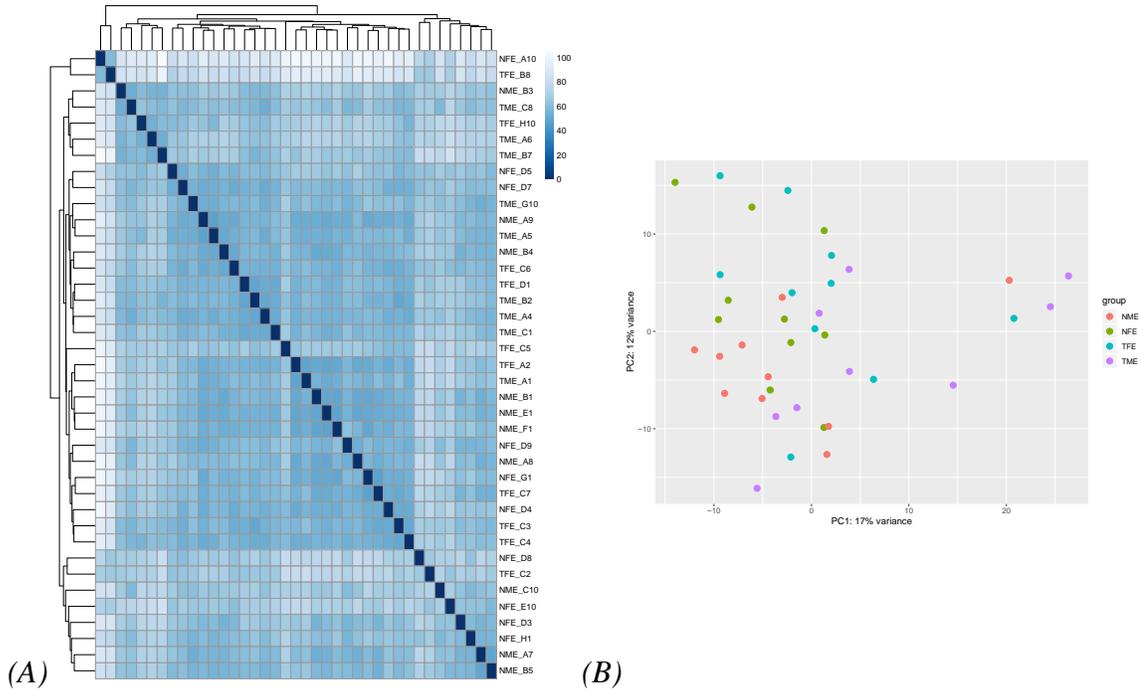

(A)   (B)

Figure 3: *(A)* **Heatmap and** *(B)* **PCA scatterplot of eyes of naïve and trained individuals. While there is not a large difference between trained and naïve expression patterns, there is a difference between male and female expression patterns. (N = naïve, T = trained, M = male, F = female, E = eye, B = brain)**

A difference can be seen between males and females (3B). PC1 and PC2 accounted for about 29 percent of variation. There does not look to be a large difference between trained and naïve individuals due to either PC1 or PC2, as there are no distinct clusters of trained or naïve individuals in the heatmap nor PCA plot.
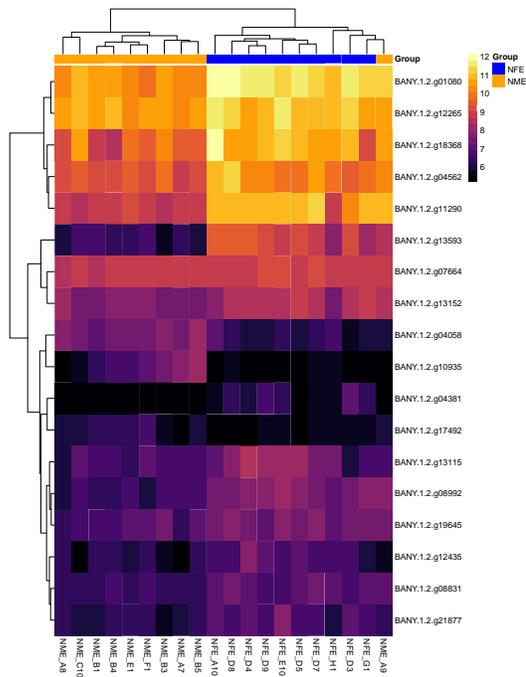
Figure 4: Heatmap of differentially expressed genes in eyes of naïve individuals. All but one male sample, sample NME_A9, clustered by sex. Females had upregulation of several genes compared to males. (blue = female, orange = male, N = naïve, M = male, F = female, E = eye)

Female and male eyes clustered accordingly to their respective sex, except for one male sample, sample NME_A9. Comparing the eyes of naïve males and females, a total of 18 genes were differentially expressed. These genes are associated with GO terms including metabolic process, endonuclease activity and RNA-dependent DNA biosynthetic process, DNA binding transcription factor activity, and integral component of membrane. Of the 18 genes differentially expressed, about 15 were upregulated in females and three upregulated in males. The five most upregulated in females were: BANY.1.2.g01080, BANY.1.2.g12265, BANY.1.2.g18368, BANY.1.2.g04562, BANY.1.2.g11290, and BANY.1.2.g13593. These genes function as endonuclease-reverse transcriptase, X-box

binding protein 1, facilitated trehalose transporter Tret-1 like, neuralized-like protein 4, and carbonyl reductase [NADPH] 3-like (Table 1). BANY.1.2.g04058, was upregulated in males and downregulated in females. This gene is an aldose 1-epimerase-like isoform X1 (Table 1).

SeqName	Description	Best.Blast.Hit	Length	pvalue	padj
BANY.1.2.g01080	endonuclease-reverse transcriptase	hypothetical protein evm_014675 [Chilo suppressalis]	4543	5.75E-08	0.0004312
BANY.1.2.g04058	aldose 1-epimerase-like isoform X1	aldose 1-epimerase-like isoform X2 [Bicyclus anynana]	9857	1.43E-07	0.00071602
BANY.1.2.g04381	circadian clock-controlled protein-like	circadian clock-controlled protein-like [Bicyclus anynana]	499	2.52E-08	0.00037805
BANY.1.2.g04562	facilitated trehalose transporter Tret1-like	facilitated trehalose transporter Tret1-like [Bicyclus anynana]	5874	1.84E-06	0.00345664
BANY.1.2.g07664	Putative uncharacterized transposon-derived protein F52C9.6	Putative uncharacterized transposon-derived protein F52C9.6 [Eumeta japonica]	28116	7.54E-07	0.00226032
BANY.1.2.g08831	insulin-like growth factor-binding protein 7	insulin-like growth factor-binding protein 7 [Galleria mellonella]	3061	5.00E-05	0.0416304
BANY.1.2.g08992	juvenile hormone resistence protein II	aryl hydrocarbon receptor nuclear translocator-like protein 1 isoform X2 [Bicyclus anynana]	56918	1.83E-05	0.01915818
BANY.1.2.g10935	fatty acyl-CoA reductase wat-like	fatty acyl-CoA reductase wat-like [Bicyclus anynana]	17582	4.32E-06	0.00720159
BANY.1.2.g11290	neuralized-like protein 4	LOW QUALITY PROTEIN: neuralized-like protein 4 [Bicyclus anynana]	12664	1.21E-06	0.00258436
BANY.1.2.g12265	X-box-binding protein 1	hypothetical protein KGM_200164 [Danaus plexippus plexippus]	7150	4.16E-05	0.03672522
BANY.1.2.g12435	reverse transcriptase	O-acyltransferase like protein-like [Bicyclus anynana]	14846	1.92E-05	0.01915818
BANY.1.2.g13115	band 7 protein AGAP004871	unnamed protein product [Leptidea sinapis]	12128	1.81E-05	0.01915818
BANY.1.2.g13152	zinc finger CCCH domain-containing protein 10	uncharacterized protein LOC112049125 isoform X1 [Bicyclus anynana]	14775	8.22E-06	0.01120861
BANY.1.2.g13593	carbonyl reductase [NADPH] 3-like	carbonyl reductase [NADPH] 3-like [Bicyclus anynana]	882	3.40E-07	0.00127663
BANY.1.2.g17492	facilitated trehalose transporter Tret1-like	facilitated trehalose transporter Tret1-like [Bicyclus anynana]	25186	1.46E-05	0.0183015
BANY.1.2.g18368	uncharacterized protein LOC112052706	uncharacterized protein LOC112052706 [Bicyclus anynana]	2284	2.35E-05	0.02198753
BANY.1.2.g19645	pollen-specific leucine-rich repeat extensin-like protein 2	pollen-specific leucine-rich repeat extensin-like protein 2 [Bicyclus anynana]	721	5.21E-06	0.00781887
BANY.1.2.g21877	2-acylglycerol O-acyltransferase 2-A-like	2-acylglycerol O-acyltransferase 2-A-like isoform X2 [Bicyclus anynana]	7048	9.09E-07	0.0022722

Table 1: Table of differentially expressed genes in eyes of naïve males and females

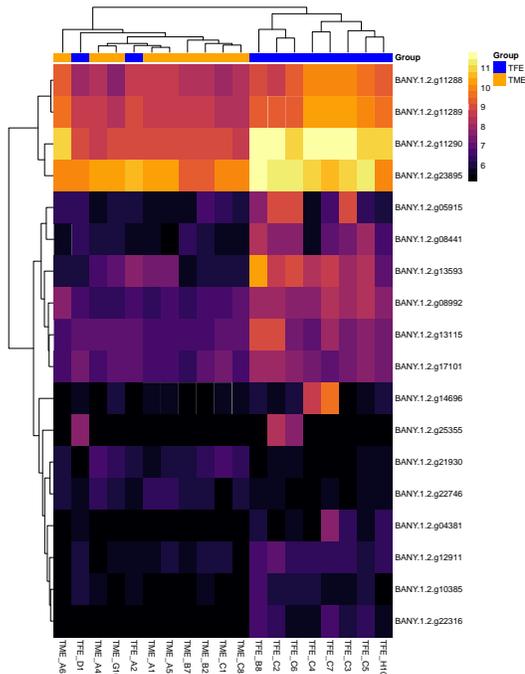*Eyes of trained males and females had distinct expression patterns*

A total of 18 genes were differentially expressed in the eyes of trained males versus females. GO terms associated with these genes are RNA directed DNA polymerase activity, extracellular region, integral component of membrane, microtubule motor activity, DNA-binding transcription factor activity, and RNA-directed DNA polymerase activity. Besides female samples TFE_D1 and TFE_A2, male and female samples grouped by sex. The two most visibly upregulated genes in females were: BANY.1.2.g11290 and BANY.1.2.g23895. The descriptions of these genes are neuralized-like protein 4 and tetratricopeptide repeat protein 4, respectively (Table 2).

SeqName	Description	Best.Blast.Hit	Length	pvalue	padj
BANY.1.2.g04381	circadian clock-controlled protein-like	circadian clock-controlled protein-like [Bicyclus anynana]	499	4.08E-06	0.00847761
BANY.1.2.g05915	circadian clock-controlled protein-like	uncharacterized protein LOC112044125 [Bicyclus anynana]	10473	1.39E-05	0.01779873
BANY.1.2.g08441	pupal cuticle protein PCP52-like	pupal cuticle protein PCP52-like [Bicyclus anynana]	6512	2.21E-09	3.67E-05
BANY.1.2.g08992	juvenile hormone resistence protein II	aryl hydrocarbon receptor nuclear translocator-like protein 1 isoform X2 [Bicyclus anynana]	56918	1.98E-06	0.00656637
BANY.1.2.g10385	reverse transcriptase	nose resistant to fluoxetine protein 6-like isoform X2 [Bicyclus anynana]	17254	1.54E-05	0.0182408
BANY.1.2.g11288	Putative uncharacterized transposon-derived protein F52C9.6	Putative uncharacterized transposon-derived protein F52C9.6 [Eumeta japonica]	15566	4.30E-05	0.03968608
BANY.1.2.g11289	neuralized-like protein 4	LOW QUALITY PROTEIN: neuralized-like protein 4 [Bicyclus anynana]	15021	1.13E-05	0.01564234
BANY.1.2.g11290	neuralized-like protein 4	LOW QUALITY PROTEIN: neuralized-like protein 4 [Bicyclus anynana]	12664	2.46E-08	0.00020394
BANY.1.2.g12911	venom allergen 5-like	venom allergen 5-like [Bicyclus anynana]	5247	9.65E-07	0.00534317
BANY.1.2.g13115	band 7 protein AGAP004871	unnamed protein product [Leptidea sinapis]	12128	2.74E-05	0.02846338
BANY.1.2.g13593	carbonyl reductase [NADPH] 3-like	carbonyl reductase [NADPH] 3-like [Bicyclus anynana]	882	1.55E-06	0.00645554
BANY.1.2.g14696	nose resistant to fluoxetine protein 6-like	uncharacterized protein LOC112050180 [Bicyclus anynana]	9189	3.53E-06	0.00838262
BANY.1.2.g17101	pancreatic lipase-related protein 2	pancreatic lipase-related protein 2 isoform X2 [Bicyclus anynana]	25309	2.50E-05	0.02765032
BANY.1.2.g21930	glycine-rich protein 5-like	glycine-rich protein 5-like [Bicyclus anynana]	2017	9.87E-06	0.01538347
BANY.1.2.g22316	trichohyalin-like	trichohyalin-like [Bicyclus anynana]	4004	5.98E-06	0.01104059
BANY.1.2.g22746	dynein heavy chain 6, axonemal	dynein heavy chain 6, axonemal-like [Bicyclus anynana]	15212	3.51E-05	0.03433083
BANY.1.2.g23895	tetratricopeptide repeat protein 4	tetratricopeptide repeat protein 4 [Bicyclus anynana]	7853	2.62E-06	0.00723876
BANY.1.2.g25355	reverse transcriptase	uncharacterized protein LOC112044210 [Bicyclus anynana]	2399	1.02E-05	0.01538347

Table 2: Table of differentially expressed genes in eyes of trained individuals
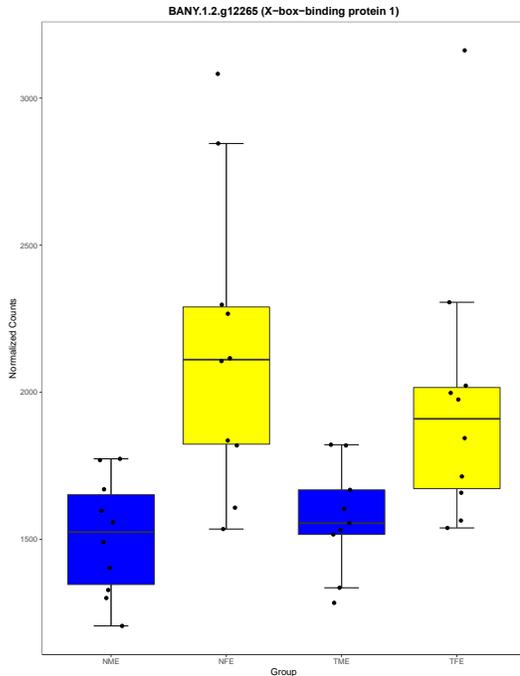
Figure 6: Differentially expressed gene BANY.1.2.g12265, X-box binding protein 1, in eyes of naïve and trained individuals. This gene was upregulated in females compared to males. (yellow = female, blue = male, N = naïve, T = trained, M = male, F = female, E = eye)

  BANY.1.2.g12265 was upregulated in the eyes of females. This gene controls an X-box binding protein. It is associated with GO term DNA-binding transcription factor activity and regulation of transcription. Naïve and trained males and naïve and trained females had similar expression of this gene. Males and females differentially expressed this gene. Because it was differentially expressed between males and females but not naïve and trained, this indicates an effect of sex but not of training.

BANY.1.2.g19645 (pollen−specific leucine−rich repeat extensin−like protein 2)
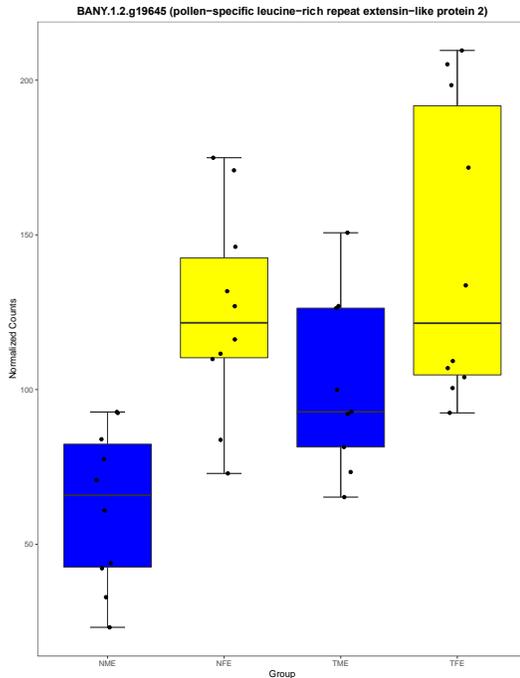
Figure 7: Differentially expressed gene BANY.1.2.g19645, pollen-specific leucine-rich repeat extension-like protein 2, in eyes of naïve and trained individuals. This was upregulated in trained males versus naïve males, as well as in females versus males. (yellow = female, blue = male, N = naïve, T = trained, M = male, F = female, E = eye)

BANY.1.2.g19645 serves as a pollen-specific leucine-rich repeat extension-like protein. It was upregulated in trained males compared to naïve males. It was upregulated in females compared to males. As this gene was differentially expressed between males and females as well as between naïve and trained males, this suggests an interactive effect of sex and training.

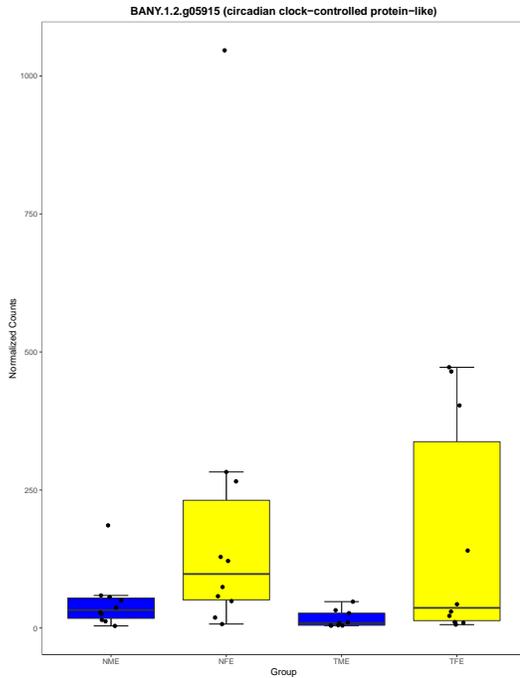**BANY.1.2.g05915 (circadian clock−controlled protein−like)**

Figure 8: Differentially expressed gene BANY.1.2.g05915, circadian clock-controlled protein-like, in eyes of naïve and trained individuals. Females had an upregulation and males had a down regulation of this gene. (yellow = female, blue = male, N = naïve, T = trained, M = male, F = female, E = eye)

The function of BANY.1.2.g05915 involves circadian clock-controlled behavior. It was greatly downregulated in the eyes of males. Both naïve and trained males had little expression of this gene, while both naïve and trained females had an upregulation of this gene. It was differentially expressed between males and females, but not naïve and trained, indicating an effect of sex but not of training.
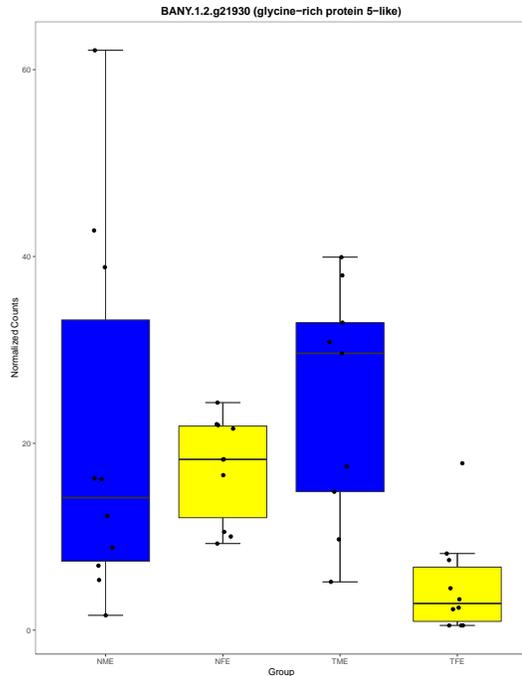
Figure 9: Differentially expressed gene BANY.1.2.g21930, glycine-rich protein 5-like, in eyes of naïve and trained individuals. This gene was greatly downregulated in trained females compared to the other treatment groups. (yellow = female, blue = male, N = naïve, T = trained, M = male, F = female, E = eye)

   BANY.1.2.g21930 is a gene related to glycine-rich protein. It was downregulated in the eyes of trained females. Naïve males, trained males, and naïve females had comparable levels of expression, with males having a greater range of expression and naïve males having the widest range. There was differential expression between trained males and females as well as between naïve and trained females, showing an interactive effect of sex and training.

Brains of naïve and trained males and females did not cluster completely by sex or treatment
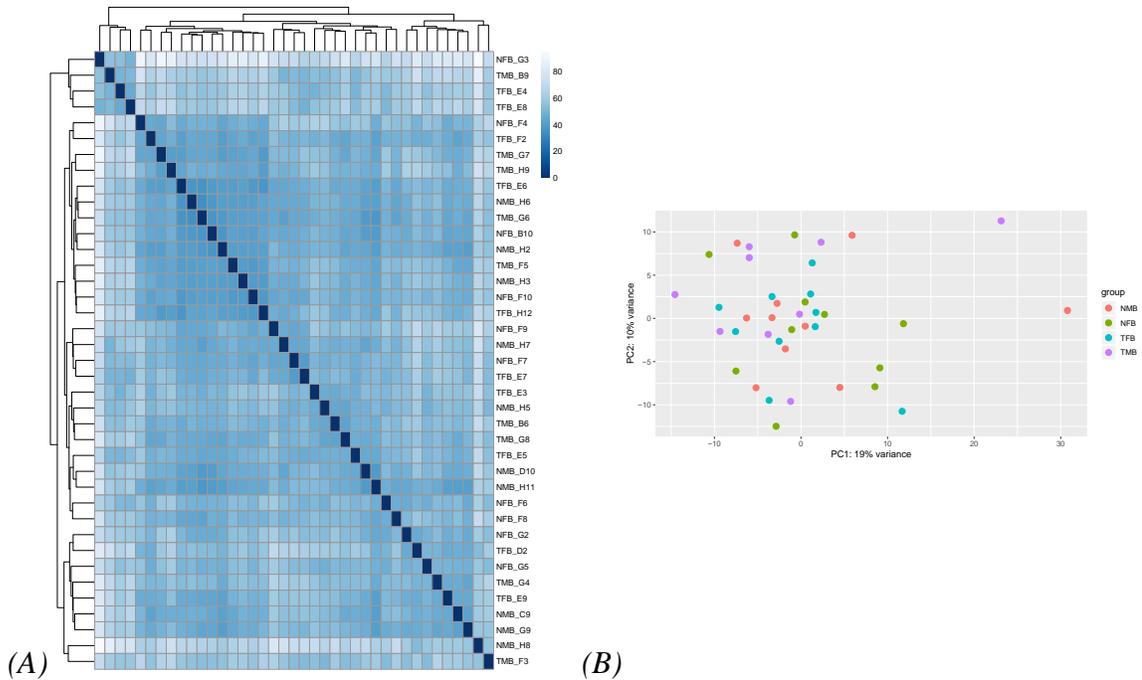
Figure 10: *(A)* **Heatmap and** *(B)* **PCA scatterplot of brains of naïve and trained individuals. There is a difference in expression pattern between males and females. (N = naïve, T = trained, M = male, F = female, B = brain)**

 While there are no distinct clusters between males and females nor trained and naïve seen in the heatmap, there are clusters of males and females seen in the PCA plot. PC1 and PC2 accounted for approximately 29 of variation (10B).

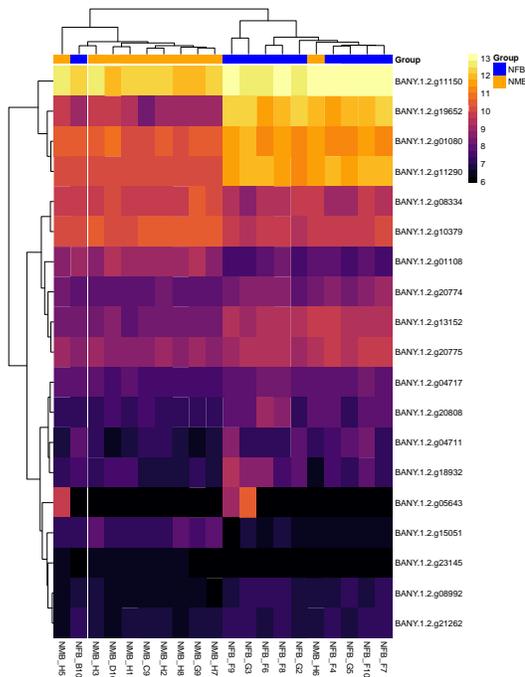*Brains of naïve males and females had distinct expression patterns*

Figure 11: Heatmap of differentially expressed genes in brains of naive individuals. Female sample NFB_B10 clustered with males and male sample NMB_H8 clustered with females; otherwise, female and male brain gene expression clustered accordingly to their respective sex. Females had upregulation of several genes. (blue = female, orange = male, N = naïve, , M = male, F = female, B = brain)

There were a total of 19 genes differentially expressed in the brains of naïve females versus males. GO terms involved are catalytic activity, DNA binding, RNA binding, ATP binding, and integral component of membrane. Aside from female NFB_B10 that clustered with males and male sample NMB_H8 that clustered with females, males and females clustered by sex. The three most upregulated in females were: BANY.1.2.g19652, BANY.1.2.g01080, and BANY.1.2.g11290. The purpose of these genes include endonuclease-reverse transcriptase and neuralized-like protein 4 (Table 3). Both males and females had high expression of BANY.1.2.g11150, a gene with the

function of cyclic nucleotide-gated channel rod photoreceptor subunit alpha-like (Table

3).

SeqName	Description	Best.Blast.Hit	Length	pvalue	padj
BANY.1.2.g01080	endonuclease-reverse transcriptase	hypothetical protein evm_014675 [Chilo suppressalis]	4543	1.73E-06	0.00514389
BANY.1.2.g01108	protein Teyrha-meyrha isoform X1	uncharacterized protein LOC112044210 [Bicyclus anynana]	21856	9.31E-07	0.00460022
BANY.1.2.g04711	acyl-CoA synthetase short-chain family member 3, mitochondrial	acyl-CoA synthetase short-chain family member 3, mitochondrial [Bicyclus anynana]	10225	5.89E-05	0.04894681
BANY.1.2.g04717	uncharacterized protein LOC112043382 isoform X2	uncharacterized protein LOC112043382 isoform X2 [Bicyclus anynana]	819	4.01E-05	0.04201166
BANY.1.2.g05643	uncharacterized protein LOC114248481	uncharacterized protein LOC114248481 [Bombyx mandarina]	19564	9.62E-09	0.00014271
BANY.1.2.g08334	succinate dehydrogenase assembly factor 3, mitochondrial	succinate dehydrogenase assembly factor 3, mitochondrial [Bicyclus anynana]	1064	5.03E-06	0.0106495
BANY.1.2.g08992	juvenile hormone resistence protein II	aryl hydrocarbon receptor nuclear translocator-like protein 1 isoform X2 [Bicyclus anynana]	56918	6.18E-05	0.04894681
BANY.1.2.g10379	helicase SKI2W	helicase SKI2W [Bicyclus anynana]	22875	6.38E-06	0.01183456
BANY.1.2.g11150	cyclic nucleotide-gated channel rod photoreceptor subunit alpha-like	cyclic nucleotide-gated channel rod photoreceptor subunit alpha-like [Bicyclus anynana]	6266	1.47E-06	0.01978753
BANY.1.2.g11290	neuralized-like protein 4	LOW QUALITY PROTEIN: neuralized-like protein 4 [Bicyclus anynana]	12664	3.99E-07	0.0029563
BANY.1.2.g13152	zinc finger CCCH domain-containing protein 10	uncharacterized protein LOC112049125 isoform X1 [Bicyclus anynana]	14775	1.69E-06	0.00514389
BANY.1.2.g15051	protein henna	protein henna [Bicyclus anynana]	8632	1.11E-05	0.0165014
BANY.1.2.g18932	RNA-directed DNA polymerase from mobile element jockey-like	hypothetical protein B5V51_14575 [Heliothis virescens]	40360	6.27E-05	0.04894681
BANY.1.2.g19652	uncharacterized protein LOC112044338	uncharacterized protein LOC112044338 [Bicyclus anynana]	774	1.87E-05	0.02304812
BANY.1.2.g20774	PAN2-PAN3 deadenylation complex subunit PAN3	PAN2-PAN3 deadenylation complex subunit PAN3, partial [Bicyclus anynana]	13682	4.25E-05	0.04201166
BANY.1.2.g20775	endonuclease-reverse transcriptase	PAN2-PAN3 deadenylation complex subunit PAN3, partial [Bicyclus anynana]	18905	2.43E-05	0.02768347
BANY.1.2.g20808	collagen alpha-1(IV) chain-like	collagen alpha-1(IV) chain-like [Bicyclus anynana]	9104	5.52E-05	0.04894681
BANY.1.2.g21262	sex peptide receptor	sex peptide receptor isoform X1 [Bicyclus anynana]	3401	7.46E-06	0.01229812
BANY.1.2.g23145	serine--pyruvate aminotransferase, mitochondrial-like	serine--pyruvate aminotransferase, mitochondrial-like isoform X2 [Bicyclus anynana]	5649	5.01E-06	0.0106495

Table 3: Table of differentially expressed genes in brains of naïve individuals

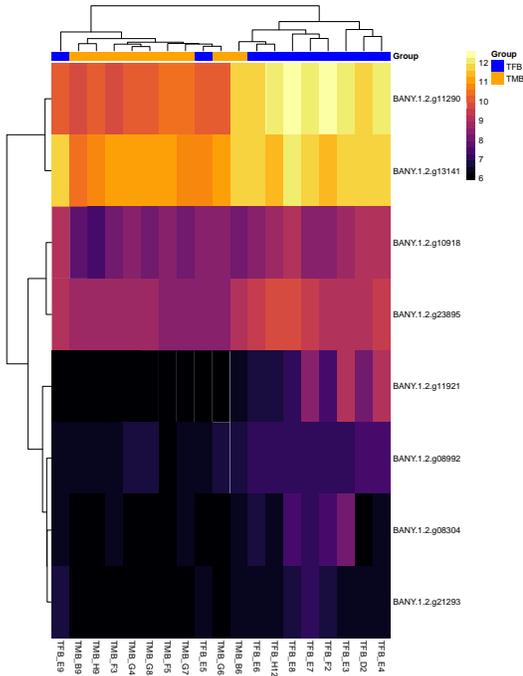*Brains of trained males and females had distinct expression patterns*

Figure 12: Heatmap of differentially expressed genes in brains of trained individuals. Females had upregulation of eight genes compared to males. (blue = female, orange = male, T = trained, M = male, F = female, B = brain)

Eight genes were differentially expressed in the brains of trained males compared to trained females. GO terms related to these genes are oxidoreductase activity, DNA-binding transcription factor activity, integral component of membrane, lipid transporter activity, and Rab guanyl-nucleotide exchange factor activity. The two most upregulated genes in females were BANY.1.2.g11290 and BANY.1.2.g13141. These genes act as neuralized-like protein 4 and DENN domain-containing protein 1A-like isoform X5, respectively (Table 4).

SeqName	Description	Best.Blast.Hit	Length	pvalue	padj
BANY.1.2.g08304	Lian-Aa1 retrotransposon protein	uncharacterized protein LOC112046286 [Bicyclus anynana]	2448	9.91E-06	0.02556592
BANY.1.2.g08992	juvenile hormone resistence protein II	aryl hydrocarbon receptor nuclear translocator-like protein 1 isoform X2 [Bicy	56918	5.66E-06	0.01929482
BANY.1.2.g10918	transmembrane protein 135-like	transmembrane protein 135-like [Bicyclus anynana]	11504	3.68E-07	0.00284655
BANY.1.2.g11290	neuralized-like protein 4	LOW QUALITY PROTEIN: neuralized-like protein 4 [Bicyclus anynana]	12664	2.40E-07	0.00284655
BANY.1.2.g11921	vitellogenin-like	vitellogenin-like [Bicyclus anynana]	7876	6.23E-06	0.01929482
BANY.1.2.g13141	DENN domain-containing protein 1A-like isoform X5	DENN domain-containing protein 1A-like isoform X5 [Bicyclus anynana]	36158	1.20E-05	0.0265209
BANY.1.2.g21293	RNA-directed DNA polymerase from mobile element jockey-like	uncharacterized protein LOC112057116 [Bicyclus anynana]	5994	4.88E-06	0.01929482
BANY.1.2.g23895	tetratricopeptide repeat protein 4	tetratricopeptide repeat protein 4 [Bicyclus anynana]	7853	2.39E-05	0.04624092

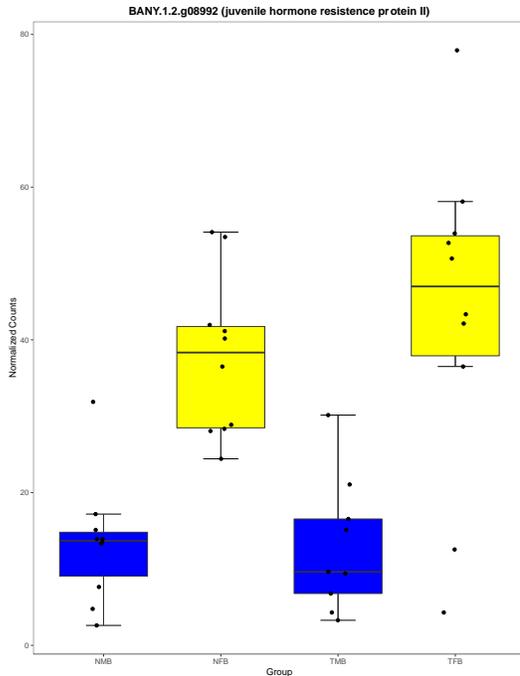**Table 4: Table of differentially expressed genes of brains of trained individuals**

Figure 13: Differentially expressed gene BANY.1.2.g08992, juvenile hormone resistence protein II, in brains of naïve and trained individuals. Females had an upregulation of this gene. (yellow = female, blue = male, N = naïve, T = trained, M = male, F = female, B = brain)

BANY.1.2.g08992 is a gene for juvenile hormone resistence protein II It is associated with GO terms DNA-binding transcription factor activity, nucleus, transcription factor complex, cytoplasm, regulation of transcription and DNA-templated, and protein dimerization activity. Naïve and trained males had very similar expression patterns for this gene. Females had upregulation of this compared to males. It was differentially expressed between males and females, but not naïve and trained, indicating an effect of sex but not of training.
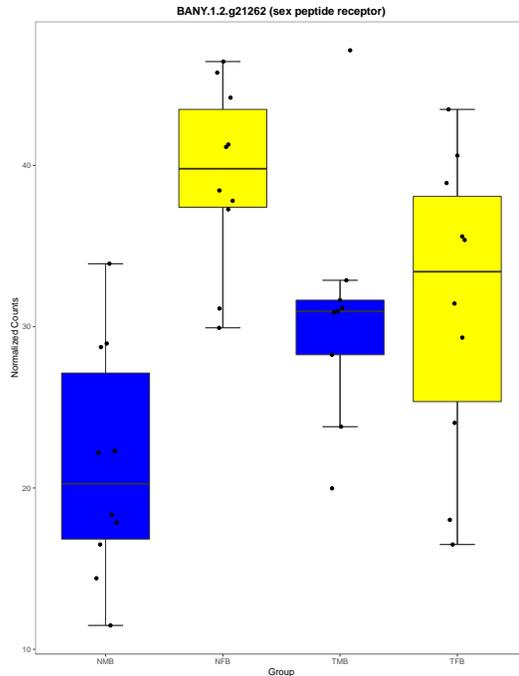
Figure 14: Differentially expressed gene BANY.1.2.g21262, sex peptide receptor, in brains of naïve and trained individuals. This was upregulated in females compared to males, and in trained males compared to naïve males. (yellow = female, blue = male, N = naïve, T = trained, M = male, F = female, B = brain)

BANY.1.2.g21262 is a gene for sex peptide receptor. It would be expected that a gene controlling reproductive behavior would be differentially expressed between the sexes, and it was a result that we found. This gene is associated with GO terms protein-coupled receptor signaling pathway, G protein-coupled peptide receptor activity, and integral component of membrane. This gene was upregulated in naïve females compared to naïve males, and in trained males compared to naïve males. Females looked to have higher expression of this gene than males. BANY.1.2.g21262 was differentially expressed between males and females as well as between naïve and trained, suggesting an interactive effect of sex and training.
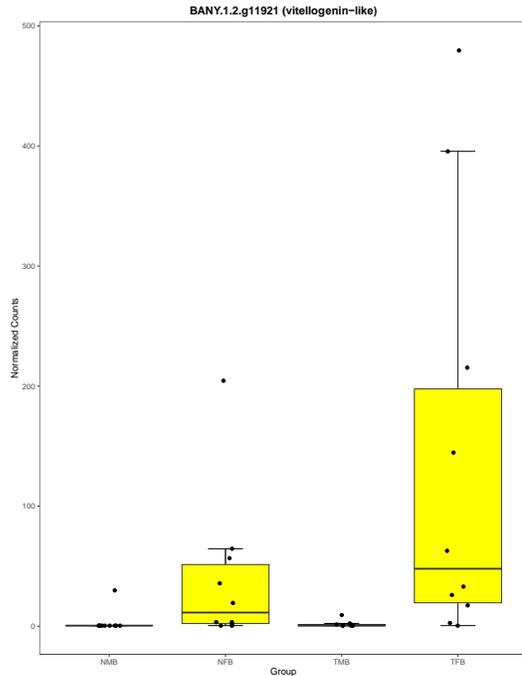
Figure 15: Differentially expressed gene BANY.1.2.g11921, vitellogenin-like, in brains of naïve and trained individuals. Females had a much greater expression of this gene, while males had little to no expression of this gene. (yellow = female, blue = male, N = naïve, T = trained, M = male, F = female, B = brain)

BANY.1.2.g11921 is a vitellogenin-like gene. This gene is associated with GO terms lipid transporter activity and lipid transport. Females greatly upregulated this gene compared to males who had almost no expression of this gene. In trained females there was a wide range in expression pattern. It was differentially expressed between males and females, but not naïve and trained, showing an effect of sex but not of training.

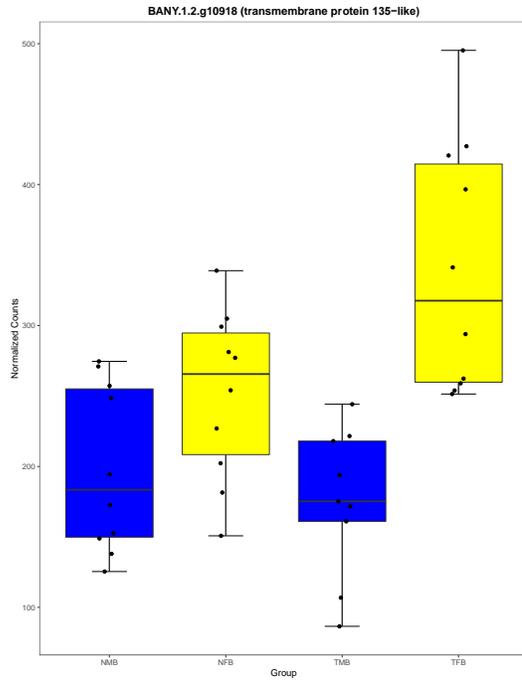**BANY.1.2.g10918 (transmembrane protein 135−like)**

Figure 16: Differentially expressed gene BANY.1.2.10918, transmembrane protein 135-like, in brains of naïve and trained individuals. Females upregulated this gene compared to the other treatment groups, with trained females having the greatest expression of this gene. (yellow = female, blue = male, N = naïve, T = trained, M = male, F = female, B = brain)

BANY.1.2.g10918 is associated with GO term integral component of membrane. Females had greater expression of this gene compared with males, with trained females having the greatest expression out of the treatment groups. Naïve and trained males had like expression patterns. BANY.1.2.g10918 was differentially expressed between males and females as well as between naïve and trained females, indicating an interactive effect of sex and training.

Discussion

We had three hypotheses. Hypothesis one stated that if we saw genes that are differentially expressed in males and females, but not trained and naïve, then that suggests that there is an effect of sex but not an effect of training. Hypothesis two stated that if we saw genes that are differentially expressed in trained and naïve individuals, but not males and females, then that suggests that there is an effect of training but not an effect of sex. Hypothesis three stated that if we saw genes that are differentially expressed in males and females and trained and naïve, then that suggests that there is an interactive effect of sex and training on brain gene expression.

We had data that supported all three hypotheses. We found instances of differential gene expression between males and females and/or naïve and trained. This provides evidence that there are differences between male and female neural processes. This also provides evidence that there are differences between processing in eyes and brain. This means that differences in learning are influenced by genes expressed due to sex and/or an interaction of sex and social experience. Gene regulation is occurring in both the eyes and brains of *B. anynana* in response to social stimuli. Learning and social behavior are neurally active processes.

Our results are consistent with those of the study previously mentioned investigating swordtail fish brain gene expression in response to sexual and social stimuli. Like the swordtail fish, *B. anynana* had differential brain gene expression accordingly to sex – males and females had differences in gene expression – and social exposure – trained and naïve individuals had differences in gene expression (Cummings et al 2008). Learning

more about the genes differentially expressed between sex and social condition can give us more insight into how these genes control behavior and so mate choices.

We were able to characterize genes expressed using GO terms, which took into account three main characteristics: its molecular function, the biological process in which it participates, and its cellular location. A majority of the differentially expressed genes are involved in cellular functioning, response to environmental factors, or behavior. So, the dissimilarities between *B. anynana* males and females in what and how they learn are due to the differences in the genes and the up or downregulation of these genes each express. Though octopamine and choline o-acetyltransferase, as mentioned previously that these genes would be paid particular attention to, were not differentially expressed, ten octopamine genes and two choline o-acetyltransferase genes were expressed in the head and eye.

X-box binding protein 1 gene is an important regulator of the unfolded protein response. It has been found that silencing this gene in adult animals causes chronic ER stress and dopaminergic neuron degeneration in substantia nigra pars compacta (Valdés et al 2014). This indicates that there is a functional requirement for this gene in preserving protein homeostasis in nigral dopaminergic neurons. For *B. anynana*, this would suggest that the X-box binding protein 1 gene is critical in cell stress and protein function in their eyes. This gene, BANY.1.2.g12265, may play a bigger role in females than males as it was upregulated in females compared to males (Figure 6).

Circadian clock-controlled protein-like gene, BANY.1.2.g05915, most likely influences behavior based on circadian rhythms. Clock-controlled genes regulate gene expression and transcription in *Drosophila melanogaster* (Fernanda et al 2002). These genes peak throughout the day, regulating gene expression in a tissue-specific manner as well as processes such as signal transduction, protein stability, and heme metabolism. Certain behaviors of *B. anynana* such as courting and mating, and possibly even learning, is likely to be affected by time of day. Females had an upregulation of this gene, and so time of day may affect female behaviors and cellular processes more than males (Figure 8).

Juvenile Hormone is involved in metamorphosis and reproduction in insects (Riddiford 1994). Analogs of this hormone have been synthesized that have potent effects on the development of insects. Insects have evolved resistance to some insecticides; for example, the Met (Methoprene-tolerant) mutation in *Drosophila melanogaster* permits resistance to Methoprene, an analog of Juvenile Hormone, or Juvenile Hormone III (Shemshedini and Wilson 1990). Juvenile hormone resistance protein II, BANY.1.2.g08992, most likely developed in *B. anynana* for resistance against insecticides to help ensure regular development occurred. It makes sense that this gene was differentially expressed in brains (Figure 13).

The sex peptide receptor is essential in mating behavior. In *Drosophila melanogaster*, mating status is relevant to female adaptive food choices, and mating status is dependent on the sex peptide receptor (Riberio and Dickson 2010). The sex peptide receptor gene

controls the sex peptide receptor, so in *B. anynana* BANY.1.2.g21262 (Figure 14) is essential in courting and mating interactions. Trained males and females had similar expression of this gene as would be expected. However, naïve females had the highest expression of this gene out of the treatment groups which we would not have expected to see as they were isolated.

Vitellogenin is the egg-yolk precursor protein and is widely used as a bioindicator of estrogens in the environment. It has been seen that vitellogenin-like protein levels are increased in freshwater gastropods that have been exposed to harmful chemicals in the environment (Gagnaire et al 2009). As *B. anynana* are exposed to endocrine disruptors in the environment, their vitellogenin-like protein levels will rise. Vitellogenin-like gene, BANY.1.2.g11921, was upregulated in females and downregulated, if at all expressed, in males (Figure 15) which is an anticipated result as vitellogenin is found only in females.

A study looking at differential gene expression in the heads of *B. anynana* found several eye development and eye pigment genes that are sex-specifically expressed; female vision genes are more plastic (Macias-Munoz et al 2016). This study and ours differ in that we had a different time point (our butterflies are a couple of hours older), our sample size is larger, we dissected out the eyes and brains, and we used a different RNA sample prep kit. However, the study points out a possible reason for the genes that we found to be differentially expressed between males and females. In the genes we highlighted, we saw that most were upregulated in females. This could have been due in part to plastic vision genes in females.

Conclusion

  We wanted to explore what is behind the differences in learning between male and female *B. anynana*, specifically if these differences are associated with differential gene expression. We found that eyes and brains have distinct expression patterns, and that males and females have distinct expression patterns. There are differences in gene expression between naïve male and females eyes, between trained male and female eyes, between naïve male and female brains, and between trained male and female brains. In total, we found 63 differentially expressed genes. A number of these differentially expressed genes stand out, such as ones that control sex peptide receptor, juvenile hormone resistance, and circadian clock-controlled rhythms. There was evidence to support all three of our hypotheses; there is an effect of sex but not of training on some genes, an effect of training but not of sex on others, and an interactive effect of sex and training on an additional set of genes. The differences in learning, then, in *B. anynana* are associated with the differences in gene regulation in the eyes and brains of males and females in response to social stimuli.

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